Methods in Molecular Biology 1378

Springer Protocols

Uttam Garg *Editor*

# Clinical Applications of Mass Spectrometry in Biomolecular Analysis

## Methods and Protocols

Humana Press

# METHODS IN MOLECULAR BIOLOGY

*Series Editor*
**John M.Walker**
**School of Life and Medical Sciences**
**University of Hertfordshire**
**Hatfield, Hertfordshire, AL10 9AB, UK**

For further volumes:
http://www.springer.com/series/7651

# Clinical Applications of Mass Spectrometry in Biomolecular Analysis

## Methods and Protocols

Edited by

**Uttam Garg**

*Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA*

Humana Press

*Editor*
Uttam Garg
Department of Pathology and Laboratory
Medicine, Children's Mercy
Hospitals and Clinics
Kansas City, MO, USA

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-3181-1 ISBN 978-1-4939-3182-8 (eBook)
DOI 10.1007/978-1-4939-3182-8

Library of Congress Control Number: 2015957377

Springer New York Heidelberg Dordrecht London


Printed on acid-free paper

Humana Press is a brand of Springer
Springer Science+Business Media LLC New York is part of Springer Science+Business Media (www.springer.com)

# Dedication

This book is dedicated to my wife Jyotsna and daughters Megha and Mohini who are my inspiration to keep moving forward in life.

# Preface

Clinical laboratory applications of mass spectrometry are growing rapidly. This has been made possible with the advent of benchtop compact, relatively inexpensive, and user-friendly systems. This versatile technology offers a number of advantages including increased sensitivity and specificity, multiple component analysis, and no need of specialized reagents. In fact the technique is a must for the measurement of many clinically relevant analytes in the fields of drug analysis, endocrinology, and inborn errors of metabolism. This volume *Clinical Applications of Mass Spectrometry in Biomolecular Analysis* provides stepwise instructions for the analysis of a number of analytes of clinical importance. Each chapter provides a brief introduction about the analyte followed by stepwise instructions on the analytical protocol.

I am grateful to my colleagues who contributed to the contents of this book. I am hopeful that the readers will find this book useful.

***Kansas City, MO*** ***Uttam Garg***

# Contents

*Dedication* . . . . . *v*
*Preface* . . . . . *vii*
*Contributors* . . . . . *xiii*

1 Mass Spectrometry in Clinical Laboratory: Applications in Biomolecular Analysis . . . . . 1
***Uttam Garg and Yan Victoria Zhang***

2 Quantification of Free Carnitine and Acylcarnitines in Plasma or Serum Using HPLC/MS/MS. . . . . 11
***David Scott, Bryce Heese, and Uttam Garg***

3 Quantification of Arginine and Its Methylated Derivatives in Plasma by High-Performance Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) . . . . . 21
***Faye B. Vicente, Gina Vespa, Alan Miller, and Shannon Haymond***

4 Quantitation of Albumin in Urine by Liquid Chromatography Tandem Mass Spectrometry . . . . . 31
***Hemamalini Ketha and Ravinder J. Singh***

5 Quantitation of Aldosterone in Serum or Plasma Using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). . . . . 37
***J. Grace Van Der Gugten and Daniel T. Holmes***

6 Quantification of Five Clinically Important Amino Acids by HPLC-Triple TOF™ 5600 Based on Pre-column Double Derivatization Method . . . . . 47
***Shuang Deng, David Scott, and Uttam Garg***

7 Sensitive, Simple, and Robust Nano-Liquid Chromatography-Mass Spectrometry Method for Amyloid Protein Subtyping . . . . . 55
***Drew Payto, Courtney Heideloff, and Sihe Wang***

8 Quantitation of Ubiquinone (Coenzyme $Q^{10}$) in Serum/Plasma Using Liquid Chromatography Electrospray Tandem Mass Spectrometry (ESI-LC-MS/MS) . . . . . 61
***Richard E. Mathieu Jr. and Catherine P. Riley***

9 Quantitative Analysis of Salivary Cortisol Using LC-MS/MS . . . . . 71
***Yan Victoria Zhang***

10 Quantification of Dihydroxyacetone Phosphate (DHAP) in Human Red Blood Cells by HPLC-TripleTOF 5600™ Mass Spectrometer . . . . . 81
***Shuang Deng, David Scott, Douglas Myers, and Uttam Garg***

11 Simultaneous Quantitation of Estradiol and Estrone in Serum Using Liquid Chromatography Mass Spectrometry . . . . . 87
***Catherine P. Riley, Richard E. Mathieu Jr., and Carmen Wiley***

12 Direct Measurement of Free Estradiol in Human Serum and Plasma by Equilibrium Dialysis-Liquid Chromatography-Tandem Mass Spectrometry . . . . . 99
***Julie A. Ray, Mark M. Kushnir, Alan L. Rockwood, and A. Wayne Meikle***

13 Quantification of γ-Aminobutyric Acid in Cerebrospinal Fluid Using Liquid Chromatography-Electrospray Tandem Mass Spectrometry . . . . . 109
***Erland Arning and Teodoro Bottiglieri***

14 Quantitation of Insulin Analogues in Serum Using Immunoaffinity Extraction, Liquid Chromatography, and Tandem Mass Spectrometry . . . . . 119
***J. Grace Van Der Gugten, Sophia Wong, and Daniel T. Holmes***

15 Quantitation of Insulin-Like Growth Factor 1 in Serum by Liquid Chromatography High Resolution Accurate-Mass Mass Spectrometry. . . . . 131
***Hemamalini Ketha and Ravinder J. Singh***

16 Quantitation of Free Metanephrines in Plasma by Liquid Chromatography-Tandem Mass Spectrometry. . . . . 139
***Courtney Heideloff, Drew Payto, and Sihe Wang***

17 Quantification of Metanephrine and Normetanephrine in Urine Using Liquid Chromatography-Tandem Mass Spectrometry . . . . . 149
***Jessica Gabler and Sihe Wang***

18 High-Throughput Analysis of Methylmalonic Acid in Serum, Plasma, and Urine by LC-MS/MS. Method for Analyzing Isomers Without Chromatographic Separation. . . . . 159
***Mark M. Kushnir, Gordon J. Nelson, Elizabeth L. Frank, and Alan L. Rockwood***

19 Quantitation of 5-Methyltetrahydrofolate in Cerebrospinal Fluid Using Liquid Chromatography-Electrospray Tandem Mass Spectrometry . . . . . 175
***Erland Arning and Teodoro Bottiglieri***

20 Quantitative Organic Acids in Urine by Two Dimensional Gas Chromatography-Time of Flight Mass Spectrometry (GCxGC-TOFMS) . . . . . 183
***Lawrence Sweetman, Paula Ashcraft, and Jeanna Bennett-Firmin***

21 High Sensitivity Measurement of Pancreatic Polypeptide and Its Variant in Serum and Plasma by LC-MS/MS. . . . . 199
***Hernando Escobar, Mark M. Kushnir, Alan L. Rockwood, and A. Wayne Meikle***

22 Quantitation of Parathyroid Hormone in Serum or Plasma by Liquid Chromatography-Tandem Mass Spectrometry . . . . . 211
***Hemamalini Ketha and Ravinder J. Singh***

23 Determination of Phenylalanine and Tyrosine by High Performance Liquid Chromatography-Tandem Mass Spectrometry . . . . . 219
***Judy Peat and Uttam Garg***

24 Urine Purine Metabolite Determination by UPLC-Tandem Mass Spectrometry . . . . . 227
***Qin Sun***

25 Urine Pyrimidine Metabolite Determination by HPLC Tandem Mass Spectrometry . . . . . 237
***Qin Sun***

26 Quantitation of Plasma Renin Activity in Plasma Using Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) . . . . . 243
***J. Grace Van Der Gugten and Daniel T. Holmes***

27 Quantitation of S-Adenosylmethionine and S-Adenosylhomocysteine in Plasma Using Liquid Chromatography Electrospray Tandem Mass Spectrometry . . . . . 255
***Erland Arning and Teodoro Bottiglieri***

28 A Simple, High-Throughput Method for Analysis of Ceramide, Glucosylceramide, and Ceramide Trihexoside in Dried Blood Spots by LC/MS/MS . . . . . 263
***Wei-Lien Chuang, Joshua Pacheco, and Kate Zhang***

29 Quantification of Dehydroepiandrosterone, 11-Deoxycortisol, 17-Hydroxyprogesterone, and Testosterone by Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS) . . . . . 273
***Ada Munar, Clint Frazee, and Uttam Garg***

30 Urinary Succinylacetone Analysis by Gas Chromatography-Mass Spectrometry (GC-MS) . . . . . 281
***Hongjie Chen and Chunli Yu***

31 Quantification of 1,25-Dihydroxyvitamin D2 and D3 in Serum Using Liquid Chromatography-Tandem Mass Spectrometry . . . . . 291
***Jonathon Mahlow, Dustin R. Bunch, and Sihe Wang***

32 High-Throughput Serum 25-Hydroxy Vitamin D Testing with Automated Sample Preparation . . . . . 301
***Judy Stone***

33 Quantitation of 25-OH-Vitamin-D2 and 25-OH-Vitamin-D3 in Urine Using LC-MS/MS . . . . . 321
***Dean C. Carlow, Ryan C. Schofield, and Michelle Denburg***

*Index* . . . . . *331*

# Contributors


Erland Arning • *Center of Metabolomics, Institute of Metabolic Disease, Baylor Research Institute, Dallas, TX, USA*

Paula Ashcraft • *Department of Mass Spectrometry, Center of Metabolomics, Baylor Research Institute, Dallas, TX, USA*

Jeanna Bennett-Firmin • *Department of Mass Spectrometry, Center of Metabolomics, Baylor Research Institute, Dallas, TX, USA*

Teodoro Bottiglieri • *Center of Metabolomics, Institute of Metabolic Disease, Baylor Research Institute, Dallas, TX, USA*

Dustin R. Bunch • *Department of Laboratory Medicine, Cleveland Clinic, Cleveland, OH, USA; Department of Chemistry, Cleveland State University, Cleveland, OH, USA*

Dean C. Carlow • *Department of Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA*

Hongjie Chen • *Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA*

Wei-Lien Chuang • *Genzyme Corporation, a Sanofi Company, Framingham, MA, USA*

Michelle Denburg • *The Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA*

Shuang Deng • *Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA*

Hernando Escobar • *ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories, Salt Lake City, UT, USA*

Elizabeth L. Frank • *ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories, Salt Lake City, UT, USA; Department of Pathology, University of Utah, Salt Lake City, UT, USA*

Clint Frazee • *Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA*

Jessica Gabler • *ThermoFisher Scientific, West Palm Beach, Fl, USA*

Uttam Garg • *Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA; University of Missouri School of Medicine, Kansas City, MO, USA*

J. Grace Van Der Gugten • *Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada; Department of Pathology and Laboratory Medicine, St. Paul's Hospital, Vancouver, BC, Canada*

Shannon Haymond • *Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA; Department of Pathology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA*

Bryce Heese • *Department of Pediatrics, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA*

Courtney Heideloff • *Department of Clinical Pathology, Cleveland Clinic, Cleveland, OH, USA*

Daniel T. Holmes • *Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada; Department of Pathology and Laboratory Medicine, St. Paul's Hospital, Vancouver, BC, Canada*
Hemamalini Ketha • *Departement of Pathology, University of Michigan Health System, Ann Arbor, MI, USA*
Mark M. Kushnir • *ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories, Salt Lake City, UT, USA*
Jonathon Mahlow • *Department of Laboratory Medicine, Cleveland Clinic, Cleveland, OH, USA*
Richard E. Mathieu Jr. • *Pathology Associates Medical Laboratories, Spokane, WA, USA*
A. Wayne Meikle • *ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, USA; Department of Pathology,University of Utah, Salt Lake City, UT, USA; Department of Medicine,University of Utah, Salt Lake City, UT, USA*
Alan Miller • *Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA*
Ada Munar • *Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA*
Douglas Myers • *Department of Pediatrics, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA*
Gordon J. Nelson • *ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, USA*
Joshua Pacheco • *Genzyme Corporation, a Sanofi Company, Framingham, MA, USA*
Drew Payto • *Department of Clinical Pathology, Cleveland Clinic, Cleveland, OH, USA*
Judy Peat • *Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA*
Julie A. Ray • *ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT, USA*
Catherine P. Riley • *National Reference Laboratory, R&D Section, Pathology Associates Medical Laboratories, Spokane, WA, USA*
Alan L. Rockwood • *ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories, Salt Lake City, UT, USA; Department of Pathology,University of Utah School of Medicine, Salt Lake City, UT, USA*
Ryan C. Schofield • *Department of Laboratory Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA*
David Scott • *Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, MO, USA*
Ravinder J. Singh • *Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA*
Judy Stone • *Toxicology/Mass Spectrometry Laboratory, Center for Advanced Laboratory Medicine, University of California San Diego, San Diego, CA, USA*
Qin Sun • *Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA*
Lawrence Sweetman • *Department of Mass Spectrometry, Center of Metabolomics, Baylor Research Institute, Dallas, TX, USA*
Gina Vespa • *Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA*
Faye B. Vicente • *Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA*
Sihe Wang • *Department of Laboratory Medicine, Cleveland Clinic, Cleveland, OH, USA*

Carmen Wiley • *Pathology Associates Medical Laboratories (PAML), Spokane, WA, USA*
Sophia Wong • *Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC, Canada; Department of Pathology and Laboratory Medicine, St. Paul's Hospital, Vancouver, BC, Canada*
Chunli Yu • *Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA*
Kate Zhang • *Genzyme Corporation, a Sanofi Company, Framingham, MA, USA*
Yan Victoria Zhang • *Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY, USA*

# Chapter 1

# Mass Spectrometry in Clinical Laboratory: Applications in Biomolecular Analysis

**Uttam Garg and Yan Victoria Zhang**

## Abstract

Mass spectrometry (MS) is a technique that can identify analytes on the basis of mass-to-charge ($m/z$) ratio. Although this technique has been used in research and specialized clinical laboratories for decades, however, in recent years, MS has been increasingly used in routine clinical laboratories. MS, especially when coupled to gas chromatography or liquid chromatography, provides very specific and often sensitive analysis of many analytes. Other advantages of MS include simultaneous analysis of multiple analytes (>100) and generally without need for specialized reagents. Commonly measured analytes by MS include drugs, hormones, and proteins.

**Key words** Clinical laboratory, Mass spectrometry, Liquid chromatography, Gas chromatography, Tandem mass spectrometry, Endocrinology, Newborn screening, Hormones and proteins

## 1 Introduction

Use of mass spectrometry in clinical laboratories is growing rapidly. Once considered too specialized and costly for routine use, mass spectrometry has made its way in many routine clinical laboratories [1, 2]. This has been made possible with the advent of bench-top, inexpensive, and user-friendly mass spectrometry systems. The major advantages of mass spectrometry include increased specificity, simultaneous analysis of multiple analytes, and generally no need for specialized reagents. Mass spectrometry is a preferred technology for the measurement of many laboratory analytes particularly drugs and hormones. Other well established clinical applications of mass spectrometry are in the field of biochemical genetics such as newborn screening and inherited metabolic disorders. In recent years, clinical applications in the areas of protein analysis and fast pathogen identification have emerged.

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_1, 

## 2 Fundamentals of Mass Spectrometry

It is beyond the scope of this chapter to include detailed description of mass spectrometry. Here, a brief description on the fundamentals of mass spectrometry is provided. Mass spectrometry is an analytical technique that is based on the principle that charged particles moving through an electric or magnetic field can be separated by their mass-to-charge ($m/z$) ratios. A typical mass spectrometric analysis can be divided into following steps:

- Sample preparation, which can vary from minimal preparation to elaborative liquid–liquid or solid phase extraction.
- Introduction and separation of analyte(s) of interest by chromatographic system, if needed.
- Introduction of sample into mass spectrometer. Although sample can be introduced directly into a mass spectrometer, it is generally achieved through gas or liquid chromatographic (GC or LC) systems.
- Ionization of analyte molecules. Ionization can be achieved through various techniques such as electron impact, chemical, electrospray, atmospheric-pressure chemical, and matrix-assisted laser desorption.
- Separation of ionized molecules. Separation of ions is achieved by mass analyzers. Most commonly used mass analyzer in clinical laboratories is quadrupole. In recent years, ion-trap and time-of-flight mass analyzers are also being used particularly for screening large number of analytes.
- Detection of ionized molecules.
- Data analysis.

Schematic diagram of a mass spectrometer with different options is shown in Fig. 1.

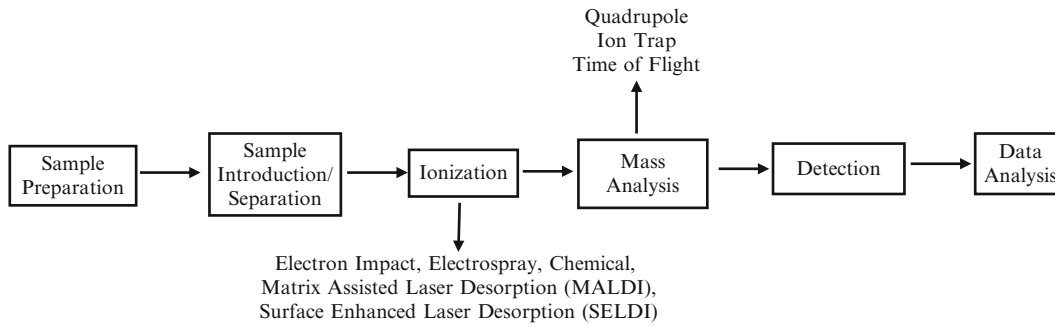



**Fig. 1** Schematic diagram of a mass spectrometry analysis

### 2.1 GC-MS

GC-MS has been used in clinical laboratories for several decades. A typical GC-MS uses helium gas, sample injection port, capillary column, mass spectrophotometer, vacuum system, detector, and a data analysis system. Typical analysis involves analyte extraction from a sample, concentration of the extract, sample derivatization if the compound of interest is not volatile or is heat-labile, and injection of sample in GC-MS. Once analyte ions are fragmented, either selected ions can be analyzed (selected ion monitoring) or all fragments can be analyzed (total ion monitoring) to produce a mass spectrum. Selected ion monitoring is more sensitive than total ion monitoring, and is generally used in quantitative analysis. Total ion monitoring is very useful in the identification of unknown compounds. It is like a fingerprint since a specific mass spectrum will be produced by the fragmentation of a specific analyte. GC-MS is suitable for analysis of small molecules that are volatile, nonpolar, and thermally stable.

### 2.2 LC-MS

In recent years LC-MS, particularly LC-MS/MS, has gained popularity and has become the method of choice, particularly for the analysis of hormones and proteins. Wide array of analytes can be measured by LC-MS/MS as compared to GC-MS. Analytes that are heat labile and difficult to derivative are more suited for LC-MS analysis. Furthermore, sample preparation is generally less involved as compared to GC-MS. Disadvantages of LC-MS/MS are less reproducible mass spectra, higher maintenance and cost as compared to GC-MS.

### 2.3 Time-of-Flight (TOF)-MS

In TOF-MS, an ion's mass-to-charge ratio is determined by calculating the time required by the ion to travel a fixed distance into a flight tube. Under a fixed electrical field, lighter ions travel faster than the heavier ions. The major advantages of TOF-MS analyzers are high mass resolution and exact mass measurements. For example, at $m/z$ of 100 and resolution of 2 ppm, mass error is less than $m/z$ 0.0002. This provides adequate information to assign initial molecular formulae to a compound for further identification and confirmation. High-resolution TOF-MS is being used in drug and metabolites screening and identification. Matrix-assisted laser desorption ionization (MALDI)-TOF is being increasingly used in the identification of proteins and bacteria.

## 3 Clinical Applications

MS is increasingly being used in clinical laboratory for the analysis of wide array of analytes. Most common applications of MS are in the fields of therapeutic drug monitoring, toxicology, endocrinology, and inborn error of metabolism. In recent years, emerging applications include pathogen identification, proteomics, and genomics.

### 3.1 Therapeutic Drug Monitoring and Toxicology

In clinical laboratory, immunoassays are the commonly used methods for therapeutic drug monitoring. Immunoassays are also commonly used for screening few drugs of abuse. Since immunoassays are not available for a number of drugs and they are non-specific, MS is used for the assay of these drugs and confirmation of immunoassay-positive results for drugs of abuse. Although GC-MS is still widely used in therapeutic drug monitoring and toxicology, LC-MS/MS is being increasingly used due to ease of sample preparation. Using GC-MS or LC-MS/MS, 100–1000s of drugs and toxins can be screened in a single analytical run.

### 3.2 Endocrinology

Although immunoassays remain mainstay for the determination of hormones, MS is increasingly used for the assay of hormones [3, 4]. Immunoassays have inherent degree of non-specificity and variability among different assays particularly for steroid hormones and catecholamines and their metabolites [5]. For example, steroid hormone immunoassays generally overestimate real concentrations, and there is a significant inter-laboratory variability [6, 7]. This makes difficult to follow-up a patient over long period of time due to assay changes within a laboratory or use of different immunoassays among different laboratories. Although both GC-MS and LC-MS/MS are used for the determination of hormone assay, the later is becoming a preferred technique due to ease of sample preparation. Table 1 lists commonly assayed hormones using mass spectrometry.

### 3.3 Inborn Error of Metabolism

Mass spectrometry has shown great promise in the screening and confirmation of inborn error of metabolism. LC-MS/MS is widely used in newborn screening to detect wide array of metabolic disorders including disorders of amino acids, organic acids, and fatty acids metabolism [8, 9]. GC-MS has been used for several decades and still remains the most commonly used technique for the analysis of urine organic acids. HPLC coupled with spectrophotometry had been the mainstay method for analysis of amino acids. This method is very time-consuming and is being replaced by LC-MS/MS. Other commonly measured analytes for the detection of metabolic disorders include acylcarnitines, bile acids, purines, and pyrimidines. Tables 2 and 3 list the disorders screened by tandem mass spectrometry in newborn screening, and other metabolic disorders and metabolites diagnosed/assayed by mass spectrometry.

### 3.4 Other Emerging Applications

Until recently, most of the clinical applications of MS have been in the analysis of small molecules. However, in recent years, applications of MS are expanding in the analysis of large molecules such as proteins, lipids, polysaccharides, and DNA [10–12]. For example, matrix-assisted laser desorption ionization (MALDI) mass spectrometry is now commonly used for rapid bacterial identification [13–15]. Protein profiling is showing great promise in the area of cancer detection [10, 11, 16].

**Table 1**
**Hormones assays by mass spectrometry**

| |
|---|
| • Aldosterone |
| • Androstenedione |
| • Catecholamines |
| • Coenzyme Q |
| • Cortisol |
| • Corticosterone |
| • Cortisone |
| • Deoxycortisol |
| • Dehydroepiandrosterone |
| • 1,25-Dihydroxyvitamin D |
| • Estrone |
| • Estradiol |
| • 17-Hydroxyprogesterone |
| • 17-Hydroxypregnenolone |
| • 25-Hydroxyvitamin D |
| • Insulin |
| • Pancreatic polypeptide |
| • Parathyroid hormone (PTH) |
| • Metanephrines |
| • Testosterone |

## 4 Introducing Mass Spectrometry in the Clinical Laboratory

Before bringing mass spectrometry into the laboratory, both patient care needs and financial considerations should be assessed. In addition, mass spectrometers need specific infrastructure to accommodate special needs such as consistent electric supply, high purity gases, and ventilation. A dedicated electricity supply and an uninterrupted power supply (UPS) are needed to protect the instrument. Other major challenge is acquiring staff that is already trained in mass spectrometry or training staff to implement this technology in clinical laboratories.

Implementation of mass spectrometry depends on specific needs. Sometimes primary driving force for adoption of mass spectrometry is substantial cost savings over other methods such as immunoassays. Other times, the driving force is to provide analytically superior results (e.g., steroid hormone analysis or confirmation

**Table 2**
**Metabolic disorders screened by tandem mass spectrometry through newborn screening**

| |
|---|
| *Organic acidurias*<br>• Isovaleric acidemia<br>• Glutaric aciduria 1 and 2<br>• 3-Hydroxy-3-methylglutaric aciduria<br>• Multiple carboxylase deficiency<br>• Methylmalonic acidemia due to mutase deficiency<br>• 3-Methylcrotonyl CoA carboxylase deficiency<br>• Methylmalonic acidemia due to cobalamin A and B defects<br>• Propionic acidemia<br>• 3-Ketothiolase |
| *Fatty acids oxidation defects*<br>• Medium chain acyl-CoA dehydrogenase<br>• Very long chain acyl-CoA dehydrogenase<br>• Long chain 3-hydroxy-acyl-CoA dehydrogenase<br>• Trifunctional protein deficiency<br>• Carnitine uptake defect<br>• Short chain acyl-CoA dehydrogenase |
| *Amino acids/urea cycle*<br>• Phenylketonuria<br>• Maple syrup urine disease<br>• Homocystinuria<br>• Citrullinemia<br>• Argininosuccinic aciduria<br>• Tyrosinemia |

of immunoassay-positive drugs of abuse results) or develop mass spectrometry methods due to lack of other methods. Given many choices of instrument availability and need for current and future tests selection, implementing mass spectrometry in a clinical laboratory could be challenging, and needs careful financial and human resources evaluation [17–24]. Major considerations in implementing mass spectrometry are listed in Table 4.

While modern mass spectroscopy companies provide high-quality products, consulting with colleagues and site visits can help narrow down the choices of vendors for further investigation. Analyzing small set of test samples on different platforms can provide better insight into the capabilities of different instruments. Before final selection, instrument service availability and response time should be considered seriously. It is not uncommon that method to be implemented in the laboratory is already published. Despite this, reproducing method and its validation can be a substantial investment.

Mass spectrometric assays are considered high-complexity assays under CLIA, and laboratory-developed tests (LDTs) by FDA. Laboratory is responsible for developing and evaluating performance characteristics of the assays. A number of CLSI guidelines

**Table 3**
**Other metabolic disorders/metabolites commonly diagnosed/assayed by mass spectrometry**

| |
|---|
| • Confirmation of disorders in Table 2 |
| • Many other disorders of organic, amino, and fatty acids not listed above under screening |
| • Acylcarnitines |
| • S-adenosylhomocysteine |
| • S-adenosylmethionine |
| • G-Aminobutyric acid |
| • Bile acids synthesis |
| • Cholesterol synthesis |
| • Disorders of glycosylation |
| • Glycosphingolipids |
| • Neurotransmitters synthesis |
| • Lysosomal disorders |
| • Pterins |
| • Purine and pyrimidine disorders |
| • Succinylacetone |

are available as a reference for mass spectrometry [25], and for evaluation of other components of method development such as limit of detection (LoD), limit of quantitation (LoQ), accuracy, precision, analytical measurement range, and reference intervals. Sample preparation and use of isotope-labeled compounds as internal standards are unique features to mass spectrometry assays. Isotope-labeled compounds behave very similar to the analytes and thus reduce the variability in sample extraction and analysis. A mass difference between the analyte of interest and the internal standard of at least 3 mass units is desirable, although a difference of at least 5 is preferred to eliminate cross-talk. Once the mass spectrometry is introduced, instruments, methods, and users need ongoing support and upgrades.

## 5 Conclusion

In conclusion, in recent years, mass spectrometry has emerged as an important tool in the clinical laboratory. Its current major applications are in therapeutic drug monitoring, metabolic screening, and endocrinology. Its applications are increasing in the areas of protein profiling, microorganism identification, and biomarkers discovery.

**Table 4**
**Major steps in implementing mass spectrometry in a clinical laboratory**

*Clinical needs*
- Primary consideration
- Reduce turn-around time
- Control over sample handling process

*Instrument selection*
- Based on intended analyses and economics
- Site visit and communication with colleagues and vendor
- Service availability and response time for service requests

*Assay selection*
- Based on type of instrumentation, analytes, and clinical needs
- Literature search and communication with colleagues
- Consider lab staff experience and training

*Financial justification*
- Key is to have an institutionally acceptable return on investment (ROI)
- Benefits include bringing test in-house and reduce send-out costs
- Primary investment is instrument itself and
- Other investment considerations should include
  - Service contract
  - Infrastructure renovation
  - Cost for interfacing to the LIS if desirable
  - Ongoing operating cost (e.g., high-grade reagents, gas)

*Infrastructure planning*
- Space for Instrumentation and HPLC
- Nitrogen gas dewars or nitrogen generator
- Ventilation and noise blocking
- Lab space re-arrangements (e.g., fixed vs. movable bench)
- Dedicated electric system and uninterrupted power supply
- IT support and data backup

*Staff and personnel training*
- Essential for a successful implementation
- Is an ongoing process
- Onsite training with manufacturers
- Online training courses
- Conferences workshops, symposia, and short courses

*Method development and validation*
- Meets CLIA requirements for high complex testing
- Use highest grade reagents available (MS grade or at least HPLC grade)
- Choose proper internal standards
- Validation shall include
  - Precision
  - Accuracy
  - Analytical sensitivity (LoQ)
  - Reportable range
  - Specificity and interference

## References


1. Hammett-Stabler CA, Garg U (2010) The evolution of mass spectrometry in the clinical laboratory. Methods Mol Biol 603:1–7
2. Strathmann FG, Hoofnagle AN (2011) Current and future applications of mass spectrometry to the clinical laboratory. Am J Clin Pathol 136:609–616
3. Pagotto U, Fanelli F, Pasquali R (2013) Insights into tandem mass spectrometry for the laboratory endocrinology. Rev Endocr Metab Disord 14:141
4. Vogeser M, Parhofer KG (2007) Liquid chromatography tandem-mass spectrometry (LC-MS/MS)--technique and applications in endocrinology. Exp Clin Endocrinol Diabetes 115:559–570
5. Soldin SJ, Soldin OP (2009) Steroid hormone analysis by tandem mass spectrometry. Clin Chem 55:1061–1066
6. Albrecht L, Styne D (2007) Laboratory testing of gonadal steroids in children. Pediatr Endocrinol Rev 5(Suppl 1):599–607
7. Handelsman DJ, Wartofsky L (2013) Requirement for mass spectrometry sex steroid assays in the Journal of Clinical Endocrinology and Metabolism. J Clin Endocrinol Metab 98: 3971–3973
8. Garg U, Dasouki M (2006) Expanded newborn screening of inherited metabolic disorders by tandem mass spectrometry: clinical and laboratory aspects. Clin Biochem 39:315–332
9. Jones PM, Bennett MJ (2002) The changing face of newborn screening: diagnosis of inborn errors of metabolism by tandem mass spectrometry. Clin Chim Acta 324:121–128
10. Jimenez CR Verheul HM (2014) Mass spectrometry-based proteomics: from cancer biology to protein biomarkers, drug targets, and clinical applications. Am Soc Clin Oncol Educ Book e504–10
11. Li Y, Song X, Zhao X, Zou L, Xu G (2014) Serum metabolic profiling study of lung cancer using ultra high performance liquid chromatography/quadrupole time-of-flight mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 966:147–153
12. Whiteaker JR (2010) The increasing role of mass spectrometry in quantitative clinical proteomics. Clin Chem 56:1373–1374
13. Ho YP, Reddy PM (2011) Advances in mass spectrometry for the identification of pathogens. Mass Spectrom Rev 30:1203–1224
14. Lagace-Wiens P (2015) Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS)-based identification of pathogens from positive blood culture bottles. Methods Mol Biol 1237:47–55
15. Luan J, Yuan J, Li X, Jin S, Yu L, Liao M, Zhang H, Xu C, He Q, Wen B et al (2009) Multiplex detection of 60 hepatitis B virus variants by maldi-tof mass spectrometry. Clin Chem 55:1503–1509
16. Kriegsmann J, Kriegsmann M, Casadonte R (2014) MALDI TOF imaging mass spectrometry in clinical pathology: a valuable tool for cancer diagnostics (Review). Int J Oncol 46: 893–906
17. Clarke W, Rhea JM, Molinaro R (2013) Challenges in implementing clinical liquid chromatography-tandem mass spectrometry methods--the light at the end of the tunnel. J Mass Spectrom 48:755–767
18. Roux A, Lison D, Junot C, Heilier JF (2011) Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: a review. Clin Biochem 44:119–135
19. Wu AH, French D (2013) Implementation of liquid chromatography/mass spectrometry into the clinical laboratory. Clin Chim Acta 420:4–10
20. Armbruster DA, Overcash DR, Reyes J (2014) Clinical chemistry laboratory automation in the 21st century - Amat Victoria curam (victory loves careful preparation). Clin Biochem Rev 35:143–153
21. Vogeser M, Kirchhoff F (2011) Progress in automation of LC-MS in laboratory medicine. Clin Biochem 44:4–13
22. Vogeser M, Seger C (2010) Pitfalls associated with the use of liquid chromatography-tandem mass spectrometry in the clinical laboratory. Clin Chem 56:1234–1244
23. Carvalho VM (2012) The coming of age of liquid chromatography coupled to tandem mass spectrometry in the endocrinology laboratory. J Chromatogr B Analyt Technol Biomed Life Sci 883–884:50–58
24. Himmelsbach M (2012) 10 years of MS instrumental developments--impact on LC-MS/MS in clinical chemistry. J Chromatogr B Analyt Technol Biomed Life Sci 883–884:3–17
25. CLSI (2014) Liquid chromatography-mass spectrometry methods; Approved guideline. Document C62-A, vol 62-A. CLSI, Wayne, PA

# Chapter 2

# Quantification of Free Carnitine and Acylcarnitines in Plasma or Serum Using HPLC/MS/MS

**David Scott, Bryce Heese, and Uttam Garg**

## Abstract

Acylcarnitines are formed by esterification between fatty acids CoA or organic acids CoA molecules and carnitine. In various fatty acids oxidation defects and organic acidurias, there is increased concentration of corresponding acylcarnitines. Abnormalities in specific acylcarnitines are used in the diagnosis of fatty acids oxidation defects and organic acidurias. Most commonly used method for the assay of acylcarnitines is HPLC-tandem mass spectrometry (HPLC/MS/MS). A HPLC/MS/MS method is described for the quantification of number of acylcarnitines. The method involves butylation of carnitine/acylcarnitines using acidified butanol, HPLC flow injection, and measurement of acylcarnitines using precursor ion scan and multiple reactions monitoring (MRM).

**Key words** Fatty acid oxidation defects, Organic acidemia, Organic acidurias, HPLC, Mass spectrometry, Medium chain acyl-CoA dehydrogenase deficiency, Inborn error of metabolism, Inherited metabolic disorders

## 1 Introduction

Inborn metabolic disorders, although individually rare, are collectively quite common. It is estimated that inborn metabolic disorders have frequency of 1:500. A number of organic acidurias and most fatty acids oxidation defects can be diagnosed through the analysis of plasma acylcarnitine profile [1–5]. Organic acidurias are a diverse group of disorders commonly characterized by episodes of acidosis, vomiting, lethargy, coma, seizures, hypotonia, hypertonia, and developmental delay [6]. There are more than 12 fatty acids oxidation defects. Common clinical features of fatty acids oxidation defects include hypoglycemia, liver disease, cardiomyopathy, and sudden unexpected death [2].

HPLC-tandem mass spectrometry (HPLC/MS/MS) is the method of choice for the measurement of carnitine and acylcarnitines, although other methods such as gas chromatography–mass spectrometry and high-performance liquid chromatography have

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_2, 

been described [1, 4, 7, 8]. HPLC/MS/MS method described here for the quantification of a number of acylcarnitines involves butylation of acylcarnitines using acidified butanol, HPLC flow injection, and measurement of acylcarnitines by precursor ion scan and multiple reactions monitoring (MRM). Deuterated internal standards are used for quantification of carnitine and acylcarnitines.

## 2 Materials

### 2.1 Samples

Collect 0.5 mL blood in red top (plain) or green top (heparin) tube. Centrifuge blood at 1200 × *g* for 7 min. Separate serum or plasma and refrigerate at 4 °C. Samples are stable for 2 months when refrigerated.

### 2.2 Solvents and Reagents

1. Formic Acid, 88 % ACS (Fisher).
2. UTAK blank human serum (UTAK Laboratories).
3. 3 N HCl in butanol (Regsil).
4. Mobile Phase (80:20 acetonitrile:water with 0.01 % formic acid): To a 1 L volumetric flask, add 800 mL acetonitrile, 114 μL 88 % formic acid, and then fill to the mark with deionized water. Stable for 1 year at room temperature.
5. Sample reconstitution solution (80:20 acetonitrile:water): To a 100 mL volumetric flask, add 80 mL acetonitrile and then fill to the mark with deionized water. Stable for 1 year at room temperature.

### 2.3 Internal Standards and Quality Controls

1. Internal standard mixture (NSK-B from Cambridge Isotope Laboratories): Dissolve in 200 mL of methanol. Concentrations of various acylcarnitines are listed in Table 1.
2. Quality controls:

**Table 1**
**Concentrations of internal standards**

| Compound | Concentration (nmol/mL) |
|---|---|
| D9-L-Carnitine (C0) | 0.760 |
| D3-Acetycarnitine (C2) | 0.190 |
| D3-Propionylcanitine (C3) | 0.038 |
| D3-Butyrylcarnitine (C4) | 0.038 |
| D9-Isovalerylcarnitine (C5) | 0.038 |
| D3-Octanoylcarnitine (C8) | 0.038 |
| D9-Tetradecanoylcarnitine (C14) | 0.038 |
| D3-Hexadecanoylcarnitine (C16) | 0.076 |

**Table 2**
**Preparation of stock carnitine/acylcarnitine solutions**

| Compound | M.W. | Amount in vial (μg) | Reconstitute vial with this amount of methanol (mL) | Resulting concentration (nmol/mL) |
|---|---|---|---|---|
| C0 | 198 | 5000 | 2.52 | 10,000 |
| C2 | 240 | 5000 | 2.1 | 10,000 |
| C3 | 254 | 5000 | 1.97 | 10,000 |
| C4 | 268 | 5000 | 1.86 | 10,000 |
| C5 | 282 | 5000 | 1.77 | 10,000 |
| C8 | 324 | 5000 | 1.54 | 10,000 |
| C14 | 408 | 5000 | 1.22 | 10,000 |
| C16 | 436 | 5000 | 1.15 | 10,000 |

**Table 3**
**Preparation of primary combo carnitine/acylcarnitine solution**

| Compound | Stock solution (μL) | Resulting concentration (nmol/mL) |
|---|---|---|
| C0 | 800 | 4000 |
| C2 | 400 | 2000 |
| C3 | 20 | 100 |
| C4 | 10 | 50 |
| C5 | 10 | 50 |
| C8 | 10 | 50 |
| C14 | 10 | 50 |
| C16 | 10 | 50 |

QS this combo carnitine/acylcarnitine solution to 2 mL using methanol (730 μL)

(a) Prepare stock of carnitine/acylcarnitine compounds (Cambridge Isotopes) according to Table 2. Stable for 2 years at −20 °C.

(b) Use stock carnitine/acylcarnitine compounds to prepare primary combo carnitine/acylcarnitine solution (Table 3). Stable for 2 years at −20 °C.

(c) Use primary combo carnitine/acylcarnitine solution to prepare working controls as shown in Table 4. Stable for 1 year at −20 °C.

**Table 4**
**Preparation of working quality controls**

| | QC1 | QC2 | QC3 | QC4 |
|---|---|---|---|---|
| UTAK serum (μL) | 200 | 995 | 980 | 950 |
| Primary combo (μL) | 0 | 5 | 20 | 50 |
| 0.9 % Saline (μL) | 800 | 0 | 0 | 0 |
| Resulting concentrations | Below normal limit | Approximately the upper limit of normal values | Approximately 4× upper limit of normal | Approximately >9× upper limit of normal |

### 2.4 Analytical Equipment and Supplies

1. MS/MS 4000Q TRAP (AB Sciex).
2. Prominence HPLC (Shimadzu).
3. Dry block at 60 °C.
4. Sample evaporator, Turbovap (Zymark).
5. Fume Hood.

## 3 Methods

### 3.1 Stepwise Procedure

1. To a 1.5 mL microcentrifuge tube, add 20 μL sample or control.
2. Add 400 μL of working internal standard.
3. Close cap, vortex, and allow to stand for 5 min.
4. Centrifuge for 5 min at 12,000 × *g* for 10 min.
5. Transfer 300 μL of supernatant into a 13 × 100 mm tube.
6. Evaporate to dryness under a stream of nitrogen at 40 °C (*see* **Note 1**).
7. To the resulting residue, add 100 μL of 3 N HCl in butanol.
8. Cap tubes and incubate in dry block for 20 min at 60 °C.
9. Evaporate to dryness under a stream of nitrogen at 40 °C (*see* **Notes 2** and **3**).
10. Reconstitute with 900 μL of 80 % acetonitrile/water.
11. Transfer sample to autosampler vial.
12. Inject 50 μL onto HPLC/MS/MS.

### 3.2 Instrument Operating Conditions

See Tables 5, 6, 7, 8, and 9 for HPLC and mass-spectrometer conditions.

### 3.3 Data Analysis

1. Chemoview software (AB Sciex) is used for data analysis (*see* **Notes 4** and **5**).
2. Quantification of the various acylcarnitine species is based on the ratio of the analyte's response (intensity) to the response

**Table 5**
**HPLC operating conditions**

| Time (min)[a] | Flow rate (mL/min) |
|---|---|
| 0.15 | 0.30 |
| 0.16 | 0.03 |
| 3.50 | 0.03 |
| 3.51 | 1.00 |
| 4.00 | 1.00 |
| 4.01 | 0.03 |
| 5.00 | 0.30 |
| 6.00 | Stop |

[a]Will vary for different instruments depending on factors such as length/diameter of tubing and dead volume

**Table 6**
**MS source parameters for both precursor ion scan and MRM**

| | |
|---|---|
| CUR | 25 |
| CAD | Medium |
| TEM | 400 |
| GS1 | 45 |
| GS2 | 45 |
| Ihe | ON |
| IS | 4000 |
| EP | 10 |
| CXP | 3 |

**Table 7**
**MS setting for MRM transitions**

| Compound | MRM transition | DP | CE | Internal standard | MRM transition | DP | CE |
|---|---|---|---|---|---|---|---|
| C0, Free carnitine | 218.2 → 159 | 60 | 20 | D9 C0 | 227.2 → 159 | 60 | 20 |
| C2, Acetylcarnitine | 260.2 → 141 | 51 | 21 | D3 C2 | 263.2 → 141 | 58 | 21 |
| C3, Propionylcarnitine | 274.2 → 141 | 61 | 23 | D3 C3 | 277.2 → 141 | 60 | 23 |
| C4, Isobutyryl/butyrylcarnitine | 288.2 → 141 | 60 | 24 | D3 C4 | 291.2 → 141 | 60 | 24 |

Dwell time for all the compounds was 50 ms

**Table 8**
**Transitions for acylcarnitines and internal standards**

| Compound | Precursor ion | Internal standard | Precursor ion |
|---|---|---|---|
| C5:1, Tiglylcarnitine | 300.2 → 85.1 | D9 C5 | 311.3 → 85.1 |
| C5, Isovaleryl/2-methylbutyryl/Pivaloyl | 302.2 → 85.1 | D9 C5 | 311.3 → 85.1 |
| C4-OH, 3-OH-butyrylcarnitine | 304.2 → 85.1 | D9 C5 | 311.3 → 85.1 |
| C6, Hexanoylcarnitine | 316.3 → 85.1 | D9 C5 | 311.3 → 85.1 |
| C5-OH, 3-OH-isovaleryl/2-methyl-3-OH-butyryl | 318.2 → 85.1 | D9 C5 | 311.3 → 85.1 |
| C6-OH, 3-OH-hexanoylcarnitine | 332.3 → 85.1 | D3 C8 | 347.3 → 85.1 |
| C8:1, Octenoylcarnitine | 342.3 → 85.1 | D3 C8 | 347.3 → 85.1 |
| C:8, Octanoylcarnitine | 344.3 → 85.1 | D3 C8 | 347.3 → 85.1 |
| C3-DC, Malonylcarnitine | 360.3 → 85.1 | D3 C8 | 347.3 → 85.1 |
| C10:1, Decenoylcarnitine | 370.3 → 85.1 | D3 C8 | 347.3 → 85.1 |
| C10, Decanoylcarnitine | 372.3 → 85.1 | D3 C8 | 347.3 → 85.1 |
| C4-DC, Methylmalonylcarnitine | 374.3 → 85.1 | D3 C8 | 347.3 → 85.1 |
| C5-DC, Glutarylcarnitine | 388.3 → 85.1 | D3 C8 | 347.3 → 85.1 |
| C12:1, Dodecenoylcarnitine | 398.3 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C12, Dodecanoylcarnitine | 400.3 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C6-DC, 3-methyl-glutarylcarnitine | 402.3 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C12-OH, 3-OH-dodecanoylcarnitine | 416.3 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C14:2, Tetradecadienoylcarnitine | 424.3 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C14:1, Tetradecenoylcarnitine | 426.4 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C14, Tetradecanoylcarnitine | 428.4 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C14:1-OH, 3-OH-tetradecenoylcarnitine | 442.4 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C14-OH, 3-OH-tetradecanoylcarnitine | 444.4 → 85.1 | D9 C14 | 437.4 → 85.1 |
| C16:1, Hexadecenoylcarnitine | 454.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C16, Hexadecanoylcarnitine | 456.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C16:1-OH, 3-OH-hexadecenoylcarnitine | 470.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C16-OH, 3-OH-hexadecanoylcarnitine | 472.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C18:2, Linoleylcarnitine | 480.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C18:1, Oleylcarnitine | 482.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C18, Stearoylcarnitine | 484.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C18:2-OH, 3-OH-linoleylcarnitine | 496.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C18:1-OH, 3-OH-oleylcarnitine | 498.4 → 85.1 | D3 C16 | 459.4 → 85.1 |
| C18-OH, 3-OH-stearoylcarnitine | 500.4 → 85.1 | D3 C16 | 459.4 → 85.1 |

**Table 9**
**MS setting for precursor ion scans**

| | |
|---|---|
| Polarity | Positive |
| Scan mode | Profile |
| Resolution Q1 | Unit |
| Resolution Q3 | Unit |
| Setting time | 5.0 ms |
| MR pause | 5.0 ms |
| MCA | No |
| Step size | 0.10 Da |
| Center/width | No |
| Scanning range | 210–550 |
| Scan time | 6 s |
| DP range | 45–65 |
| CE range | 30–55 |

(intensity) of an appropriate isotopically labeled internal standard using the following calculations:

$$\text{Unknown conc}(\text{nM}) = \frac{\text{Intensity Analyte} \times \text{IS conc}(\text{nM}) \times \text{Dilution Factor}(21)}{\text{Intensity IS}}$$

3. Intensities used for quantification of C0, C2, C3, and C4 are obtained through MRM mode (Table 7). Intensities for all other acylcarnitine quantifications are obtained through precursor ion mode (Tables 8 and 9).
4. While analyzing data, it is important to evaluate interferences (*see* **Notes 6–8**).
5. Typical total ion chromatogram is shown in Fig. 1.
6. Typical precursor ion spectrum is shown in Fig. 2.

## 4 Notes

1. Drying time is ~5 min. May vary with nitrogen flow rate and type of equipment.
2. Drying time is ~10 min. May vary with nitrogen flow rate and type of equipment.
3. Make sure that extract is completely dry.
4. External calibration is not used in this assay. Chemoview software is used only for quantification. Chromatographic review

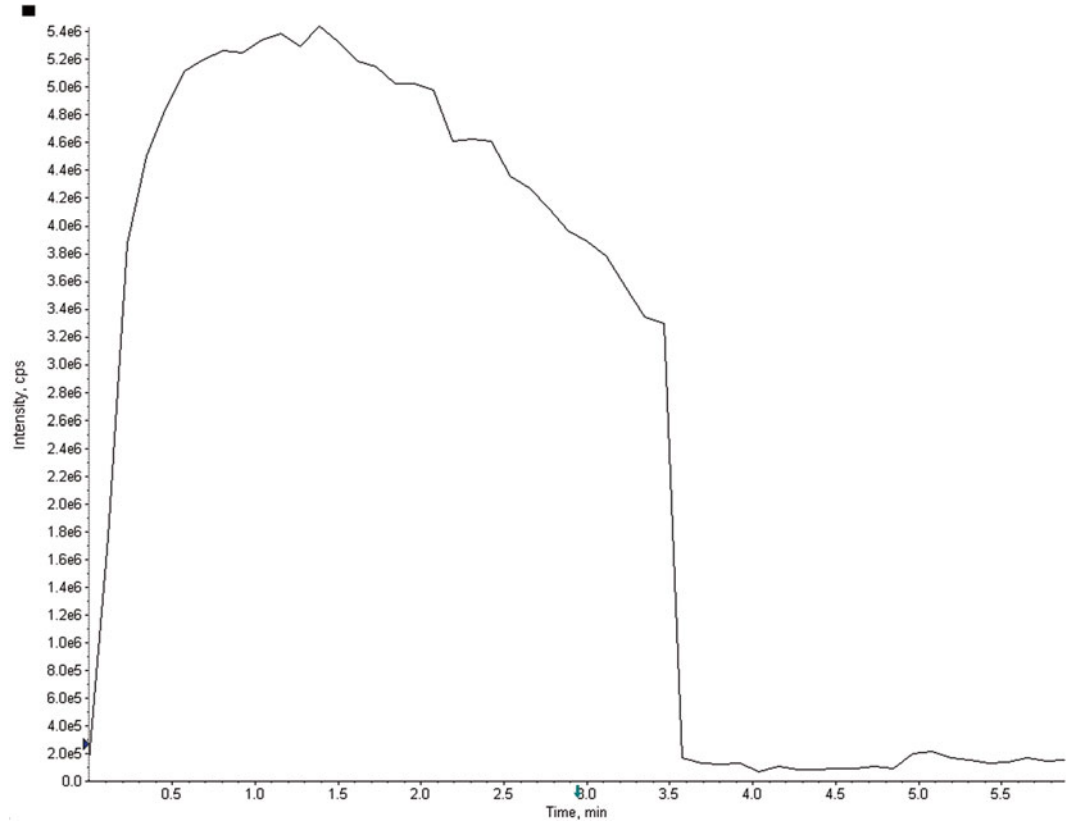



**Fig. 1** Total ion chromatogram for carnitine/acylcarnitines

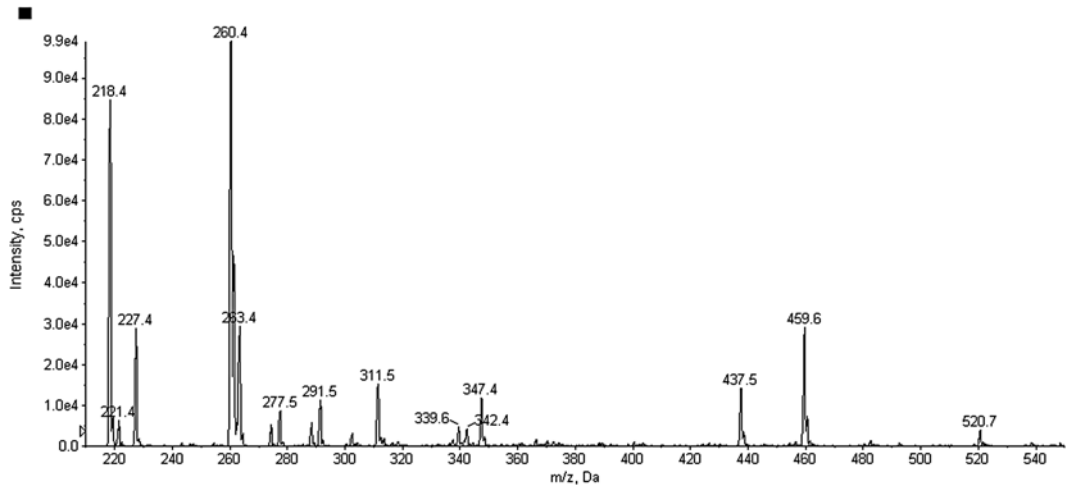



**Fig. 2** Typical precursor ion spectrum from a healthy individual

is performed on the Analyst Software (AB Sciex). Ion suppression is also monitored in Analyst Software (AB Sciex) by comparing highest intensity of the total ion chromatogram (TIC) to the lowest intensity of the TIC. The highest intensity should be at least ten times greater than the lowest intensity.

5. Carryover is monitored by running the internal standard preparation after the most concentrated control (QC4). This injection of the internal standard should have C0 and C2 values of less than 1.0 nmol/mL. All other acylcarnitines should be less than 0.1 nmol/mL.
6. In precursor ion scan, glutamate interferes with C2 quantification due to $260 \rightarrow 85$ transition. This interference is avoided by using C2 MRM of $260 \rightarrow 141$.
7. Cefotaxime interferes with C16:1-OH due to $470 \rightarrow 85$ transition.
8. Isotope of formiminoglutamate (FIGLU) with $m/z$ of 288 interferes with C4. This is avoided by using MRM $288 \rightarrow 141$ for C4.

## References


1. Naylor EW, Chace DH (1999) Automated tandem mass spectrometry for mass newborn screening for disorders in fatty acid, organic acid, and amino acid metabolism. J Child Neurol 14(Suppl 1):S4–S8
2. Rinaldo P, Cowan TM, Matern D (2008) Acylcarnitine profile analysis. Genet Med 10: 151–156
3. Schmidt-Sommerfeld E, Penn D, Duran M, Bennett MJ, Santer R, Stanley CA (1993) Detection of inborn errors of fatty acid oxidation from acylcarnitine analysis of plasma and blood spots with the radioisotopic exchange-high-performance liquid chromatographic method. J Pediatr 122:708–714
4. Smith EH, Matern D (2010) Acylcarnitine analysis by tandem mass spectrometry. Curr Protoc Hum Genet Chapter 17, Unit 17 8 1–20
5. Chace DH, Kalas TA, Naylor EW (2003) Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. Clin Chem 49:1797–1817
6. Seashore, MR (1993) The organic acidemias: an overview. GeneReviews® [Internet]. http://www.ncbi.nlm.nih.gov/books/NBK1134/
7. Kobayashi H, Hasegawa Y, Endo M, Purevsuren J, Yamaguchi S (2007) ESI-MS/MS study of acylcarnitine profiles in urine from patients with organic acidemias and fatty acid oxidation disorders. J Chromatogr B Analyt Technol Biomed Life Sci 855:80–87
8. Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, van Gennip AH (1999) Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidaemias and fatty acid oxidation defects. J Inherit Metab Dis 22:302–306

# Chapter 3

# Quantification of Arginine and Its Methylated Derivatives in Plasma by High-Performance Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

**Faye B. Vicente, Gina Vespa, Alan Miller, and Shannon Haymond**

## Abstract

Arginine is the substrate for nitric oxide synthases (NOS), thus the production of nitric oxide (NO) is based on arginine availability. Arginine is methylated through the activity of protein arginine methyltransferases (PRMT1 and PRMT2), to form asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA). These compounds have gained interest in recent years due to their influence on NO production rates and association with cardiovascular and renal diseases. The accurate and precise measurement of arginine and its methylated derivatives is needed for research studies investigating their role(s) in NO bioavailability and development of disease. We describe a high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for quantifying arginine, ADMA, and SDMA requiring only 50 μL of plasma. The sample preparation involves addition of internal standards (ADMA-$d_7$ for ADMA and SDMA, and $^{13}C_6$ -arginine for arginine) prior to protein precipitation with LCMS grade acetonitrile. Samples are centrifuged and supernatant is dried under nitrogen gas at 50 °C. Samples are reconstituted with mobile phase (ammonium acetate—formic acid—water). Arginine, ADMA, and SDMA are separated using an isocratic HPLC method on a 3 μM silica analytical column. MS/MS detection is performed in the multiple-reaction monitoring (MRM) mode and the transitions monitored are $m/z$ 203 to $m/z$ 70 for ADMA and SDMA, $m/z$ 210 to $m/z$ 77 for ADMA-$d_7$, $m/z$ 175 to $m/z$ 70 for arginine, and $m/z$ 181 to $m/z$ 74 for $^{13}C_6$-arginine.

**Key words** Arginine, Asymmetric dimethylarginine, Symmetric dimethylarginine, Mass spectrometry, Liquid chromatography, Plasma, Quantification

## 1 Introduction

Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are the major products of arginine methylation. N-monomethylarginine (NMMA) is an intermediate in this pathway so its concentration is significantly less than that of ADMA and SDMA in plasma. These compounds have gained recent interest due to their role in NO production and evidence that imbalanced NO synthesis leads to loss of vascular "protection", which results in endothelial dysfunction and oxidative stress [1]. Nitric oxide is

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_3, 

produced from L-arginine in a reaction catalyzed by three distinct isoforms of NO synthase (NOS). NMMA and ADMA directly inhibit NOS, whereas SDMA may limit NO production by competitively inhibiting the cellular uptake of arginine. Recent reports indicate increased concentrations of methylarginine compounds are associated with many pathological conditions, including cardiovascular disease, renal failure, pulmonary hypertension, septic shock, and preeclampsia [2, 3]. The actions of methylated arginines and their contribution to evolution of disease are poorly understood. Investigation into the metabolism of arginine and its methylated derivatives requires accurate and precise measurement of their concentrations in biological samples. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is well suited for this purpose, as it enables simultaneous quantitation of arginine and the methylarginines with high sensitivity, specificity, and precision.

This chapter describes a LC-MS/MS method for quantifying arginine, ADMA, and SDMA in 50 μL of plasma.

## 2 Materials

### 2.1 Samples

Plasma collected using K2-EDTA anticoagulant. Samples are stable for 1 month when frozen at −20 °C for up to two freeze/thaw cycles.

### 2.2 Solvents and Reagents

1. Clinical Laboratory Reagent Water (CLRW) obtained from Millipore Milli-Q Integral 5 Water Purification System.
2. Ammonium Acetate HPLC grade, 1 M, prepared with Special Reagent Water. Stable at 4 °C for 1 month.
3. Mobile Phase A and Purge Solvent (2 mM ammonium acetate/0.1 % (v/v) formic acid in CLRW): Add 2 mL of 1 M ammonium acetate solution and 1 mL formic acid to 1 L water. Stable at room temperature for 2 weeks.
4. Mobile Phase B (2 mM ammonium acetate/0.1 % (v/v) formic acid in methanol): Add 2 mL of 1 M ammonium acetate solution and add 1 mL formic acid to 1 L methanol. Stable at room temperature for 2 weeks.
5. Column Wash Solvent (50 % methanol in water): Mix 500 mL of water and 500 mL of methanol in a 1-L solution bottle. Stable at room temperature for 1 month.
6. Needle Wash Solvent (100 % methanol): Stable at room temperature for 1 month.
7. Charcoal dextran stripped human serum.
8. Phosphate Buffered Saline (PBS), 0.138 M NaCl, 0.0027 M KCl.

### 2.3 Standards and Calibrators

1. Primary standards: $N^G$,$N^G$-Dimethylarginine (ADMA) dihydrochloride ($C_8H_{18}N_4O_2 \cdot 2HCl$), $N^G$,$N^{G\prime}$-Dimethyl-L-arginine di(*p*-hydroxyazobenzene-*p*′-sulfonate) salt (SDMA) ($C_8H_{18}N_4O_2 \cdot 2C_{12}H_{10}N_2O_4S$), L-arginine ($C_6H_{14}N_4O_2$) (Sigma Aldrich Co.).
2. ADMA Calibrator Stock Solutions (23.3–727 μmol/L primary standard in CLRW):
   (a) Add 10 mg ADMA primary standard to 50-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA standard stock solution 1 is 727 μmol/L. Stable at −70 °C for 2 years (*see* **Note 1**).
   (b) Add 4 mL of the ADMA standard solution 1 to 25-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA standard stock solution 2 is 116 μmol/L. Stable at −70 °C for 2 years.
   (c) Add 2 mL of standard stock solution 2 to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA standard stock solution 3 is 23.3 μmol/L. Stable at −70 °C for 2 years.
3. SDMA Calibrator Stock Solutions (66.2–1380 μmol/L primary standard in CLRW):
   (a) Add 10 mg SDMA primary standard to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA standard stock solution 1 is 1380 μmol/L. Stable at −70 °C for 2 years (*see* **Note 1**).
   (b) Add 4 mL of the SDMA standard solution 1 to 25-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA standard stock solution 2 is 221 μmol/L. Stable at −70 °C for 2 years.
   (c) Add 3 mL of standard stock solution 2 to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA standard stock solution 3 is 66.2 μmol/L. Stable at −70 °C for 2 years.
4. Arginine Calibrator Stock Solution (5473 μmol/L primary standard in CLRW): Add 25 mg arginine primary standard to 25-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at −70 °C for 2 years (*see* **Note 1**).
5. Calibrators (0–2.33 μmol/L ADMA, 0–4.42 μmol/L SDMA, 0–230 μmol/L Arginine in CLRW): Prepare Calibrators 1–5 by diluting the standard stock solution(s) according to Table 1. Working calibrators 1, 2, 3, and 5 are made according to Table 1 using 50-mL volumetric flask(s). Working calibrator 4 is made according to Table 1 using a 100-mL volumetric flask. Working calibrator 5 is CLRW. The calibrators are stable for 2 years when stored at −70 °C (*see* **Note 2**).

**Table 1**
**Preparation of calibrators**

| Calibrator | Volume of standard stock solution (mL) | Volume of special reagent water (mL) | Final concentration (μmol/L) |
|---|---|---|---|
| 1 | 1.0 (ADMA stock solution 2)<br>1.0 (SDMA stock solution 2)<br>2.0 (Arginine stock solution) | 46.0 | 2.33 (ADMA)<br>4.42 (SDMA)<br>230 (Arginine) |
| 2 | 0.5 (ADMA stock solution 2)<br>0.5 (SDMA stock solution 2)<br>1.0 (Arginine stock solution) | 48.0 | 1.16 (ADMA)<br>2.21 (SDMA)<br>115 (Arginine) |
| 3 | 1.0 (ADMA stock solution 3)<br>1.0 (SDMA stock solution 3)<br>0.5 (Arginine stock solution) | 47.5 | 0.47 (ADMA)<br>1.32 (SDMA)<br>57.4 (Arginine) |
| 4 | 0.5 (ADMA stock solution 3)<br>0.5 (SDMA stock solution 3)<br>0.5 (Arginine stock solution) | 98.5 | 0.12 (ADMA)<br>0.33 (SDMA)<br>28.7 (Arginine) |
| 5 | 0 | 50 | 0 (ADMA)<br>0 (SDMA)<br>0 (Arginine) |

6. HPLC/MS Check Stock Solution (34.9 μmol/L ADMA, 11.0 μmol/L SDMA, 2872 μmol/L Arginine in CLRW): Add 3 mL of ADMA standard stock solution 2, 0.5 mL of SDMA standard stock solution 2, and 5 mL of arginine standard stock solution to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at −70 °C for 2 years.
7. HPLC/MS Check Standard (0.55 μmol/L ADMA, 0.11 μmol/L SDMA, 28.7 μmol/L Arginine in CLRW): Add 0.5 mL of HPLC/MS Check stock solution to 50-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at −70 °C for 2 years.

### *2.4 Internal Standard and Quality Controls*

1. Primary internal standards (I.S.): asymmetric dimethylarginine hydrochloride (ADMA-$d_7$) ($C_8H_{12}ClD_7N_4O_2 \cdot H_2O$), $^{13}C_6$-L-arginine hydrochloride ($^{13}C_6H_{15}ClN_4O_2$) (Cambridge Isotope Laboratories).
2. Quality Control Stock solutions: Primary standards are separately weighed or from different lots than those used to prepare calibrator stock solutions.
3. ADMA-$d_7$ I.S. Stock Solution (379 μmol/L primary I.S. in CLRW): Add 5 mg ADMA-$d_7$ primary I.S. to 50-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at −70 °C for 2 years (*see* **Note 1**).
4. $^{13}C_6$-Arginine I.S. Stock Solution (923 μmol/L primary I.S. in CLRW): Add 10 mg $^{13}C_6$-arginine primary I.S. to 50-mL

volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at –70 °C for 2 years (*see* **Note 1**).

5. I.S. Working Solution (1.90 μmol/L ADMA-$d_7$, 36.9 μmol/L $^{13}C_6$-Arginine in CLRW): Add 0.5 mL of ADMA-$d_7$ I.S. stock solution and 4 mL of $^{13}C_6$-Arginine I.S. stock solution to 100-mL volumetric flask, bring to volume with CLRW and mix well by inversion. Stable at –70 °C for 2 years (*see* **Note 3**).
6. ADMA Quality Control Stock Solutions (7.27–3635 μmol/L primary standard in CLRW):
   (a) Add 10 mg ADMA primary standard to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA quality control stock solution 1 is 3635 μmol/L. Stable at –70 °C for 2 years (*see* **Note 1**).
   (b) Add 5 mL of previous ADMA quality control stock solution 1 to 50-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA quality control stock solution 2 is 364 μmol/L. Stable at –70 °C for 2 years.
   (c) Add 2 mL of ADMA quality control stock solution 2 to 100-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This ADMA quality control stock solution 3 is 7.27 μmol/L. Stable at –70 °C for 2 years.
7. SDMA Quality Control Stock Solutions (2.76–138 μmol/L primary standard in CLRW):
   (a) Add 10 mg SDMA primary standard to 100-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA quality control stock solution 1 is 138 μmol/L. Stable at –70 °C for 2 years (*see* **Note 1**).
   (b) Add 2 mL of previous SDMA quality control stock solution 1 to 100-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This SDMA quality control stock solution 2 is 2.27 μmol/L. Stable at –70 °C for 2 years.
8. Arginine Quality Control Stock Solutions (2297–4594 μmol/L primary standard in CLRW).
   (a) Add 20 mg of arginine primary standard to 25 mL volumetric flask, bring to volume with CLRW and mix well by inversion. This arginine quality control stock solution 1 is 4594 μmol/L. Stable at –70 °C for 2 years (*see* **Note 1**).
   (b) Add 5 mL of the previous arginine quality control stock solution 1 to 10-mL volumetric flask, bring to volume with CLRW and mix well by inversion. This arginine quality control stock solution 2 is 2297 μg/mL. Stable at –70 °C for 2 years.

**Table 2**
**Preparation of quality controls**

| Quality control | Volume of control stock solution (mL) | Volume of charcoal dextran stripped serum (mL) | Final concentration (μmol/L) |
|---|---|---|---|
| Low | 1.0 (ADMA stock solution 3)<br>3.0 (SDMA stock solution 2)<br>0.5 (Arginine stock solution 2) | 20.5 | 0.29 (ADMA)<br>0.33 (SDMA)<br>66.4 (Arginine) |
| High | 2.0 (ADMA stock solution 3)<br>5.0 (SDMA stock solution 2)<br>2.0 (Arginine stock solution 2) | 16.0 | 0.58 (ADMA)<br>0.55 (SDMA)<br>200 (Arginine) |

9. Quality Controls (0.29–0.58 μmol/L ADMA, 0.33–0.55 μmol/L SDMA, 45.9–184 μmol/L, Arginine in charcoal stripped serum).
   (a) The levels of ADMA, SDMA, and arginine in the serum or plasma are predetermined by using this protocol or by an external laboratory using LC-MS/MS technology and are added to the final concentrations of the quality controls.
   (b) Prepare low and high controls by diluting quality control stock solutions as shown in Table 2. For each dilution step: Add appropriate amount of quality control stock solution(s) for each ADMA, SDMA, and arginine as shown in Table 2 to 25-mL volumetric flask(s) and bring to volume with serum (or plasma). Mix well by inversion after each dilution step. Stable at −70 °C for 2 years (*see* **Notes 2** and **4**).

### 2.5 Supplies and Equipment

1. Analytical Column: Phenomenex Luna Silica, 3 μm 100 × 4.6 mm I.D.
2. Guard Column: Phenomenex Luna Silica, 4 × 3.0 mm I.D.
3. Waters 2795 Alliance HT Separation Module with Micromass Quattro Micro API equipped with MassLynx.
4. Thermo Scientific Reacti-Therm III Heating/Stirring Module.

## 3 Methods

### 3.1 Stepwise Procedure

1. Ensure the instrument is properly tuned and verify system performance (*see* **Notes 5** and **6**).
2. Pipette 50 μL of sample (calibrators, quality controls, PBS blank, and patient plasma) to labeled 1.5-mL microcentrifuge tubes.
3. Add 50 μL of the internal standard solution.
4. Cap and vortex mix tubes briefly and let stand for 10 min at room temperature.
5. Add 500 μL of acetonitrile to each tube, cap and vortex mix for 7–10 s.

6. Let the samples stand for 15 min at room temperature.
7. Centrifuge at 13,440 rcf for 5 min at room temperature.
8. Transfer supernatant into labeled 13 × 100 mm glass culture tubes.
9. Using the Thermo Scientific Reacti-Therm III Heating/Stirring Module, dry samples gently under nitrogen gas at 50 °C until completely dry (*see* **Note 7**).
10. Reconstitute the supernatant by adding 1.0 mL mobile phase A.
11. Cap the tubes and vortex mix thoroughly for 7–10 s.
12. Centrifuge at 1430–1500 × *g* for 5 min.
13. Transfer solution to appropriately labeled autosampler vials.
14. Inject 10 μL of sample onto LC-MS/MS.

### 3.2 Analysis

1. Instrumental operating parameters are given in Table 3.
2. Analyze the data using the QuanLynx software (Waters Corporation).

**Table 3**
**LC-MS/MS operating conditions**

| | | | |
|---|---|---|---|
| A. HPLC[a] | | | |
| Column temperature | | 10 °C ±5 | |
| Flow rate | | 0.375 mL/min | |
| Isocratic program | | Time (min) | |
| | | 0.00–9.00 | |
| | | 95 % Mobile Phase A | |
| B. MS/MS tune settings[b] | | | |
| Capillary voltage (kV) | | 0.25 | |
| Source temperature (°C) | | 130 | |
| Desolvation temperature (°C) | | 450 | |
| Cone gas (L/h) | | 30 | |
| Desolvation gas (L/h) | | 650 | |
| Collision gas pressure (mbar) | | 4.17 e–3 | |
| LM1 resolution | | 13.5 | |
| HM1 resolution | | 13.5 | |
| Ion energy 1 | | 0.1 | |
| MS/MS entrance | | –2 | |
| MS/MS exit | | 2 | |
| LM2 resolution | | 11.3 | |
| HM2 resolution | | 11.3 | |
| Ion energy 2 | | 2.4 | |
| C. MRM method settings[b] | | | |
| Cone (V) | Collision (eV) | Inter-channel delay (s) | Inter-scan delay (s) |
| 27 | 23 | 0.03 | 0.03 |

[a]The total run time is 9.0 min. Solvent flow was diverted from the source to waste at 0–2 min and at 8.5–9.0 min
[b]Tune and MRM settings may vary slightly between instruments

**Table 4**
**Precursor and product ions for ADMA, SDMA, ADMA-$d_7$, arginine, and $^{13}C_6$-arginine**

| Analyte | Precursor ion $(M+H)^+$ | Product ion |
|---|---|---|
| ADMA | 203.10 | 69.95 |
| SDMA | 203.10 | 69.95 |
| ADMA-$d_7$ | 210.15 | 76.95 |
| Arginine | 175.10 | 69.90 |
| $^{13}C_6$-Arginine | 181.10 | 73.95 |

3. With each analytical run, a 5-point standard calibration curve is created by linear regression of the analyte/I.S. peak area ratio with the origin included using the quantifying ions indicated in Table 4. The concentrations of the controls and unknown samples are determined from the curve.
4. The expected retention times for ADMA, SDMA, and arginine are 6.77 min (acceptable range: 6.43–7.11 min), 6.04 min (acceptable range: 5.74–6.32 min), and 4.27 min (acceptable range: 4.20–4.45 min), respectively. The expected retention times for ADMA-$d_7$ and $^{13}C_6$-Arginine are 6.77 min (acceptable range: 6.43–7.11 min) and 4.26 min (acceptable range: 4.20–4.45 min), respectively. Representative ion chromatograms for ADMA, SDMA, arginine, and I.S. are shown in Fig. 1.
5. Verify the performance during the analytical run by monitoring the internal standard peak area. An acceptable limit should be defined during method development or validation. We determined 1500 to be the minimum acceptable IS peak area in our method. Re-inject the sample if the internal standard peak area is below the acceptance limit of 1500. If after re-injection, the internal standard peak area is still below 1500, determine the signal-to-noise ratio of the analyte peak. Signal-to-noise ratio greater than 10 is acceptable for reporting.
6. Evaluate for carryover effects in the PBS blank injected after Calibrator 1. Carryover is significant when ADMA, SDMA, and arginine concentrations in the PBS blank is greater than the limit of detection levels 0.01, 0.03, and 1.15 μmol/L, respectively, and in the low quality control is greater than the two standard deviations of the target value and/or assigned mean. If carryover is significant, troubleshoot and perform corrective action. Repeat the evaluation to demonstrate that carryover is no longer detected.
7. Run is acceptable if the calculated concentrations in the control samples are within two standard deviations of the target values and/or assigned means.

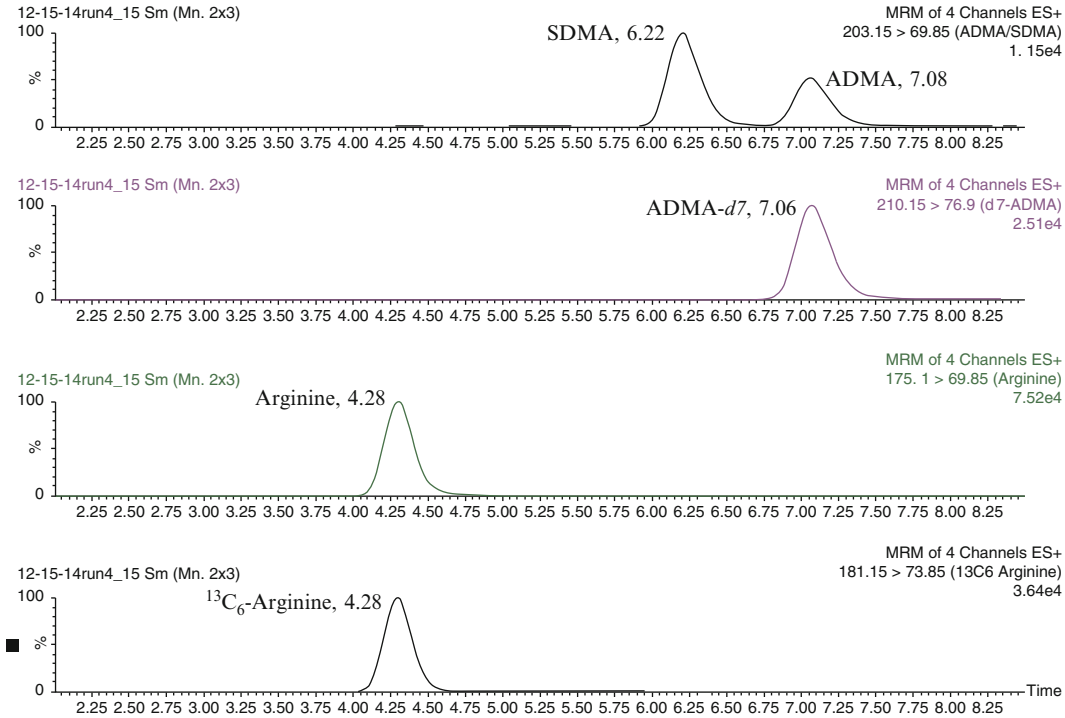



**Fig. 1** Representative LC-MS/MS ion chromatograms of SDMA (0.71 μmol/L), ADMA (0.69 μmol/L), ADMA-$d_7$ (1.9 μmol/L), arginine (62.5 μmol/L), and $^{13}C_6$-arginine (36.1 μmol/L) in a plasma sample

8. The method is linear from 0.10 to 2.15 μmol/L for ADMA, 0.10–6.00 μmol/L for SDMA, and 5.8–230.0 μmol/L for arginine. The low limit of quantitation of ADMA, SDMA, and arginine is 0.10, 0.10, and 5.8 μmol/L, respectively. The intra- and inter-day precision is <5 %.
9. No significant ion suppression was found in charcoal-stripped serum (used for preparing quality controls) and plasma pools.

## 4 Notes

1. When preparing standard and I.S. stock solution(s), completely dissolve the solid material in small amount of CLRW in volumetric flask(s) before bringing to volume. Briefly sonicate to accelerate the dissolution process.
2. Calibrators and quality controls are pre-aliquoted and stored in −70 °C until use. Pipette 125 μL of the calibrator and quality controls solutions into 1.2-mL cryogenic vials. Opened calibrator vials are for one time use only. Opened quality control vials are stable for 7 days at −20 °C.
3. Working I.S. solution is pre-aliquoted and stored in −70 °C until use. Pipette 2 mL of the solution into 2.0-mL cryogenic vials.

Opened vials are for one time use only. When more than one vial of the solution is required to prepare a batch of samples, combine and mix well before use.

4. Charcoal dextran stripped serum is preferred for preparing quality controls because it contains minimal level of endogenous ADMA, SDMA, and arginine. However, pooled or single-donor serum and/or EDTA plasma obtained from healthy volunteers may also be used. The serum or plasma can be diluted with PBS to reduce the concentration of endogenous ADMA, SDMA, and arginine. The percentage of PBS compared to serum or plasma should not exceed 50 %.
5. System check: To verify system performance before running patient samples, inject the HPLC/MS check standard solution after a water blank. Verify that the analytes retention times are within their respective acceptable limits and that the signal-to-noise (peak-to-peak) of the ADMA and arginine peaks is greater than 100 and greater than 10 for the SDMA peak. The HPLC/MS check standard solution is pre-aliquoted and stored in −70 °C until use. Opened vials are for one time use only. New columns are prepared by flushing with 10 mL of 100 % isopropanol alcohol at 0.400 mL/min followed by 50 mL of 100 % methanol at 0.400 mL/min.
6. Tuning the mass spectrometer: To adjust the mass spectrometer parameters for optimum sensitivity and stability of ions measured, tuning solutions of ADMA, SDMA, ADMA-$d_7$, arginine, and $^{13}C_6$-arginine (12, 40, 10, 4, and 4 μg/mL in CLRW, respectively) are infused into the ion source at 10 μL/min while solvent from the HPLC consisting of 95 % Mobile Phase A and 5 % Mobile Phase B is introduced via a peak "tee" connector at 0.375 mL/min. After analytical runs are completed, the column is flushed for 60 min at a flow rate of 0.200 mL/min and stored with 70 % methanol in water.
7. Apply low nitrogen gas flow during the drying step. Analyte may be lost at higher gas flow rates.

## References

1. Förstermann U, Münzel T (2006) Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 113(13):1708–1714
2. Brinkmann SJ, de Boer MC, Buijs N, van Leeuwen PA (2014) Asymmetric dimethylarginine and critical illness. Curr Opin Clin Nutr Metab Care 17(1):90–97
3. Schepers E, Speer T, Bode-Böger SM, Fliser D, Kielstein JT (2014) Dimethylarginines ADMA and SDMA: the real water-soluble small toxins? Semin Nephrol 34(2):97–105

# Chapter 4

# Quantitation of Albumin in Urine by Liquid Chromatography Tandem Mass Spectrometry

**Hemamalini Ketha and Ravinder J. Singh**

## Abstract

Urinary excretion of human serum albumin (HSA), a 6.65 kDa monomeric protein, is a sensitive marker of renal damage associated with many diseases including diabetes mellitus. Albumin is synthesized by the liver and functions as a transport protein for fat-soluble hormones and drugs and for maintaining plasma colloid osmotic pressure and pH. Albumin is not filtered at the glomerulus and its presence in the urine at concentration above 30 mg/day is suggestive of glomerular damage. Early diagnosis of microalbuminuria (30–300 mg/24 h urine albumin excretion or 30–300 mg/g creatinine in random collections) has prognostic value for monitoring disease progression and early clinical management of diabetic nephropathy in prediabetic patients. Current methods for quantitation of urine albumin are based on immunoassays or size exclusion high-performance liquid chromatography coupled with UV detection (SEC-HPLC-UV). Studies have demonstrated discordance between the existing methods. It has been suggested that while immunoassays underestimate albumin in urine, SEC-HPLC-UV method overestimates albumin as it cannot separate co-eluting interferences. This chapter describes a liquid chromatography tandem mass spectrometry LC-MS/MS candidate reference method for albumin quantitation.

**Key words** LC-MS/MS, Albumin, Microalbuminuria, Microalbumin

## 1 Introduction

Albumin, a 6.65 kDa monomeric protein, is the most abundant plasma protein in humans. Human liver synthesizes approximately 15 g (~200 mg/kg) albumin per day. Primary functions of albumin include maintenance of plasma oncotic pressure and blood pH, transport of fat soluble hormones, unconjugated bilirubin and drugs [1]. Albumin synthesis is regulated by nutritional status, colloid osmotic pressure, cytokines, and hormones. Colloid osmotic pressure is a form of osmotic pressure exerted by proteins, primarily albumin, which is responsible for about 80 % of the total colloid osmotic pressure exerted by blood plasma on the interstitial fluid. Physiological states that cause a reduction in plasma albumin including proteinuria and malnutrition lead to reduction of plasma oncotic pressure and increased capillary filtration and result in edema.

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_4, 

Presence of protein in the urine (proteinuria) is a marker of degree of renal damage in kidney disease [2, 3]. Proteinuria has been classified as glomerular, tubular, overflow, or post-renal based on the site of the filtration defect. In adults with normal kidney function, albumin is not filtered via glomerular filtration. Therefore, albuminuria is also termed as glomerular proteinuria and quantitation of urine albumin is clinically useful for assessment of glomerular permeability. The presence of 30–300 mg/24 h urine albumin excretion or 30–300 mg/g creatinine in random collections is termed as microalbuminuria and greater than 300 mg in a 24 h urine collection is termed as albuminuria. It is recommended to confirm the presence of microalbuminuria by repeating the 24 h urine measurement over a 2- to 3-month period. Microalbuminuria is an important prognostic marker of diabetic nephropathy and is an adverse predictor of glycemic control in prediabetic patients. Untreated diabetic nephropathy can lead to renal failure in 5–7 years. On the other hand, timely diagnosis and treatment of microalbuminuria can help slow the progression of diabetic nephropathy [3].

FDA-cleared immunoassays and size-exclusion high-performance liquid chromatography coupled with UV detection (SEC-HPLC-UV)-based assay (Accumin™, AusAm Biotechnologies) are used for albumin measurements [4, 5]. A study by Sviridov et al. suggested that SEC-HPLC-UV cannot separate transferrin, α1 acid glycoprotein, and α1 antitrypsin from albumin leading to overestimation compared to the immunoassay [6]. Due to discordance between the two methods, liquid chromatography tandem mass spectrometry (LC-MS/MS)-based methods have been developed [7, 8]. This chapter describes a candidate reference LC-MS/MS method for human albumin in urine.

## 2 Materials

### 2.1 Samples

Urine is an acceptable sample type for this method. Samples have to be maintained frozen prior to analysis and up to three freeze–thaw cycles are acceptable.

### 2.2 Reagents and Buffers

1. Phosphate Buffered Saline (PBS) (Roche). Store at room temperature. Expiration: until date printed on box. Dilute appropriately with water (stable at room temperature for 3 months).
2. Mobile Phase A (water, 0.1 % formic acid): Stable at room temperature for 1 month.
3. Mobile Phase B (acetonitrile, 0.1 % formic acid): Stable at room temperature for 1 month.
4. Clinical Laboratory Reagent Water (CLRW).

**Table 1**
**Dilution scheme for preparing HSA calibrator samples**

| Calibrator concentration mg/L | Intermediate 2 g/L Std (mL) | PBS (mL) |
|---|---|---|
| 0 | 0 | 250 |
| 12 | 1.5 | 248.5 |
| 24 | 3 | 247 |
| 48 | 6 | 244 |
| 96 | 12 | 238 |
| 210 | 26.25 | 223.75 |
| 420 | 52.5 | 197.5 |

5. Human serum albumin (HSA) (Sigma-Aldrich) for preparation of calibrators.
6. Bovine serum albumin (BSA) (Sigma-Aldrich) for preparation of internal standard.

### 2.3 Calibrators, Internal Standard, and Quality Control Samples

1. Dissolve 2 g HSA in 1 L PBS, Label as Intermediate 2 g/L Std. Store at −80 °C for 2 years. A dilution scheme for preparation of calibrators has been shown in Table 1.
2. Dissolve 1 g BSA in 1 L CLRW water to make a working internal standard solution 1 g/L. Store at −80 °C for 2 years (*see* **Note 1**).
3. Three levels of quality control samples (Low: 30–50 mg/L; Medium: 90–120 mg/L; High: 350–380 mg/L are prepared by pooling patient samples at appropriate concentrations). Samples can be aliquoted and stored at −80 °C for 2 years.

### 2.4 Supplies and Equipment

1. Transfer pipettes, vortex, and microtiter plate shaker.
2. Incubator shaker that can shake at 250 rpm.
3. Analytical column: 2.0 cm × 2.1 mm, 5 μm Supelco Discovery® BIO wide-pore C-8 column (Chromtech).
4. Thermo-Cohesive HPLC system (Thermo Scientific).
5. Applied Biosystems API 5000 triple quadrupole mass spectrometer.

## 3 Method

### 3.1 Sample Preparation

1. Thaw the patient samples, calibrators, controls, blanks, and IS until they reach room temperature.
2. Pipette 0.1 mL of each of samples, calibrators, controls, and blanks into individual well of the 96-well plate.

3. Add 20 μL IS (BSA) into each well. Cover the plate with an adhesive plate seal and incubate at ambient temperature on the plate shaker for 15 min with constant shaking.
4. The prepared samples can be stored in a refrigerator until analyzed.

### 3.2 HPLC Conditions

1. Mobile phase A: water, 0.1 % formic acid; mobile phase B: acetonitrile, 0.1 % formic acid (*see* **Note 2**).
2. Flow rate: 400 μL/min.
3. The injected extract is separated on a wide pore C8, size 2 cm × 2.1 mm, 5 μm analytical column. During the first 5 min, the organic mobile phase content is increased from 5 to 30 % with a linear gradient, and the flow diverted to waste with the help of a multiplexing HPLC system (Cohesive Technologies Inc.). During the next 4 min, albumin is eluted by an increasing gradient of the organic phase from 30 to 95 %, and redirecting sample flow to the mass spectrometer in the relevant acquisition LC time frame (ABI Sciex API 5000 LC-MS/MS system).

### 3.3 Mass Spectrometer Conditions (See Notes 3–5)

The *m/z* of albumin and the respective IS for quantitative analysis have been shown in Table 2. Representative fragmentation pattern and total ion chromatograms have been shown in Fig. 1.

### 3.4 Data Analysis and Quantitation

1. Data are quantitated using the Analyst™ Software (version 1.4.1 or higher) (Applied Biosystems).
2. Calibration curves are established for every day of analytical run based on linear fit regression of IS peak area/IS concentration (*x*-axis) vs. analyte concentration/IS peak area (*y*-axis). Peak area ratios are then used to calculate the analyte concentration by the software.
3. Area of Q1/Q3 pair 685.1/913.2 is used for quantitation and 698.5/930.9 is used as the IS.
4. Calibration curve is acceptable if correlation coefficient is ≥0.99. If one of the calibrators is out of range by greater

**Table 2**
**MRMs for human albumin (analyte) and bovine serum albumin (IS)**

| Ion | Q1 Mass (amu) | Q3 Mass (amu) | Time (ms) |
|---|---|---|---|
| Human albumin, analyte | 685.1 ($b_{24}^{4+}$)[a] | 913.2 ($b_{24}^{3+}$)[a] | 150 |
| Bovine serum albumin, IS | 698.5 ($b_{24}^{4+}$)[a] | 930.9 ($b_{24}^{3+}$)[a] | 150 |

[a]b*n*; *n* = number of amino acids

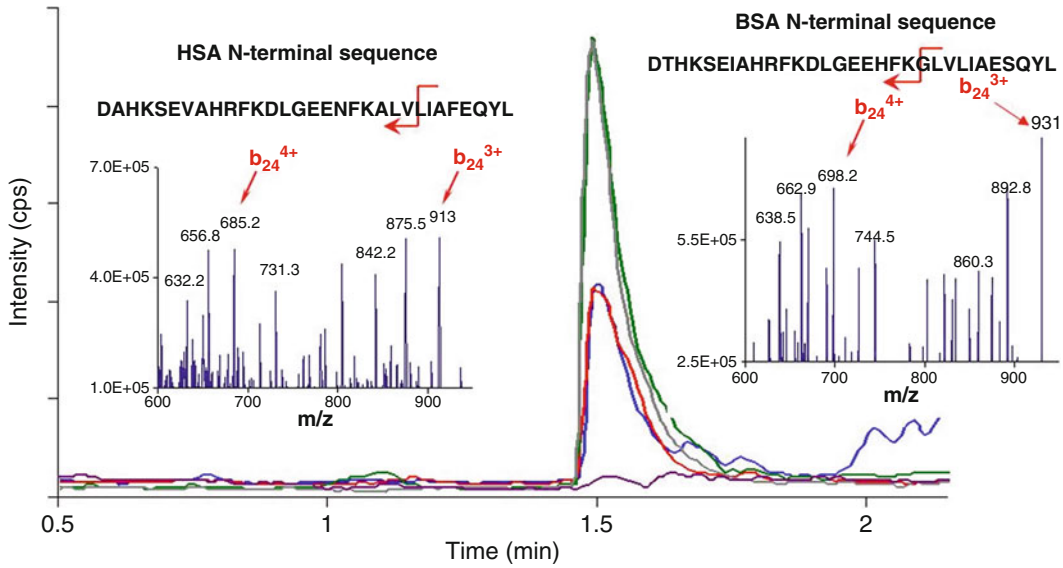



**Fig. 1** Total ion chromatogram of a microalbuminuric patient urine sample. *x*-axis shows elution time and the *y*-axis shows signal intensity in counts per second. The internal standard (BSA) 24 amino acid N-terminal fragments in their 3+ charge and 4+ charge states are depicted in *green* and *gray*, respectively. The corresponding 3+ and 4+ fragments of the albumin in the patient sample are shown in *blue* and *red*, respectively. The *purple* trace shows the result of ion suppression test where the analyte was injected directly into the mobile phase. The *inset* shows the corresponding ion scan mass spectra of the N-terminal fragments obtained for HSA and BSA. Reproduced with permission from ref. [8]

than ± 20 % of the expected value then the curve may be plotted after eliminating the calibrator. The lowest and the highest calibrators may not be removed as they will affect the LOQ and reportable range.

5. QC values are acceptable if found to be with ± 2SD of the established mean for the lot.
6. The chromatographic retention times are monitored closely. A ± 0.03 min shift in retention time on each LC system is considered acceptable.

## 4 Notes

1. N15-labeled HSA has also been used as an internal standard for quantitation [7].
2. Mobile phase gradients may need to be optimized with other organic solvent like methanol to achieve best signal-to-noise ratios.
3. Mass spectrometer source and lens parameters need to be optimized on every instrument as they differ between instruments of even the same manufacturer.

4. MS/MS parameters should be optimized for HSA and BSA by infusing a stock solution through a "tee" into a 250 μL/min flow stream consisting of 40 % mobile phase A. We observed that declustering potential of 350 V could facilitate N-terminal fragmentation of albumin.
5. Ion suppression on the instrument can be assessed as follows. Connect a syringe pump via a tee to the column effluent and infuse HSA and BSA stock solution directly into the electrospray source until a constant response is obtained. Then a stripped serum blank and a pooled serum control extracted and prepared after complete sample work-up is injected onto the column to observe the effect of matrix suppression on the response for the continuously infused HSA or BSA.

## References

1. Rothschild MA, Oratz M, Schreiber SS (1988) Serum albumin. Hepatology 8:385–401
2. Remuzzi G, Macia M, Ruggenenti P (2006) Prevention and treatment of diabetic renal disease in type 2 diabetes: the BENEDICT study. J Am Soc Nephrol 17:S90–S97
3. Perkins BA, Krolewski AS (2005) Early nephropathy in type 1 diabetes: a new perspective on who will and who will not progress. Curr Diab Rep 5:455–463
4. Owen WE, Roberts WL (2005) Performance characteristics of an HPLC assay for urinary albumin. Am J Clin Pathol 124:219–225
5. Busby DE, Bakris GL (2004) Comparison of commonly used assays for the detection of microalbuminuria. J Clin Hypertens (Greenwich) 6:8–12
6. Sviridov D, Meilinger B, Drake SK, Hoehn GT, Hortin GL (2006) Coelution of other proteins with albumin during size-exclusion HPLC: implications for analysis of urinary albumin. Clin Chem 52:389–397
7. Singh R, Crow FW, Babic N, Lutz WH, Lieske JC, Larson TS, Kumar R (2007) A liquid chromatography-mass spectrometry method for the quantification of urinary albumin using a novel 15N-isotopically labeled albumin internal standard. Clin Chem 53:540–542
8. Babic N, Larson TS, Grebe SK, Turner ST, Kumar R, Singh RJ (2006) Application of liquid chromatography-mass spectrometry technology for early detection of microalbuminuria in patients with kidney disease. Clin Chem 52:2155–2157

# Chapter 5

# Quantitation of Aldosterone in Serum or Plasma Using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

**J. Grace Van Der Gugten and Daniel T. Holmes**

## Abstract

Accurate determination of serum and plasma aldosterone is essential for screening, diagnosis, and subtype classification of primary aldosteronism (PA). Its measurement is also used in the investigation of adrenal incidentaloma, adrenal carcinoma, Addison's disease, congenital adrenal hyperplasia, renal artery stenosis, and renal tubular channelopathies. We describe a simple and robust method for the accurate and precise measurement of aldosterone in serum or plasma using liquid chromatography and tandem mass spectrometry (LC-MS/MS). After addition of internal standard, aldosterone is extracted from serum samples using supported liquid extraction (SLE) with methyl-t-butyl ether (MtBE). The MtBE is evaporated to dryness and sample is reconstituted with mobile phase before injection onto the LC-MS/MS and quantitation using an 8-point calibration curve. The assay calibration range is approximately 50–6500 pmol/L (0.16–234 ng/dL) with total imprecision between 6.8 and 4.1 % for concentrations between about 50 and 1000 pmol/L respectively.

**Key words** Primary aldosteronism, Mineralocorticoid hypertension, Secondary hypertension, Hypokalemia, Aldosterone, Plasma renin activity, Mass spectrometry

## 1 Introduction

Primary aldosteronism (PA) is caused by adrenal aldosterone production which is independent of or out of proportion to stimulation by angiotensin II as produced by the renin–angiotensin system [1, 2]. Under the action of excess plasma aldosterone, affected individuals develop hypertension which is characteristically resistant to commonly employed antihypertensives and a minority of more severely affected patients may develop hypokalemia and metabolic alkalosis. Causes of PA include (bilateral) idiopathic adrenal hyperplasia, aldosterone-producing adenoma, and a number of hereditary forms, the most common of which is Glucocorticoid Remediable Aldosteronism also known as Familial Hyperaldosteronism Type I.

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_5, 

Aldosterone is a difficult analyte owing to its low concentration and the presence of metabolites that cross-react with immunoassay methods, particularly in the setting of renal impairment [3]. These analytical problems have meant that aldosterone immunoassays may produce results that differ by a factor of 2 on identical samples [4]. LC-MS/MS offers a means to alleviate these analytical issues because of the increased specificity it affords.

While the reference method for serum aldosterone has been gas chromatography and mass spectrometry after derivatization [5], the availability of high-sensitivity triple quadrupole mass spectrometers has made LC-MS/MS methods without chemical derivatization possible. Sample preparation approaches have included protein precipitation followed by online [6, 7] or offline [8] solid phase extraction (SPE), liquid–liquid extraction (LLE) [9–11] and more recently supported liquid extraction (SLE) [12, 13]. The SLE methods offer an advantage over LLE methods in that they require approximately half the initial sample volume in order to achieve the same signal amplitude [12]. As with the majority of other methods [6–13], electrospray ionization (ESI) in negative mode is used. We present a method for serum aldosterone from 250 μL of serum. The method is suitable for PA screening (i.e. the calculation of the aldosterone:plasma renin activity ratio) and has adequate analytical sensitivity for aldosterone analysis after salt-loading (e.g. by saline suppression or fludrocortisone). Analysis of adrenal venous samples can be achieved after appropriate sample dilution and cortisol analysis can be "multiplexed" as needed for this purpose. The sample preparation can be easily modified for the analysis of urine samples after acid hydrolysis.

## 2 Materials

### 2.1 Samples

1. Serum from gel-free red-top tubes. EDTA plasma and heparin plasma collected in gel-free tubes are expected to be suitable samples. Interferences from gel-containing sample collection tubes are well-known to cause interferences with LC-MS/MS steroid methods and have been observed to affect this assay also.
2. Supine sampling: Based on the 20 min in vivo half-life of aldosterone [14], equilibration of levels requires at least 90 min of supine posture prior to collection.
3. Upright sampling: This is the preferred specimen type. Patient should be upright (sitting, standing, walking) for about 2 h prior to collection, followed by 5–15 min seated. It is preferable to collect the sample by mid-morning [1].

### 2.2 Solvents and Reagents

1. 1 M ammonium acetate solution: Weight 7.7 g of ammonium acetate and add deionized water to a total volume of 100 mL. Estimated stability: 1 year at 2–8 °C.

2. Methyl-t-butyl ether (MtBE) OmniSolv®HPLC grade (EMD Millipore. Billerica, MA).
3. Mass Spect Gold Steroid Free Serum (SFS) (Golden West Biologicals. Temecula, CA).
4. Mobile Phase A (2 mM ammonium acetate in water): Add 2 mL of 1 M ammonium acetate solution to 1 L of deionized water. Estimated stability: at least 3 months at room temperature.
5. Mobile Phase B (2 mM ammonium acetate in methanol): Add 2 mL of 1 M ammonium acetate solution to 1 L of methanol. Estimated stability: at least 3 months at room temperature.
6. 1:1 methanol:water: Mix equal volumes of water and methanol in a suitable storage container. Estimated stability: at least 6 months at room temperature.
7. Reconstitution Solvent (20:80 methanol:water with 2 mM ammonium acetate): Mix 50 mL of methanol with 200 mL of water. Add 0.50 mL of 1 M ammonium acetate solution. Estimated stability: up to 6 months at 2–8 °C.

### 2.3 Internal Standards and Standards

1. Primary standard: 100 μg/mL aldosterone in acetonitrile (Cerilliant. Round Rock, TX).
2. Primary internal standard (IS): d7-aldosterone (IsoSciences, King of Prussia, PA (*see* **Note 1**)).
3. Aldosterone working solution: Dilute the 100 μg/mL aldosterone stock solution 100-fold to 1000 ng/mL by aliquoting 50 μL of the 100 μg/mL aldosterone stock solution to a 5 mL class A volumetric flask containing approximately 4 mL of acetonitrile. Bring to volume and mix well before transferring to an appropriate glass storage container. Store at −70 °C. Estimated stability: at least 1 year at −70 °C.
4. Aldosterone high spiking solution (234 ng/mL or 649 nmol/L): Add 0.234 mL of the 1000 ng/mL aldosterone working solution to a 10 mL class A volumetric flask containing approximately 5 mL of acetonitrile. Bring to volume with acetonitrile, mix well, and transfer to an appropriate glass storage container. Store at −70 °C. Estimated stability: at least 1 year at −70 °C.
5. Aldosterone low spiking solution (23.4 ng/mL or 64.9 nmol/L): Add 1 mL of the high spiking solution to a 10 mL class A volumetric flask containing approximately 5 mL of acetonitrile. Bring to volume with acetonitrile, mix well, and transfer to an appropriate glass storage container. Store at −70 °C. Estimated stability: at least 1 year at −70 °C.
6. IS stock solution (100 μg/mL or 273 μmol/L): Dissolve 1 mg of d7-aldosterone powder into 1 mL of acetonitrile in a new glass vial. Transfer contents to a 10 mL class A volumetric flask. Wash the original vial five times with 1 mL acetonitrile transferring

washing solution to the 10 mL volumetric flask. Bring the 10 mL volumetric flask to volume with acetonitrile and mix well before transferring to an appropriate glass storage container. Store at −70 °C. Estimated stability: at least 1 year at −70 °C.

7. IS working solution: transfer 500 µL of the 100 µg/mL d7-aldosterone stock solution to a 25 mL class A volumetric flask containing approximately 10 mL of acetonitrile. Bring to 25 mL volume with acetonitrile and mix well before transferring to an appropriate glass storage container. Store at −70 °C. Estimated stability: at least 1 year at −70 °C.
8. IS tubes preparation: Aliquot 200 µL of the IS working solution prepared above to 16×100 mm glass tubes. Allow to evaporate (in fume hood) to dryness. Cap and store at −70 °C. Estimated stability: at least 1 year at −70 °C.

### 2.4 Calibrators and Controls

1. Controls used are Lyphochek Immunoassay Plus Controls, levels 1, 2 and 3 (BioRad, Montreal, QC). The target value is set based on the mean value of 20 points over ten runs. Controls are run in duplicate distributed throughout each analytical run.
2. An in-house patient pool is prepared approximately once a year by pooling discarded, anonymized, and previously analyzed specimens. The patient pool target value is set based on the mean value of 20 points over ten runs. Patient pools are run in duplicate distributed throughout each analytical run.
3. Calibrators are prepared in-house using the aldosterone spiking solutions and SFS according to details provided in Table 1.

**Table 1**
**Volumes required to prepare final calibrant solutions**

| Calibrator level | Spiking solution | Volume of spiking solution (µL) | Final volume (mL) | Final concentration (ng/dL) | Final concentration (pmol/L) |
|---|---|---|---|---|---|
| 1 | Low | 35 | 50 | 1.6 | 45 |
| 2 | Low | 80 | 50 | 3.7 | 104 |
| 3 | Low | 160 | 50 | 7.5 | 207 |
| 4 | High | 32 | 50 | 15 | 415 |
| 5 | High | 62 | 50 | 29 | 804 |
| 6 | High | 125 | 50 | 58.5 | 1620 |
| 7 | High | 250 | 50 | 117 | 3241 |
| 8 | High | 500 | 50 | 234 | 6482 |

Calibrators are prepared by placing 25 mL of SFS in a 50 mL class A volumetric flask. Spiking solution is then added and 50 mL volume is filled with SFS followed by thorough mixing. Low spiking solution is 23.4 ng/mL (64.9 nmol/L). High spiking solution is 234 ng/mL (649 nmol/L). Final concentrations of calibrators are provided in the two most commonly employed units: ng/dL and pmol/L. To convert ng/dL to pmol/L, multiply by 27.74

### 2.5 Analytical Equipment and Supplies

1. Biotage ISOLUTE® SLE+ 400 µL plates (Biotage, Charlotte, NC).
2. Vacuum manifold or positive pressure manifold, installed in robotic pipettor or manually controlled.
3. Costar™ 96 well Assay Blocks, V-bottom, 2 mL (Corning Incorporated, Corning N (*see* **Note 2**)).
4. MicroMat TFE/Silicone 96-well pre-slit square sealing mats (SUN-SRi, Rockwood, TN).
5. API-5000 or API-5500 QTRAP® triple quadrupole mass spectrometer (AB SCIEX, Concord, ON) or other mass spectrometer capable of reaching the required limit of detection, equipped with appropriate software (e.g. Analyst®).
6. Shimadzu 20AC LC System with pumps, column oven, degasser, autosampler, or equivalent system (Kyoto, Japan).
7. Analytical column: Gemini NX-C18 3 µm 110 Å 100 mm × 2.0 mm (Phenomenex, Torrance) with a SecurityGuard™ C18 guard cartridge for columns with 2.0–3.0 mm internal diameters (Phenomenex, Torrance, CA).

## 3 Methods

### 3.1 Stepwise Procedure

1. Thaw calibrators, patient pool, and patient samples. Allow an IS tube come to room temperature.
2. Centrifuge the calibrators, patient pool, and patient samples for 5 min at 2100 × *g*.
3. Add 10 mL of 1:1 methanol:water to the IS tube. Vortex mix for 45 s.
4. Add 50 µL of 0.04 µg/mL d7-aldosterone IS prepared in **step 3** to the appropriate number of wells in a Costar™ 96-well plate.
5. Add 250 µL of calibrators, controls, and serum patient samples to the wells containing IS.
6. Mix plate for 20 s at medium speed.
7. Place a new Costar™ 96-well plate in the collection position in the vacuum manifold (*see* **Note 3**).
8. Place SLE + 400 µL plate on vacuum manifold (*see* **Note 4**).
9. Add entire sample and IS (total 300 µL) to wells of SLE+ 400 µL plate.
10. Apply a short (5–10 s) burst of vacuum to apply sample to SLE sorbent.
11. Wait for 5 min.
12. Add 900 µL of MtBE to all samples in appropriately ventilated conditions (fume hood or liquid handler under negative pressure).

13. Apply short burst of vacuum.
14. Wait for 5 min.
15. Apply vacuum for 1–2 min to elute MtBE.
16. Add an additional 900 µL of MtBE to all sample wells.
17. Apply a short burst of vacuum.
18. Wait for 5 min.
19. Apply vacuum for 1–2 min to elute MtBE.
20. Remove collection plate, and evaporate to dryness at room temperature in a fume hood.
21. Reconstitute samples with 125 µL of 20:80 methanol:water with 2 mM ammonium acetate.
22. Place sealing mat on 96-well plate and seal each well manually. Use of a small rolling pin as a final sealing step is recommended.
23. Vortex the sealed plate for 1 min.
24. Inject 50 µL of sample onto LC-MS/MS. Sample chromatograms for LC-MS/MS ion chromatograms for aldosterone and IS are shown in Fig. 1 (*see* **Notes 5** and **6**).

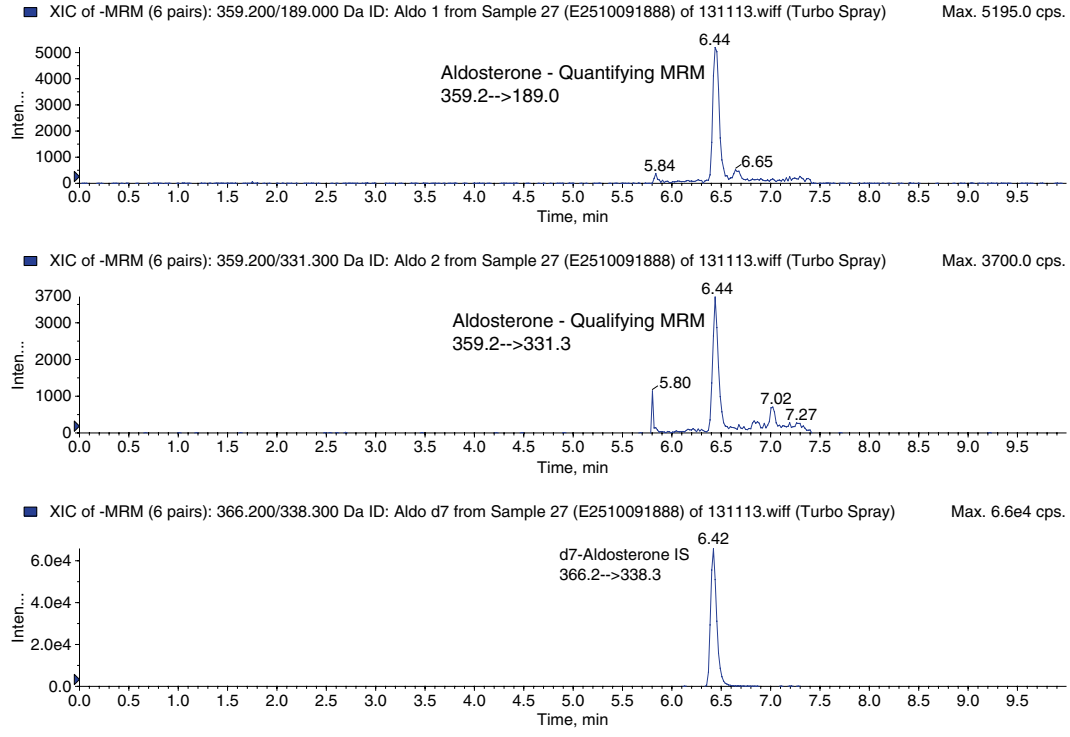



**Fig. 1** Representative chromatograms of the aldosterone quantifying multiple reaction monitoring (MRM) transition (359.2 → 189.0), qualifying MRM transition (359.2 → 331.3), and d7-aldosterone IS MRM transition (366.2 → 338.3) for a sample with an aldosterone concentration of 282 pmol/L (10.1 ng/dL). In the LC conditions provided, the expected elution time of aldosterone is approximately 6.4 min

**Table 2**
**HPLC gradient**

| Time (min) | Mobile phase A (%) |
|---|---|
| 0 | 80 |
| 1 | 80 |
| 5 | 30 |
| 6 | 30 |
| 6.5 | 10 |
| 7.5 | 10 |
| 7.6 | 80 |
| 10 | 80 |

Column is maintained at 55 °C in a column oven. Flow rate is 0.35 mL/min

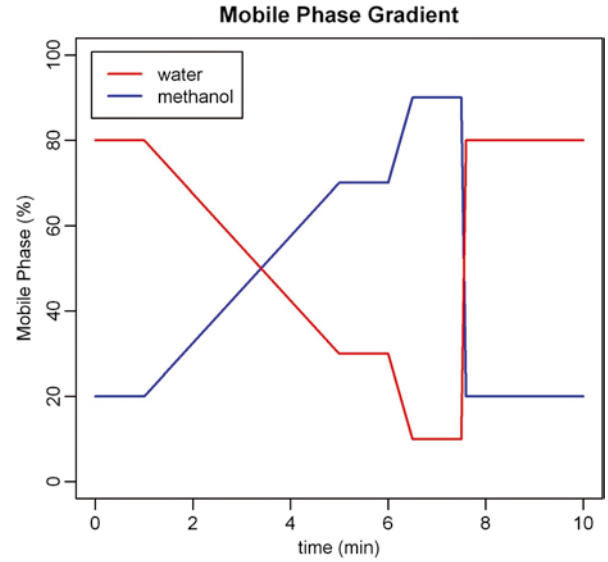



**Fig. 2** HPLC gradient

### 3.2 Analysis

1. HPLC gradient is shown in Table 2 and Fig. 2.
2. Instrument operating parameters are given in Table 3 (*see* **Note 7**).
3. Data are analyzed using Analyst® Software (AB SCIEX).
4. Standard curves are generated based on linear regression with $1/x$ weighting of the analyte/IS peak area ratio ($y$) versus analyte concentration ($x$) using the masses indicated in Table 3. Run acceptability is based on Westgard's QC rules with

**Table 3**
**Optimized instrument settings for the AB SCIEX API-5500 QTRAP® and API-5000 triple quadrupole mass spectrometers in electrospray-negative ion mode**

| | | | | | |
|---|---|---|---|---|---|
| Source parameters | | | | | |
| Curtain gas | 30 µL/min | | | | |
| Source temperature | 600 °C | | | | |
| Gas1 | 50 µL/min | | | | |
| Gas2 | 50 µL/min | | | | |
| CAD Gas | Medium/5* | | | | |
| IonSpray voltage | −4500 V | | | | |
| Entrance potential | −10 V | | | | |
| Compound-dependent parameters | | | | | |
| *Compound name* | *Q1 Mass (amu)* | *Q3 Mass (amu)* | *Declustering potential (V)* | *Collision energy (V)* | *Cell exit potential (V)* |
| Aldosterone—quantifier | 359.2 | 189.0 | −80 | −26 | −10 |
| Aldosterone—qualifier | 359.2 | 331.3 | −65/−80* | −18 | −22 |
| d7-Aldosterone IS | 366.2 | 338.3 | −80 | −20 | −22 |

All parameters are identical for both instruments except those marked (*), which are specific to the API-5000. Tune settings may vary between instruments

expected means and standard deviations of the QCs set as described in Subheading 2.4.

5. An ion ratio limit of 15 % of the quantitation to qualifying ion is used to positively identify aldosterone peaks.

## 4 Notes

1. The IS, d7-aldosterone, is no longer available from IsoSciences but remains available from Sigma Aldrich (St. Louis, MO) and Toronto Research Chemicals (Toronto, ON). IsoSciences now offers d8-aldosterone in both powder form or in solution. There is also a d4-aldosterone product from Isosciences which places deuterium atoms on completely non-exchangeable positions.
2. It should not be assumed that all polypropylene products will afford equivalent performance and recoveries. We have found the Costar brand of 96 deep square well plates to be completely satisfactory with respect to aldosterone recovery. Only square well plates have been tested.
3. Sample extraction can be done manually using a vacuum manifold or positive pressure manifold which can be obtained by a variety of manufacturers and suppliers. However, obtaining a robotic liquid handler such as a Hamilton STARlet (Hamilton Robotics Inc, Reno, NV) is highly recommended.

4. This assay can be modified to extract the aldosterone from serum using liquid–liquid extraction (LLE) as an alternative to SLE. The SLE technique uses the same principle as LLE.
5. This assay can also be adapted to analyze aldosterone in urine samples. Sample must be subjected to acid hydrolysis with the addition of 30 µL of 3.2 M HCl to 300 µL of urine. Incubate for 24 h at room temperature. Dilute 40 µL of hydrolysate in 760 µL SFS or buffer and treat mixture as a serum specimen to obtain a raw aldosterone concentration. Twenty-four hour urinary aldosterone excretion in nmol/d = (Raw Aldosterone Concentration in pmol/L) × 22 × $V_{24}$ ÷ 1000, where $V_{24}$ is the 24 h urine volume. To convert aldosterone excretion in nmol/d to µg/d, multiply by 0.36.
6. The analysis of adrenal venous samples (AVS) will frequently require samples to be run neat and at an appropriate dilution. AVS aldosterone concentrations may range from approximately $10^3$–$10^6$ pmol/L (40–40,000 ng/dL) depending on whether intravenous cosyntropin stimulation is used. We have found that analysis of neat samples along with concomitant analysis of a 50-fold dilution is adequate for reporting the majority of AVS aldosterone (and cortisol) results. Water can be used as the AVS diluent without fear of matrix effects because clinical decisions are based on *relative* aldosterone concentrations from the left and right.
7. Additional steroids can be added to this assay. In our lab, we have added cortisol to this assay in order to more efficiently deal with adrenal venous sampling collections. Although cortisol analysis is usually performed in ESI-positive mode for optimal analytical sensitivity, because of its relatively high concentration, it can be easily measured in negative ESI and included in the same aldosterone run. The elution time of cortisol in the HPLC conditions presented here is approximately 0.4 min after aldosterone. Monitored MRM transitions for cortisol are 361.3 → 282.1 (quantifier) and 361.3 → 297.0 (qualifier) with 365.3 → 127.8 for the d4-cortisol IS.

## References


1. Funder JW, Carey RM, Fardella C et al (2008) Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 93:3266–3328
2. Young WF (2007) Primary aldosteronism: renaissance of a syndrome. Clin Endocrinol (Oxf) 66:607–618
3. Jones JC, Carter GD, Macgregor GA (1981) Interference by polar metabolites in a direct radioimmunoassay for plasma aldosterone. Ann Clin Biochem 18:54–59
4. Schirpenbach C, Seiler L, Maser-Gluth C et al (2006) Automated chemiluminescence-immunoassay for aldosterone during dynamic testing: comparison to radioimmunoassays with and without extraction steps. Clin Chem 52:1749–1755
5. Siekmann L (1979) Determination of steroid hormones by the use of isotope dilution-mass

spectrometry: a definitive method in clinical chemistry. J Steroid Biochem 11:117–123

6. Taylor PJ, Cooper DP, Gordon RD et al (2009) Measurement of aldosterone in human plasma by semiautomated HPLC–tandem mass spectrometry. Clin Chem 55:1155–1162
7. Guo T, Taylor RL, Singh RJ et al (2006) Simultaneous determination of 12 steroids by isotope dilution liquid chromatography-photospray ionization tandem mass spectrometry. Clin Chim Acta 372:76–82
8. Hinchliffe E, Carter S, Owen LJ et al (2013) Quantitation of aldosterone in human plasma by ultra high performance liquid chromatography tandem mass spectrometry. J Chromatogr B 913:19–23
9. Turpeinen U, Hämäläinen E, Stenman UH (2008) Determination of aldosterone in serum by liquid chromatography–tandem mass spectrometry. J Chromatogr B 862:113–118
10. Van Der Gugten JG, Dubland J, Liu HF et al (2012) Determination of serum aldosterone by liquid chromatography and tandem mass spectrometry: a liquid–liquid extraction method for the ABSCIEX API-5000 mass spectrometry system. J Clin Pathol 65:457–462
11. Ray JA, Kushnir MM, Palmer J et al (2014) Enhancement of specificity of aldosterone measurement in human serum and plasma using 2D-LC–MS/MS and comparison with commercial immunoassays. J Chromatogr B Analyt Technol Biomed Life Sci 970:102–107
12. Van Der Gugten JG, Crawford M, Grant R et al (2012) Supported liquid extraction offers improved sample preparation for aldosterone analysis by liquid chromatography tandem mass spectrometry. J Clin Pathol 65: 1045–1048
13. Owen LJ, Keevil BG (2013) Supported liquid extraction as an alternative to solid phase extraction for LC-MS/MS aldosterone analysis? Ann Clin Biochem 50:489–491
14. Tait JF, Tait SA, Little B et al (1961) The disappearance of 7-$H^3$-D-aldosterone in the plasma of normal subjects. J Clin Invest 40: 72–80

# Chapter 6

# Quantification of Five Clinically Important Amino Acids by HPLC-Triple TOF™ 5600 Based on Pre-column Double Derivatization Method

**Shuang Deng, David Scott, and Uttam Garg**

## Abstract

Phenylalanine, tyrosine, glycine, cystine, and phosphoethanolamine are commonly measured amino acids in various physiological fluids to diagnose or follow-up various inborn errors of metabolism. The gold standard method for the amino acids quantitation has been ion exchange chromatography with ninhydrin post-column derivatization. However, this method is very laborious and time consuming. In recent years, liquid-chromatography mass spectrometry is being increasingly used for the assay of amino acids. Pre-column butyl derivatization with reverse phase chromatography has been widely used for mass spectrometry analysis of amino acids. Phosphoethanolamine is not butylated and cannot be measured by this method. Nevertheless, phosphoethanolamine can be dansyl-derivatized using dansyl chloride. We developed a double derivatization method by using butanol and dansyl chloride to derivatize carboxylic and amino groups separately, and then combining the derivatives to simultaneously measure these five amino acids using TOF-MS scan. Stable isotope-labeled internal standards were used.

**Key words** Phenylalanine, Tyrosine, Cystine, Glycine, Phosphoethanolamine, Butylation, Dansylation, HPLC-TOF-MS

## 1 Introduction

Clinically relevant amino acids are measured in physiological fluids to diagnose inborn errors of metabolism. It is common that full amino acid profile of >30 amino acids is performed in the initial diagnosis of amino acid disorders. Once an amino acid disorder is diagnosed and confirmed, the follow-up is generally done by measuring only relevant amino acid(s). Commonly, measured amino acids are phenylalanine, tyrosine, glycine, cystine, and phosphoethanolamine (PEA). Phenylalanine and tyrosine are measured for the diagnosis and follow-up of patients with phenylketonuria (PKU), the disease if untreated can cause mental retardation [1, 2]. Cystine is measured in the diagnosis and follow-up of cystinuria, a kidney stone-forming disorder [2, 3]. The increase of glycine

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_6, © Springer Science+Business Media New York 2016

concentration in plasma and cerebrospinal fluid (CSF) is the indicator of non-ketotic hyperglycinemia (NKH), a seizure disorder [4]. Urinary phosphoethanolamine (PEA) is widely used in the diagnosis of hypophosphatasia, a metabolic disorder that affects bones [5, 6].

The gold standard method for amino acid analysis has been ion exchange chromatography with ninhydrin post-column derivatization. However, this method is cumbersome and time consuming. In recent years, the reverse phase HPLC-mass spectrometry methods combined with pre-column derivatization have been used for the quantitation of amino acids [7–10]. Butylation is the most commonly used method for derivatization of amino acids. However, some amino acids including phosphoethanolamine and taurine are refractory to butylation due to lack of α-carboxylic acid group. These amino acids can be dansyl-derivatized at α-amino group using dansyl chloride. Here, we describe a double derivatization method. Butanol and dansyl chloride were selected to derivatize carboxylic and amino groups respectively. The analysis was performed using TOF-MS scan.

## 2 Materials

### 2.1 Samples

1. Plasma/Serum: Separated from 2 mL of blood in a mint green (heparin) or plain no-gel tube.
2. Urine: 3 mL random urine.
3. CSF: 1 mL CSF, non-traumatic tap.

### 2.2 Solvents and Reagents

1. Mobile phase A (2 mM ammonium formate, 0.1 % formic acid in HPLC water).
2. Mobile phase B: Acetonitrile.
3. Dansyl chloride (Sigma).
4. 3 N HCl in butanol (Regisil).
5. Sodium bicarbonate (Sigma).
6. 1 mg/mL of dansyl chloride in acetone.

### 2.3 Internal Standards and Standards

1. Stock internal standard mixture (NSK-A from Cambridge Isotope Laboratories): Dissolve in 1 mL $H_2O$. It provides concentration of 500 μM for L-Alanine (2,3,3,3-$D_4$), L-Phenylalanine (ring-$^{13}C_6$), L-Leucine (5,5,5-$D_3$), L-Valine ($D_8$). L-Arginine (4,4,5,5,-$D_4$), L-Citrulline (5,5-$D_2$), L-Tyrosine (ring-13$C_6$), L-Ornithine (5,5-$D_2$), L-Methionine (methyl-$D_3$), DL-Glutamate (2,4,4-$D_3$), L-Aspartate (2,3,3-$D_3$), and 2500 μM for L-Glycine (2-$^{13}C$, $^{15}N$).

**Table 1**
**Preparation of calibrators**

| Calibrator | Cys (μM) | Phe, Tyr, Gly, PEA (μM) |
|---|---|---|
| Blank | 0 | 0 |
| Cal 1 | 15.6 | 31.2 |
| Cal 2 | 31.2 | 62.5 |
| Cal 3 | 62.5 | 125 |
| Cal 4 | 125 | 250 |
| Cal 5 | 250 | 500 |
| Cal 6 | 500 | 1000 |
| Cal 7 | 1250 | 2500 |

**Table 2**
**Preparation of quality controls**

| Quality control | PEA (μM) | Phe, Tyr, Gly, Cys (μM) |
|---|---|---|
| QC 1 | 89.3 | 58 |
| QC 2 | 178.5 | 116 |
| QC 3 | 357 | 464 |

2. Working internal standard mixture: Dilute stock internal standards mixture 100 times in methanol.
3. 1 mM Cystine-$D_4$ internal standard (Cambridge Isotope Laboratories): Prepare in 0.1 N HCl.
4. Stock amino acid standards in 0.1 N HCl (#1700-0180, Pickering Laboratories).
5. Prepare calibrators at concentrations given in Table 1 using lithium diluent (Pickering Laboratories).
6. Quality controls: Mix 6.5 mL of amino acid standards (500 μM, Sigma) and 500 μL of 10 mM in 0.1 N HCl phosphoethanolamine (Sigma). This provides concentrations of 464 μM for Phe, Tyr, Gly and Cys, and 357 μM for PEA (QC 3). Dilute QC3 to make QC 1 and QC 2 (Table 2) (*see* **Note 1**).

### *2.4 Analytical Equipment and Supplies*

1. Triple TOF™5600 (AB Sciex).
2. Acuity UPLC (Waters).
3. Analytical Column: Kinetex C18, 100×3 mm, 2.6 μm (Phenomenex).

## 3 Methods

### 3.1 Stepwise Procedure

1. Pipette 50 μL calibrators, sample or control to 1.5 mL micro-centrifuge tubes.
2. Pipette 450 μL of working internal standard mixture and 20 μL cystine-$D_4$ internal standard.
3. Vortex tubes for 1 min and let stand for 5 min.
4. Centrifuge the tubes for 10 min at 12,000 × *g*.
5. Transfer 200 μL of supernatant to two separate disposable glass tubes (Tubes A and B).
6. Evaporate to dryness under a stream of nitrogen at 45 °C (*see* **Note 2**).
7. Add 100 μL of 3 N HCl in butanol to one disposable tube for butylation (Tube A).
8. Add 50 μL of 0.1 M $NaHCO_3$ (Tube B).
9. Add 50 μL dansyl chloride solution (Tube B).
10. Incubate both tubes A and B in dry block for 20 min at 60 °C.
11. Evaporate to dryness under a stream of nitrogen at 45 °C (*see* **Note 3**).
12. Reconstitute the residues in 500 μL mobile phase A, and vortex.
13. Combine the contents of tubes A and B, and vortex the mixture.
14. Transfer the mixture to autosampler vials, and inject 10 μL.

### 3.2 Instrument Operating Conditions

See Tables 3 and 4 for HPLC and mass spectrometer conditions.

**Table 3**
**HPLC gradient and parameters**

| Time (min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|
| 0 | 98 | 2 |
| 15 | 60 | 40 |
| 15.1 | 0 | 100 |
| 18.5 | 0 | 100 |
| 18.6 | 98 | 2 |
| 22 | 98 | 2 |

Column temperature: 50 °C. Flow rate: 0.5 mL/min

**Table 4**
**TOF-MS parameters**

| | |
|---|---|
| Ion source gas 1 (GS1) | 50 psi |
| Ion source gas 2 (GS2) | 50 psi |
| Curtain gas (CUR) | 30 psi |
| Temperature (TEM) | 550 °C |
| Ion spray voltage floating (ISVF) | 5500 V |

**Table 5**
**Compound specific parameters**

| Amino acid | Butylation (*m/z*) | Dansylation (*m/z*) | DP (v) | CE (eV) |
|---|---|---|---|---|
| Cystine | 297.09373 | – | 20 | 10 |
| Cystine-$D_4$ | 301.11883 | – | | |
| Glycine | 132.10191 | – | | |
| Glycine (2-$^{13}C$, $^{15}N$) | 134.10230 | – | | |
| Phenylalanine | 222.14886 | – | | |
| Phenylalanine (ring-$^{13}C_6$) | 228.16898 | – | | |
| Tyrosine | 238.14377 | – | | |
| Tyrosine (ring-13$C_6$) | 244.16390 | – | | |
| Phosphoethanolamine | – | 375.07742 | | |
| Aspartate (2,3,3,-$D_3$) | – | 370.11467 | | |

### 3.3 Data Analysis

1. TOF-MS is used in positive ion electrospray ionization mode. Data are collected using Analyst TF 1.6 software and quantified using MultiQuant software version 3.0 (AB Sciex).
2. Standard curves are generated based on linear regression of the analyte/IS peak area ratio ($y$) versus analyte concentration ($x$) using the quantifying ions listed in Table 5 (*see* **Notes 4** and **5**).
3. Typical TOF-MS ion-extraction chromatograms are shown in Fig. 1.
4. Typical calibration curve has a correlation ($r^2$) > 0.99.
5. Quality control samples are evaluated with each run. The acceptable results are within +/− 20 % of target values.

## 4 Notes

1. Calibrators and quality controls are prepared separately.
2. Drying time is ~5 min. May vary with nitrogen flow rate and type of equipment.

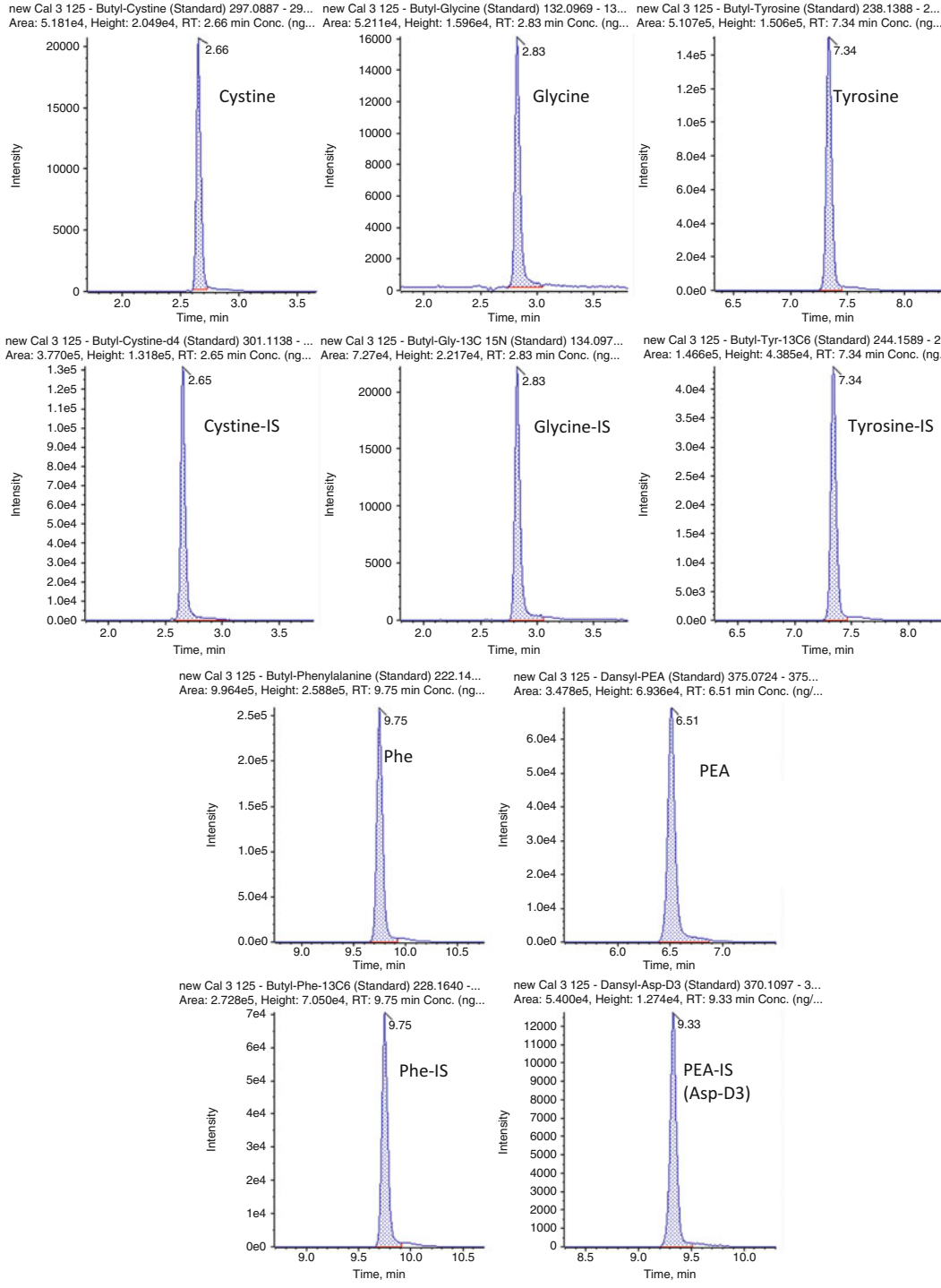



**Fig. 1** TOF-MS ion chromatograms for various amino acids. Cystine, glycine, tyrosine, and phenylalanine were measured using butylation. Phosphoethanolamine was measured using dansylation

3. Drying time is ~15 min. May vary with nitrogen flow rate and type of equipment.
4. Accuracy of the method was evaluated by comparing the method with ninhydrin HPLC amino acid analyzer. The results were within +/− 10 %.
5. Internal standard for PEA was Asp-D3 since labeled PEA was not available.

## References


1. Blaskovics ME, Schaeffler GE, Hack S (1974) Phenylalaninaemia. Differential diagnosis. Arch Dis Child 49:835–843
2. Broer S, Palacin M (2011) The role of amino acid transporters in inherited and acquired diseases. Biochem J 436:193–211
3. Mattoo A, Goldfarb DS (2008) Cystinuria. Semin Nephrol 28:181–191
4. Dinopoulos A, Matsubara Y, Kure S (2005) Atypical variants of nonketotic hyperglycinemia. Mol Genet Metab 86:61–69
5. Imbard A, Alberti C, Armoogum-Boizeau P, Ottolenghi C, Josserand E, Rigal O, Benoist JF (2012) Phosphoethanolamine normal range in pediatric urines for hypophosphatasia screening. Clin Chem Lab Med 50:2231–2233
6. Mc CR, Morrison AB, Dent CE (1955) The excretion of phosphoethanolamine and hypophosphatasia. Lancet 268:131
7. Deyl Z, Hyanek J, Horakova M (1986) Profiling of amino acids in body fluids and tissues by means of liquid chromatography. J Chromatogr 379:177–250
8. Dietzen DJ, Weindel AL, Carayannopoulos MO, Landt M, Normansell ET, Reimschisel TE, Smith CH (2008) Rapid comprehensive amino acid analysis by liquid chromatography/tandem mass spectrometry: comparison to cation exchange with post-column ninhydrin detection. Rapid Commun Mass Spectrom 22:3481–3488
9. Hardy DT, Hall SK, Preece MA, Green A (2002) Quantitative determination of plasma phenylalanine and tyrosine by electrospray ionization tandem mass spectrometry. Ann Clin Biochem 39:73–75
10. Song C, Zhang S, Ji Z, Li Y, You J (2015) Accurate Determination of Amino Acids in Serum Samples by Liquid Chromatography-Tandem Mass Spectrometry Using a Stable Isotope Labeling Strategy. J Chromatogr Sci [in press]

# Chapter 7

# Sensitive, Simple, and Robust Nano-Liquid Chromatography-Mass Spectrometry Method for Amyloid Protein Subtyping

**Drew Payto, Courtney Heideloff, and Sihe Wang**

## Abstract

Amyloidosis is a rare condition characterized by deposits of insoluble proteins in the form of β-pleated sheets. These deposits interfere with the normal structure and function of varying tissues. Thirty-one amyloid proteins have been identified, and the correct identification is critical due to the varying treatments. Immunohistochemistry, the most routine method for identification of amyloid proteins, suffers from limitations. Mass spectrometry (MS)-based methods offer better sensitivity and specificity. We describe here a sensitive, simple, and robust MS-based method for the identification of amyloid proteins. Amyloid deposits are excised from formalin-fixed tissue by laser microdissection and is put through protein extraction followed by trypsin digestion. The resulting peptides are separated by nano-liquid chromatography and analyzed by high-resolution Orbitrap mass spectrometry. The mass spectrometry data are then searched against a human protein database for identification and semi-quantification.

**Key words** Amyloidosis, Amyloidogenic protein, β-pleated sheets, Orbitrap mass spectrometry, Immunohistochemistry

## 1 Introduction

Amyloidosis is a group of rare diseases caused by extracellular protein misfolding, which generates insoluble protein deposits in the form of β-pleated sheets [1]. The fibrils generally exhibit a cross-β diffraction pattern. These fibrils bind to Congo red dye and when viewed by polarization microscopy they exhibit green, yellow, or orange birefringence color [2]. As of 2014, there are 31 known amyloidogenic proteins identified, with the most common being immunoglobulin lambda and kappa light chains, transthyretin, and serum amyloid A (SAA) which account for >90 % of the cases [3, 4]. Amyloidogenic proteins can present in various organs including but not limited to heart, liver, kidney, lung, central nervous system, skin, and cornea [3]. Amyloid deposits' clinical presentation can be very diverse ranging from asymptomatic to multiorgan

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_7, 

failure. Amyloid deposits can also be localized or systemic [5]. Treatments are available for many types of amyloidosis, however these treatments are type-specific ranging from high-dose chemotherapy to liver transplantation [5]. Due to the radically diverse and aggressive nature of these treatment options, accurate subtyping of amyloidosis is essential [6].

In clinical practice, amyloid identification is a two-step approach. The first step is to determine the presence of amyloid deposits. Congo red staining is considered the gold standard approach for identifying amyloid [5]. Once the presence of amyloid has been confirmed, subtyping must be performed to identify the amyloidogenic protein. The most routine method for this identification is immunohistochemistry staining of formalin-fixed and paraffin-embedded (FFPE) tissue, however this method is prone to limitations. One limitation is that wild-type antibodies may not cross-react with same protein in the amyloid deposit. A second limitation is the antibody availability. Due to these factors as many as 30 % of cases cannot be identified by immunohistochemistry [7–9]. Several mass spectrometry (MS) methods have been developed for the direct analysis of amyloid protein from FFPE tissue. MS methods have shown to have superior sensitivity and specificity to immunohistochemistry methods [10]. The following chapter describes a sensitive, simple, and robust proteomic method for amyloid subtyping that has been validated for clinical use.

## 2 Materials

### 2.1 Samples

Formalin-fixed and paraffin-embedded tissue (FFPE) is laser-microdissected and the excised tissue is transferred to 0.5 mL Eppendorf tubes. Microdissected samples are stored at −70 °C until analysis. FFPE samples are stable indefinitely at −20 and −70 °C.

### 2.2 Solvents and Reagents

1. 0.5 M Tris base: Weigh out 6.057 g of Tris and add to beaker containing 90 mL of clinical laboratory reagent water (CLRW). Put a mixer and stir solution. Adjust pH to 7.8 using concentrated HCl. Quantitatively transfer contents to 100 mL volumetric flask. Bring to 100 mL water. Stable for 1 year at 2–8 °C.
2. 0.1 M EDTA: Weigh out 2.9224 g of EDTA and place into a 100 mL class A volumetric flask. Bring to 100 mL with CLRW. Stable for 1 year at 2–8 °C.
3. Digestion Buffer (10 mM Tris/1 mM EDTA/0.002 % Zwittergent 3-16, pH 7.5–8.5): Pipette 1 mL of 0.5 M Tris base into 50 mL class A volumetric flask. Pipette 0.5 mL of 0.1 M EDTA into same class A volumetric. Add 10 mg of Zwittergent 3-16 to class A volumetric flask. Bring to 50 mL

with CLRW. Use HCl or KOH to pH solution between 7.8 and 8.0 if needed. Aliquot into 1.5 mL Eppendorf tubes. Stable for 5 years at −70 °C.

4. Trypsin Buffer (50 mM Acetic Acid, pH 3): Add 30 μL of glacial acetic acid to 10 mL class A volumetric flask. Bring to 10 mL with CLRW. Aliquot into glass Teflon lined screw top vials. Discard after use.
5. 20 μg/mL trypsin solution: Add 1 mL of trypsin buffer to 20 μg of trypsin. Mix vial well. Stable for 1 year at −70 °C.
6. 2.5 % Formic Acid/5 % ACN/92.5 % CLRW: Pipette 2.5 mL of formic acid and 5 mL of acetonitrile (ACN) into 100 mL class A volumetric flask. Bring to 100 mL with CLRW. Stable for 6 months at room temperature.
7. 50 mM Dithiothreitol (DTT): Weigh out 3.8 mg of DTT into 1.5 mL Eppendorf tube. Add 0.5 mL of 2.5 % Formic Acid/5 % ACN/92.5 % CLRW and vortex. Discard this solution after use.
8. Mobile Phase A (0.2 % Formic Acid in $H_2O$): Add 25 mL of Fisher Optima grade $H_2O$ to mobile phase bottle. Pipette 50 μL of formic acid into bottle. Stable for 6 months at room temperature.
9. Mobile Phase B (0.2 % Formic Acid in Acetonitrile): Add 25 mL of acetonitrile to mobile phase bottle. Pipette 50 μL of formic acid into bottle. Stable for 6 months at room temperature.

### 2.3 Injection Standards (Human Serum Albumin, HSA Digest)

A digested HSA solution (testmix) is prepared to test the instrument analytical performance before analyzing patient samples.

### 2.4 Controls

1. Each patient batch is bracketed with two extracted patient samples from the previous batch with known diagnosis. Consistent identification of the same amyloid proteins indicates acceptable quality for the current batch.
2. The presence of common endogenous amyloid proteins (serum amyloid P, apolipoprotein E, apolipoprotein A-I, and apolipoprotein A-IV) in each sample is used as internal control for the analytical processes including protein extraction, trypsin digestion, and nano-LC-MS/MS.

### 2.5 Analytical Equipment and Supplies

1. Thermo Fisher Scientific Easy-nLC 1000 HPLC.
2. Thermo Fisher Scientific Q-Exactive mass spectrometer.
3. Easy-Spray Column PepMap, C18, 3 μm, 100 Å (75 μm × 15 cm).
4. Dionex Acclaim PepMap100, C18, 3 μm, 100 Å (75 μm × 2 cm).

5. Zip Tip with 0.2 μL C18 resin.
6. Thermo Fisher Scientific Easy-nLC 1000 HPLC parameters include
   (a) Mobile phase A: CLWR + 0.2 % formic acid.
   (b) Mobile phase B: acetonitrile + 0.2 % formic acid.
   (c) Flow Rate: 2000 nL/min.
   (d) Gradient: 2–40 % B in 20 min and then 40–90 % B in 5 min.
   (e) Injection volume: 10 μL.
7. Q-Exactive (Thermo Fisher Scientific) mass spectrometer settings include:
   (a) Spray voltage: 2000 V.
   (b) Ion Polarity: Positive.
   (c) Sheath Gas Pressure: 2 units.
   (d) Ion Sweep: 0.0.
   (e) Aux Gas Pressure: 0 units.
   (f) Capillary Temperature: 250 °C.
   (g) Skimmer Offset: 0 units.
   (h) Collision Pressure: 1.5 units.
   (i) Collision Energy: 25 eV ± 10 %.
8. MS parameters include:
   (a) Full scan resolution: 35,000.
   (b) MS/MS scan resolution: 17,500.
   (c) Number of MS/MS scan per cycle: 10.
   (d) Normalized Collision Energy: 25.
   (e) Dynamic exclusion window: 12 s.

## 3 Methods

### *3.1 Stepwise Procedure (See Note 1)*

1. Turn on heating block to 99 °C.
2. Put the 0.5 mL Eppendorf sample tube in a 1.5 mL Eppendorf tube adaptor.
3. Centrifuge samples for 1 min at 15,500 × *g*.
4. Thaw Digestion Buffer on the heating block, and then invert tube to mix.
5. Add 50 μL Digestion Buffer to each tube.
6. Remove tube from holder and incubate at 99 °C for 30 min.
7. Remove tubes from heating block and flick tubes to remove condensation from cap.

8. Sonicate samples for 5 min (*see* **Note 2**).
9. Repeat **steps 7–9** two more times.
10. Put the 0.5 mL Eppendorf sample tube into a 1.5 mL Eppendorf tube adaptor.
11. Centrifuge samples for 1 min at 15,500 × *g*.
12. Add 1.2 μL of 20 μg/mL trypsin solution to each sample by inserting tip of pipette into sample solution, and then dispense trypsin.
13. Incubate at 37 °C for 18–24 h.
14. Make a fresh solution of DTT buffer 50 mM dithiothreitol (DTT) in 2.5 % formic acid/5 % ACN/92.5 %water.
15. Add 5 μL of DTT solution (made in **step 14**) to each sample by inserting tip of pipette tip into sample solution, then dispense DTT.
16. For each sample, condition a new Zip Tip three times with 10 μL of mobile phase B, then 10 μL 1:1 mix of mobile phases A:B, and then three times with 10 μL of mobile phase A.
17. Draw up and dispense 10 μL of the digested sample using the conditioned Zip Tip for a total of 50 cycles.
18. Wash the Zip Tip with 3 × 10 μL of mobile phase A.
19. Add 5 μL of 1:1 mix of mobile phases A and B into an HPLC vial.
20. Wash the Zip Tip with the above 5 μL 1:1 mix of mobile phases A:B by pumping up and down seven times.
21. Dilute the eluate with 50 μL of mobile phase A.
22. Transfer supernatant to HPLC vials.
23. Place injection vials in autosampler.
24. Inject 4 μL of injection albumin digest (test mix) to perform instrument validation.
25. Inject 10 μL of samples.

### 3.2 Analysis

1. The resulting MS raw data are searched against human IPI database using the Protein Discoverer software (Version 1.3, ThermoFisher Scientific).
2. For database search using SEQUEST, search criteria include a mass tolerance window is set at 10 ppm for the precursors and 0.05 Da for the fragments.
3. For database search using SEQUEST, oxidation of methionine and methylation of lysine are used as variable modifications.
4. All positively identified proteins are sorted by the number of matched MS/MS spectra, and used in the final reviewing process along with imaging data and clinical presentation.

## 4 Notes

1. Minimize opening and closing sample tubes throughout the process to avoid keratin contamination.
2. Sonication lasting >5 min might damage the tubes.

### References


1. Merlini G, Bellotti V (2003) Molecular mechanisms of amyloidosis. N Engl J Med 349: 583–596
2. Howie AJ, Brewer DB, Howell D, Jones AP (2008) Physical basis of colors seen in Congo red-stained amyloid in polarized light. Lab Invest 88:232–242
3. Sipe JD, Benson MD, Buxbaum JN, Ikeda S, Merlini G, Saraiva MJ, Westermark P (2014) Nomenclature 2014: amyloid fibril proteins and clinical classification of the amyloidosis. Amyloid 21:221–224
4. Magy-Bertrand N, Dupond JL, Mauny F, Dupond AS, Duchene F, Gil H, Kantelip B (2008) Incidence of amyloidosis over 3 years: the AMYPRO study. Clin Exp Rheumatol 26:1074–1078
5. Leung N, Nasr SH, Sethi S (2012) How I treat amyloidosis: the importance of accurate diagnosis and amyloid typing. Blood 120: 3206–3213
6. Picken MM (2007) New insights into systemic amyloidosis: the importance of diagnosis of specific type. Curr Opin Nephrol Hypertens 16:196–203
7. Picken MM (2007) Immunoglobulin light and heavy chain amyloidosis AL/AH: renal pathology and differential diagnosis. Contrib Nephrol 153:135–155
8. Kebbel A, Rocken C (2006) Immunohistochemical classification of amyloid in surgical pathology revisited. Am J Surg Pathol 30:673–683
9. Said SM, Reynolds C, Jimenez RE, Chen B, Vrana JA, Theis JD, Dogan A, Shah SS (2013) Amyloidosis of the breast: predominantly AL type and over half have concurrent breast hematologic disorders. Mod Pathol 26:232–238
10. Rodriguez FJ, Gamez JD, Vrana JA, Theis JD, Giannini C, Scheithauer BW, Parisi JE, Lucchinetti CF, Pendlebury WW, Bergen HR III et al (2008) Immunoglobulin derived depositions in the nervous system: novel mass spectrometry application for protein characterization in formalin-fixed tissues. Lab Invest 88:1024–1037

# Chapter 8

# Quantitation of Ubiquinone (Coenzyme $Q_{10}$) in Serum/Plasma Using Liquid Chromatography Electrospray Tandem Mass Spectrometry (ESI-LC-MS/MS)

**Richard E. Mathieu Jr. and Catherine P. Riley**

## Abstract

Dietary ubiquinone (Coenzyme $Q_{10}$) is considered an essential co-factor in the mitochondrial respiratory chain responsible for oxidative phosphorylation. This oil-soluble vitamin-like substance is mobile in cellular membranes and plays a unique role in the electron transport chain (ETC). Coenzyme $Q_{10}$ ($CoQ_{10}$) is present in most eukaryotic cells and functions as an electron carrier and an antioxidant. Although the exact role of Coenzyme $Q_{10}$ is often debated; there is a growing interest in the measurement of $CoQ_{10}$ concentrations particularly in the area of cardiovascular disease, malignancies, exercise physiology, Parkinson's disease, and patients undergoing statin drug therapies. We describe a simple method for the quantitative measurement of the ammonium adduct of Coenzyme $Q_{10}$ using a high-pressure liquid chromatography combined with positive electrospray ionization tandem mass spectroscopy (ESI-LC-MS/MS) utilizing a 3 μm PFP(2) 50 × 2.0 mm 100 Å column. A stable isotopic deuterated internal standard, in the form of Coenzyme $Q_{10}$-[$D_9$], is added to the patient serum. The extraneous proteins are precipitated from the sample with ethanol and isolation of the targeted compound is facilitated by the addition of hexane to aide in the cleanup and recovery. Quantitation occurs via a 6-point calibration that is linear from 0.16 to 6.0 μg with an observed error of 6.2 % across the analytical range.

**Key words** Coenzyme $Q_{10}$, Ubiquinone, Mass spectroscopy, LC-MS/MS

## 1 Introduction

Coenzyme $Q_{10}$ or ubiquinone is a lipophilic molecule present in all tissues and cells. It is located mainly in the inner mitochondrial membrane and is an essential component in the mitochondrial respiratory chain [1–4]. Coenzyme $Q_{10}$ is responsible for oxidative phosphorylation, functions as an electron carrier, and serves as a potent antioxidant. It is an essential component of the electron transport chain and participates in aerobic cellular respiration, generating energy in the form of ATP [3].

The measurement of Coenzyme $Q_{10}$ concentrations have been primarily performed utilizing high-pressure liquid chromatography

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_8, 

(HPLC) combined with florescence or electrochemical detection. The HPLC methodology can lend itself to extended run times, large sample volumes, subjective interpretations, and in adequate sensitivities.

Mindful of the numerous unique features and the idiosyncratic nature of the $CoQ_{10}$ molecule, we set out to improve the detection of this molecule utilizing positive electrospray ionization tandem mass spectroscopy (ESI-LC-MS/MS). Our aim was to improve the sensitivity and to simplify the extraction process to create a robust method suitable for the routine analysis of Coenzyme $Q_{10}$ from serum or plasma samples. Due to the instability and ease of degradation of the Coenzyme $Q_{10}$ molecule, the concentration of the calibration material was confirmed by measuring the absorption using a UV spectrophotometer and the respective extraction coefficient to verify the integrity of the material [5]. Six levels of calibration are prepared by serial dilution in ethanol. A stable isotopic deuterated internal standard, in the form of Coenzyme $Q_{10}$-[$D_9$], is added to 200 µL of patient serum and the proteins are precipitated using ethanol and mixed by vortexing, followed by the addition of *n*-hexane to facilitate the separation of the $CoQ_{10}$ from the precipitated proteins and the aqueous-ethanolic layer into the upper organic layer. The resuspended extraction is injected across a 3 µm PFP(2) 50 × 2.0 mm 100 Å column using 2 mM Ammonium acetate mobile phase [6, 7].

## 2 Materials

### 2.1 Samples

Serum or plasma (EDTA or heparinized). Protect from light following collection, storage, and shipment. Separate plasma or serum from cells within 1 h of collection. Samples are stable for 1 week refrigerated and 1 month when frozen at −20 °C.

### 2.2 Solvents and Reagents

1. Ethanol, 200 proof, Molecular Biology Grade 99.5 % (*see* **Note 1**).
2. Butylated hydoxyanisole (BHA) >98 %.
3. 1 M ammonium acetate solution: Prepare in deionized water.
4. 3 % Bovine Albumin: Prepare in 0.1 M phosphate buffer in 0.9 % sodium chloride.
5. Mobile Phase A (2.0 mM ammonium acetate): In a 1.0 L flask add 2.0 of 1 M ammonium acetate to 650 mL of LC/MS grade methanol. Bring the volume up by adding deionized water.
6. Mobile Phase B (2.0 mM ammonium acetate): In a 1.0 L flask add 2.0 mL of 1 M Ammonium acetate to 630 mL of LC/MS grade methanol. Bring the volume to 1.0 L by adding HPLC Grade 2-propanol.

### 2.3 Standards

1. Coenzyme $Q_{10}$, (Ubiquinone): Purity ≥98 % (HPLC Grade, Sigma-Aldrich).
2. Coenzyme $Q_{10}$ intermediate standard calibration material (50.0 μg/mL): Carefully weigh out 10.0 mg and add to 50.0 mL of HPLC Grade Hexane, swirl to mix. Add 150.0 mL of preserved reagent grade ethanol.
3. Coenzyme $Q_{10}$ working standard calibration material (6.0 μg/mL): The volume of Intermediate Standard is determined based on the spectrophotometric measurement of the intermediate standard and the extraction coefficient. The final volume of prepared working standard is 10.0 mL of preserved reagent grade ethanol.
4. Internal Standard, Coenzyme Q10-[$D_9$] (1 mg/mL): Acquired as powder from IsoSciences, King of Prussia, PA. Prepare by adding 1.0 mL of HPLC Grade *n*-Hexane to the vial.
   (a) Coenzyme $Q_{10}$ intermediate internal standard (50 μg/mL): Add the entire contents of the 1.0 mg/mL vial to 19.0 mL of preserved reagent grade ethanol.
   (b) Coenzyme $Q_{10}$ working internal standard (3.0 μg/mL): Add 600 μL of intermediate to 9.4 mL of preserved reagent grade ethanol.

### 2.4 Preparation of Calibrators and Controls

1. Calibrators: Label an appropriate number of glass tubes: *Cal 1*, *Cal 2*, *Cal 3*, *Cal 4*, *Cal 5*, and *Cal 6*. Add 200 μL of working calibration standard solution to *Cal 5* and *6*. To the tube marked Cal 5 add 200 μL of reagent grade ethanol. To the remaining tubes (4, 3, 2, and 1) add 667 μL of reagent grade ethanol. Add 333 μL of working standard calibrator to the tube marked *Cal 4*. Mix by vortexing. Draw 333 μL from the well mixed Cal 4 and add to Cal 3, serially dilute to Cal 1 discarding the final 333 μL from Cal 1 (*see* **Note 2**). Thorough mixing should occur between the transfers of the diluted calibrators prior to the transfer. Transfer 200 μL of each of the prepared calibrators to a respective clean tube; calibrators are to be extracted with the run (*see* Table 1).
2. Controls: Chromsystems coenzyme Q10 plasma control Bi-Level I+II (Chromsystems, GmbH, Gräfelfing, Germany): Control material is prepared per the manufacturer's instructions; 2.0 mL of deionized water is added to the lyophilized material and allowed to stand at least 15 min prior to use. A negative control is prepared utilizing 3.0 % bovine albumin.

### 2.5 Equipment and Supplies

1. Instrumentation: Applied Biosystems Inc. API 3200 with Shimadzu Prominence 20A HPLC system equipped with Analyst software version 1.6.1. Operating parameters are described in Table 2a, b. Parameters have been optimized specifically for the API 3200 with Shimadzu 20A Prominence

**Table 1**
**Preparation of the calibrators**

| Calibrator | Volume of stock standard (μL) | Volume of ethanol (μL) | Final concentration (μg/mL) | Final dilution |
|---|---|---|---|---|
| 6 | 200 | – | 6.0 | – |
| 5 | 200 | 200 | 3.0 | 1:2 |
| 4 | 333 | 667 | 2.0 | 1:3 |
| 3 | – | 667 | 0.67 | 1:3 |
| 2 | – | 667 | 0.22 | 1:3 |
| 1 | – | 667 | 0.07 | 1:3 |

**Table 2**
**ESI-LC-MS/MS operating conditions**

| | | | | | |
|---|---|---|---|---|---|
| A. HPLC | | | | | |
| *Column temp* | | 40 °C | | | |
| *Flow rate* | | 0.80 mL/min | | | |
| *Gradient* | *Step* | *Time (min)* | *Module* | *Event* | *Parameter (%)* |
| | 1 | 0.10 | Pumps | Pump "B" conc | 0.0 |
| | 2 | 0.50 | Pumps | Pump "B" conc | 25 |
| | 3 | 1.00 | Pumps | Pump "B" conc | 60 |
| | 4 | 1.10 | Pumps | Pump "B" conc | 60 |
| | 5 | 2.00 | Pumps | Pump "B" conc | 80 |
| | 6 | 2.10 | Pumps | Total flow | 0.85 mL/min |
| | 7 | 2.10 | Pumps | Pump "B" conc | 80 |
| | 8 | 2.80 | Pumps | Pump "B" conc | 100 |
| | 9 | 3.50 | Pumps | Pump "B" conc | 100 |
| | 10 | 3.70 | Pumps | Pump "B" conc | 0.0 |
| | 11 | 5.00 | System controller | Stop | |
| B. MS/MS settings | | | | | |
| *Scan* | | | MRM | | |
| *Polarity* | | | Positive | | |
| *Curtain gas* | | | 20.0 psi | | |
| *GS1* | | | 30.0 psi | | |
| *GS2* | | | 35.0 psi | | |
| *NC* | | | 3.00 | | |
| *CXP* | | | 7.00 | | |
| *EP* | | | 10.0 | | |
| *TEMP* | | | 400.0 | | |
| *Compound* | | *Q1 (m/z)* | *Q3 (m/z)* | *DP* | *CP (eV)* |
| Coenzyme $Q_{10}$-1 | | 880.7 | 197.3 | 50.0 | 45.0 |
| Coenzyme $Q_{10}$-2 | | 880.7 | 237.3 | 50.0 | 45.0 |
| Coenzyme $Q_{10}$-[$D_9$] | | 889.7 | 206.3 | 50.0 | 45.0 |

HPLC System; some variations would be expected as tune settings often vary slightly between instruments.

2. Column: Phenomenex LUNA 3 μm PFP(2) 50 × 2.00 mm 100 Å Column(2).
3. Spectrophotometer: Thermo Scientific Genesys 10S UV–Vis Spectrophotometer (275 nm).

## 3 Methods

### 3.1 Preparation of Standards

Each time new working standard is prepared from the intermediate stock material its concentration must be verified. This step is performed to help monitor for signs of potential degradation of the intermediate material (*see* **Note 2**). Calculate the concentration of the working standard as follows:

1. Set the spectrophotometer to a wavelength of 275 nm.
2. Pipette the intermediate standard into three cuvettes and obtain an OD reading on each sample (blanking with ethanol). Average the three results.
3. Calculate the concentration using the equation:

$$C_{(.g/mL)} = {}^{a}\text{Abs} \,/\, \varepsilon_{275}{}^{b} \times 10^{4}$$

[a]Abs = average absorbance obtained from the three replicates,

[b]$\varepsilon_{275}$ = the molar absorptivity for Coenzyme $Q_{10}$(162 dL/g/cm).

4. Calculate the volume required as follows:

$$V_s = V_i \times C_i \,/\, C_s$$

$V_s$ = volume of stock standard used to prepare intermediate standard.

$V_i$ = final volume of intermediate standard to be prepared (10 mL).

$C_i$ = target concentration of intermediate standard (6.0 μg/mL).

$C_s$ = concentration of stock standard (μg/mL).

### 3.2 Extraction and Analysis of Samples

1. Label an appropriate number of glass tubes in a manner that they can be positively identified as unique for the sample or material being tested. Place in a rack such that identifiers can be seen and matched to the sample ID.
2. Transfer 200 μL of patient sample, control, and calibrator to its corresponding tube.
3. Add 20 μL of the Coenzyme Q10 Internal Standard to each tube.

4. Add 200 µL of reagent grade ethanol to the serum to aid in precipitating the proteins from the serum, mix.
5. Add 2.0 mL of hexane to each tube; cap or cover tubes.
6. Following the addition of the hexane vortex samples for 10–15 min at a moderate speed setting. Insure that the internal standard, hexane, and sample are uniformly suspended into the solution.
7. Centrifuge the samples at >5000 × *g* for 10 min to remove the precipitated proteins from the sample.
8. Carefully remove the samples from the centrifuge so as not to disturb the separated mixture, visually inspect the tubes to insure the upper organic layer is free from particles or suspended solids. Place the samples in a rack that allows for good air movement.
9. Place the racked samples in a < −65 °C freezer for no less than 20 min. This step serves to solidify and freeze the aqueous portion of the solution.
10. Remove the tubes caps and pour off the supernatant organic layer to a clean properly labeled tube; verify that the aqueous portion is frozen and remains frozen during the pour off (*see* **Note 3**).
11. Using dry nitrogen remove the organic layer at pressure setting of 10–15 psi and a temperature of 23–42 °C for 10–12 min.
12. Insure that the tubes are dry and none of the organic solvent remains, a residual film may or may not remain, add 100 µL of reagent grade ethanol to each tube and vortex each tube 5 s.
13. Transfer reconstituted sample from the tube to appropriately labeled vials containing 150 µL spring inserts, cap vials. Samples are now ready for analysis.
14. Load samples on to the ESI-LC-MS/MS and inject 10 µL of sample.

### 3.3 Calibration Acceptability

1. Instrument settings and operating conditions are provided in the materials Subheading 2.5 and Table 2a, b.
2. Data analysis is performed using Multiquant software version 2.1 (AB Sciex Inc).
3. A unique standard curve (Calibrators 1–6) is extracted and generated with each analytical run.
4. Curves are linear via $1/X$ for the regression calculation and are generated based on the analyte/INSTD peak-area ratio using the qualifying ions from Table 2b.
5. Acceptability of the calibration is confirmed by satisfying that the following conditions are met:

(a) Abundance ratios between calibrator and internal standard (INSTD) should be within acceptable limits, ±20 %.

(b) All calibrators must have acceptable chromatography, retention time, and pattern.

(c) The correlation coefficient ($r$) for the curves generated must be ≥0.99 (*see* **Notes 4**–7).

### 3.4 Quality Control Acceptability

1. The negative control must demonstrate the presence of internal standard, meet the qualitative criteria, and have a concentration less than 0.16 mg/L.
2. The positive controls must have acceptable chromatography, retention time, ion pattern, and ratios.
3. Control values must fall within ±2SD of the target values established by the department.
4. Controls must follow accepted Westgard rules.

### 3.5 Specimen Acceptability

1. Symmetrical peak shape, no significant peak leading, tailing, or splitting.
2. There must be no interfering peaks in extracted ion chromatograms.
3. Good separation and peak resolution.
4. The retention time is consistent with the calibrators and quality control within the run.
5. A typical chromatogram is shown in Fig. 1.

## 4 Notes

1. Ethanol used in the preparation of stock calibration and internal standard material is preserved with a 0.1 % of butylated hydoxyanisole (BHA) in an effort to help stabilize the molecule during storage of the prepared reagents. Unpreserved reagent grade ethanol is used in the preparation of the calibration curve, precipitating reagent, and reconstitution solvent.
2. Protect calibration material from light and prolonged exposure to ambient temperature.
3. It is critical that the aqueous layer remain frozen during the pour. Insure that samples remain frozen during processing and if necessary split the batch into manageable portions.
4. Calibrator 1 is processed as an "Unknown" in the Multiquant software as a result of this point falling below the limit of detection (LOD) of this assay.
5. If any one point does not lie on the curve, it can be removed and the run reprocessed. If the absence of this point means that

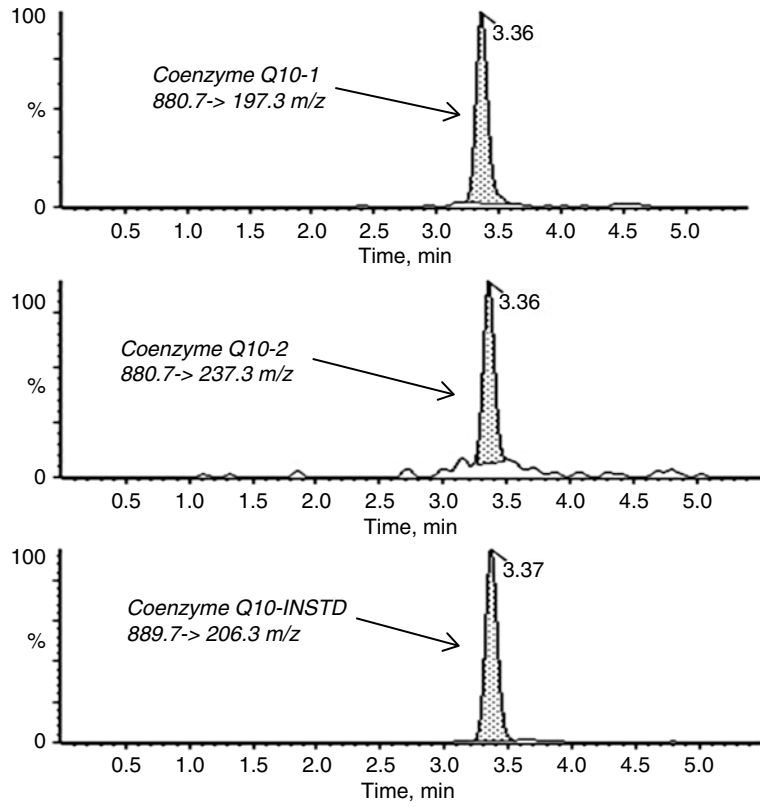



**Fig. 1** Chromatograms for Coenzyme Q10 and INSTD

the curve has a shape dissimilar to that of the generally accepted calibration curve, or more than one point appears to be anomalous, then the assay should be repeated. Points dropped from the upper or lower end of the run must be evaluated to insure that the deletion of that point does not jeopardize the upper or lower range of linearity for the analyte. If the technologist has any reason to question the integrity of the starting point, it is incumbent on them to re-extract the run.

6. Due to the instability resulting in the breakdown of calibration material due to prolonged exposure to light and ambient environments, it is incumbent to utilize the Multiquant software to overlay calibration curves against established reference curves to insure the integrity of the calibration material and closely monitor QC data for trends or bias in the material indicating that a breakdown of the material might be occurring.
7. Interference studies were performed using commonly encountered sample conditions (hemolysis, lipemia, and icterus), anticoagulants (EDTA and Heparin) and other endogenous materials (tocopherol, retinol, and phylloquinone); no interferences were observed. Ion suppression studies were performed using a sample infusion method.

## Acknowledgment

We would like to acknowledge Dawn Goertz for her help editing and directing the writing of this chapter.

### References

1. Ernster L, Dallner G (1995) Biochemical, physiological and medical aspects of ubiquinone function. Biochim Biophys Acta 1271(1): 195–204
2. Dutton PL, Ohnishi T, Darrouzet E, Leonard MA, Sharp RE, Cibney BR, Daldal F, Moser CC (2000) Coenzyme Q oxidation reduction reactions in mitochondrial electron transport. In: Kagan VE, Quinn PJ (eds) Coenzyme Q: Molecular mechanisms in health and disease. CRC Press, Boca Raton, pp 65–82
3. Bhalerao S, Clandinin TR (2012) Vitamin K2 Takes Charge. Science 336(6086):1241–1242
4. Tomasetti M, Alleva R, Borghi B, Collins AR (2001) In vivo supplementation with coenzyme Q10 enhances the recovery of human lymphocytes from oxidative DNA damage. FASEB J 15: 1425–1427
5. Kotnik D, Jazbec-Krizman P, Krizman M et al (2013) Rapid and sensitive HPLC-MS/MS method for quantitative determination of CoQ10. Research on Precision Instrument and Machinery (RPIM) 2: (10) www.Seipud.org/rpim
6. ESA Application Note 5600A: Simultaneous analysis of carotenoids, retinoids, tocopherols, vitamin K and Coenzyme Q10 in plasma; ESA Inc, 22 Alpha Rd, Chelmsford, MA 01824 10-1176P IA-2
7. Tang P, Miles MV, DeGrauw A et al (2001) HPLC Analysis of Reduced and Oxidized Coenzyme Q10 in Human Plasma. Clin Chem 47(2):256–265

# Chapter 9

# Quantitative Analysis of Salivary Cortisol Using LC-MS/MS

Yan Victoria Zhang

## Abstract

Cortisol is one of the most important glucocorticoids and plays important roles in regulating human metabolism. Midnight salivary cortisol has been shown to correlate well with free cortisol concentration in serum and is one of the first tests recommended for the diagnosis of Cushing's syndrome.

The procedure described here involves centrifugation of the saliva samples to remove solids and mucus strands before they are diluted with buffer and mixed with deuterated internal standard D4-cortisol. The samples are then subjected to reverse phase separation on a C18 column and analyzed by a tandem mass spectrometry method (LC-MS/MS). Quantification is achieved by comparing the responses of a given sample to the responses of the calibrators of known concentrations. The calibrators are prepared and analyzed along with the patient samples. Analytical specificity is ensured by using multiple reaction monitoring with fragment ions that are unique to cortisol and deuterated internal standard.

**Key words** Saliva, Cortisol, Cushing's syndrome, Multiple reaction monitoring, Liquid chromatography, Tandem mass spectrometry

## 1 Introduction

Cushing's syndrome is one of the most common diseases caused by overproduction of cortisol. Cortisol, also known as hydrocortisone, is produced in the cortex (zona fasciculata) of the adrenal gland as one of the most important glucocorticoids. It plays very important roles in regulating the metabolism of glucose and facilitating the metabolisms of carbohydrate, fat, and protein [1–4].

Cortisol follows a diurnal variation with maximum levels in early morning and minimum levels around midnight. Cushing's syndrome leads to a loss of the diurnal variation in cortisol levels, so that patients do not experience a late night decrease in plasma concentrations.

Physicians have relied on a number of primary tests for diagnosis of Cushing's syndrome [5]. Overall, levels of free cortisol are often considered most clinically relevant and are used for the diagnosis of Cushing's syndrome. Among different tests, salivary cortisol has gained significant momentum in recent years due to its

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_9, 

several unique advantages. To name a few, saliva sampling is noninvasive, salivary cortisol is relatively stable even at room temperature, and, more importantly, salivary cortisol levels correlate well with the free serum cortisol and are not affected by the different levels of corticosteroid-binding globulin or saliva flow rate [6–8]. In addition, salivary cortisol has demonstrated high levels of diagnostic capability for Cushing's syndrome with a specificity of 93–100 % and a sensitivity of 92–100 % [6, 7, 9–13]. Late-night salivary cortisol was recommended as one of the first-line diagnostic tests for Cushing's syndrome by the Endocrine Society [14].

Salivary cortisol can be measured by various methods. Radioimmunoassay (RIA) and enzyme immunoassay are the most popular early methods. The high levels of cross-reactivity to other steroids and lack of standardization among immunoassays have raised a lot of concerns for immunoassay [15, 16]. High Performance Liquid Chromatography (HPLC) methods, although offer increased specificity, do not provide enough sensitivity to measure steroids at physiological levels.

Methods using liquid chromatography and tandem mass spectrometry (LC-MS/MS) have shown the advantages of great specificity and sensitivity for cortisol [17–19]. LC-MS/MS methods have been considered as the reference and the most popular technology for measuring salivary cortisol [20–22]. Since saliva is relatively clean sample matrix, the samples can be either extracted first or "dilute and shoot." Extraction method can process samples with either off-line solid phase extraction (SPE) or online solid phase extraction [17, 23]. A simple "dilute and shoot" method may need longer LC separation time to ensure complete separation of cortisol from other compounds. We use this protocol as the example in this section to describe the analysis of cortisol.

## 2 Materials

### 2.1 Samples

Saliva samples are collected using a Salivette collection device (Sarstedt, Nümbrecht, Germany) with a minimum sample volume of 0.5 mL (*see* **Note 1**). Samples are stable at room temperature for 7 days, refrigerated 2 weeks or frozen for 12 months.

### 2.2 Solvents and Reagents

1. Cortisol, 1 mg/mL in methanol (Cerilliant, Round Top, TX).
2. Internal standard: deuterated D4Cortisol –9,11,12,12 (CDN Isotopes, Pointe Claire, Quebec, Canada).
3. Mobile Phase A: 0.1 % formic acid in water.
   Mix 990 mL of Type 1 deionized water with 1.0 mL of formic acid in a 1000 mL volumetric flask and add water to the final volume of 1000 mL. Filter through a 0.45 μm polypropylene membrane. Storage: room temperature for 1 month.

4. Mobile Phase B: 0.1 % formic acid in methanol.
   Mix 1000 mL of HPLC grade methanol with 1.0 mL formic acid. Storage: room temperature for 6 months.
5. Phosphate buffered saline: 8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4$, 0.24 g of $KH_2HPO_4$ are dissolved in 1 L Type I water and pH is adjusted to 7.4 with 0.5 M HCl or 0.5 M NaOH (*see* **Note 2**).

### 2.3 Standards and Calibrators

1. Cortisol stock solution (100 μg/mL in methanol) is prepared by diluting 1.0 mL of Cortisol (1 mg/mL) in methanol to a final volume of 10.0 mL. Store at −20 °C, expiration 1 year.
2. Calibration Standards for Cortisol are laboratory-prepared by diluting Cortisol stock solution with Phosphate Buffered Saline (PBS) to five target concentrations: 50, 100, 500, 2000, and 10,000 ng/dL. Calibrators are divided into aliquots and stored frozen at −20 °C, expiration 2 years.

### 2.4 Quality Controls and Internal Standard

3. Internal Standard stock solution (Cortisol-D4 100 μg/mL in methanol): prepare by weighing out 1.0 mg Cortisol-D4 and dissolving in 10 mL methanol. Store at −20 °C, expiration 2 years.
4. Internal Standard working solution (Cortisol-D4 500 ng/mL in methanol): dilute 50 μL Cortisol-D4 stock solution to final volume of 10.0 mL in methanol. Store at −20 °C, expiration 1 year.
5. URTS (Unextracted Retention Time Standard): Cortisol and Cortisol-D4, each at 5.0 ng/mL in 50 % methanol (*see* **Note 3**).
6. Quality Control samples are laboratory-prepared in PBS using Cortisol stock, and targeted to 100, 600, and 3000 ng/dL. Controls should be stored frozen at −20 °C and expire 1 year after preparation (*see* **Note 4**).
7. Separate cortisol stocks are prepared for dilution to calibrators and for dilution to controls.

### 2.5 Analytical Equipment and Supplies

1. Shimadzu Prominence HPLC system consisting of pumps [2], solvent degasser, autosampler, diverter valve, and system controller (Shimadzu, Kyoto, Japan).
2. AB Sciex API 5000 tandem mass spectrometer with electrospray source, running Analyst 1.5 software (ABSciex, Framingham, MA).
3. HPLC column: Onyx C18, 50 × 2.0 mm (Phenomenex, Torrance, CA).
4. Guard cartridge 5 × 3 mm (Phenomenex, Torrance, CA).
5. Salivette Cortisol collection device (Sarstedt, Nümbrecht, Germany).
6. Fixed volume pipet (200 μL) (VistaLab, Brewster, NY).

7. Electronic single channel adjustable volume pipet (200–1000 µl) (Sartorius Biohit).
8. Eppendorf-plus pipettor (Eppendorf).
9. Vortex mixer, multi-tube (VWR).
10. Autosampler vials and caps (SRI).

## 3 Methods

### 3.1 Sample Preparation Stepwise Procedure

1. Collect saliva using a "Salivette for Cortisol Testing" collection device by following the instructions (*see* **Note 1**). The samples are shipped back to the laboratory.
2. The patient Salivette is spun for 3 min in the lab at 1600 × *g* to recover saliva, and the insert is discarded.
3. Samples are prepared for injection by adding to autosampler vials in the following order: (1) 800 µL Mobile Phase A, (2) 10 µL ISTD working solution, and (3) 200 µL patient saliva, calibrator, or QC. Vials are capped, mixed, and placed in autosampler.

### 3.2 HPLC Method

1. See Table 1 for solvent composition and follow rate parameters.
2. Autosampler injects 40 µL on to the C18 Monolithic silica HPLC column equilibrated at 5 % methanol (5 % mobile phase B) and flowing initially at 1 mL/min (*see* **Note 5**).
3. After 30 s, the methanol concentration is rapidly increased to 50 % and held there while flow is decreased to 0.6 mL/min to allow for separation of cortisol peaks from other compounds.

**Table 1**
**HPLC gradient parameters**

| Time (min) | Total flow (mL/min) | Pump B concentration (%) |
|---|---|---|
| 0 | | 5 |
| 0.4 | 0.6 | 5 |
| 0.5 | 0.6 | 50 |
| 1.9 | 0.6 | 50 |
| 2.0 | 0.6 | 100 |
| 2.6 | 0.6 | 100 |
| 2.8 | 1.0 | 100 |
| 2.9 | 1.0 | 100 |
| 3.0 | 1.0 | 5 |
| 3.5 | 1.0 | 5 |

Only flows between 1.4 and 3 min are sent to MS

4. The Mobile Phase B concentration is raised to 100 % methanol at 1.0 mL/min to wash away any late-eluting components, and prepare for the next injection. At 3 min the concentration returns to 5 % B to equilibrate column for the next injection.
5. For the first 1.4 min of the run, column eluate is diverted to waste, then the diverter valve directs flow into the API-5000 until 3.0 min of run time, when flow again returns to waste.
6. Chromatography is performed at ambient temperature; Cortisol elutes at about 2.4 min, and total run time is 3.5 min.

## 4 MS Method

1. MS/MS parameters for gas, temperature, and voltage settings are summarized in Table 2. The MRM transitions and corresponding parameters for Cortisol and Internal Standard fragment ions monitored in the assay are shown in Table 3 (*see* **Note 6**).

**Table 2**
**MS/MS operating parameters**

| Ionization mode | Positive |
|---|---|
| Ion source | Turboionspray (ESI) |
| Curtain gas | 25 |
| CAD gas | Med |
| Ionspray voltage | 3000 V |
| Temperature | 500 °C |
| Gas 1 | 40 |
| Gas 2 | 40 |
| Declustering potential | 125 |
| Entrance potential | 10 |

**Table 3**
**MRM transitions and corresponding parameters**

| Compound | Q1 mass | Q3 mass | Dwell time (ms) | CE | CXP |
|---|---|---|---|---|---|
| Cortisol 1 | 363.4 | 121.1 | 100 | 36 | 18 |
| Cortisol 2 | 363.4 | 97.4 | 100 | 42 | 15 |
| D4-cortisol | 367.4 | 121.1 | 75 | 33 | 15 |

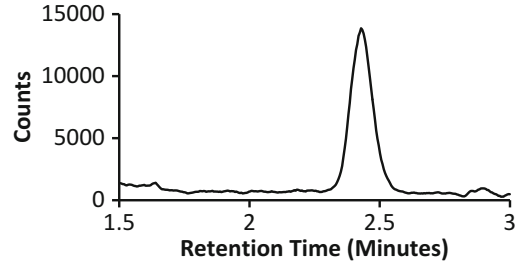



**Fig. 1** Total ion chromatogram of cortisol assay with cortisol eluted at 2.42 min

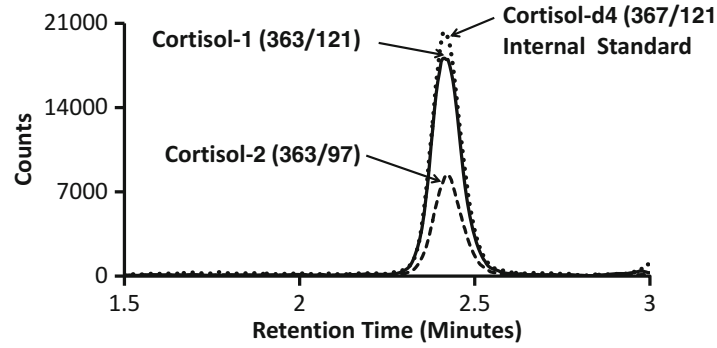



**Fig. 2** MRM transitions of cortisol (363/121 and 363/97) and the internal standard (367/121)

2. A representative LC-MS/MS total ion chromatogram of Cortisol and the MRM transitions of cortisol and the internal standard are shown in Figs. 1 and 2, respectively.
3. Two cortisol fragmentations are monitored: 363/121 defines the quantifier ion, and 363/97 the qualifier ion.

### 4.1 Data Analysis

1. The LC-MS/MS data is analyzed using the quantitation functions in Analyst Software (ABSciex).
2. The measured Cortisol peak areas corresponding to the quantifying ions and internal standards are used to construct calibration lines, by plotting the ratio of cortisol/ISTD areas against cortisol concentration. From this line, the concentration of unknown samples and controls can be determined. A least-squares regression line with 1/x weighting is employed.
3. The limit of quantitation of the method is 50 ng/dL. Patient levels (for healthy patients) are frequently below this level, and samples beyond the range of the calibration line are not likely to be encountered. A typical calibration curve has correlation coefficient ($R^2$) of >0.99 (*see* **Notes 7** and **8**).
4. Typical intra- and inter-assay imprecision is <10 %.

5. Quality control: All three levels of QC standards are run together with calibration standards and patient samples in the Salivary Cortisol assay. QC specimens are analyzed immediately after the calibrators and before the patient samples. After the patients, the calibrators and QC are injected again. QC acceptability is determined based on Westgard multi-rules (*see* **Note 4**).
6. Alternatively, if Westgard rules are not used, the analytical run may be considered acceptable if the calculated concentrations of cortisol in the controls are within +/− 20 % of target values.
7. Ion Ratio Agreement: The quantifier ion in the sample is considered acceptable if the ratios of qualifier ions to quantifying ion are within +/− 20 % of the average ion ratios for the calibrators (*see* **Note 9**).

## 5 Notes

1. The following collection instructions for the Salivary Cortisol sample are provided to the patient, along with the Salivette device(s):
   (a) Do not brush teeth before collecting sample.
   (b) Do not eat or drink for 15 min prior to sample collection.
   (c) Collect a sample between 11 p.m. and midnight or a different collection time instructed by the doctor such as 7–9 a.m. or 3–5 p.m.
2. We used PBS as dilution buffer. Mobile phase A can also be used as the dilution buffer to replace PBS. The comparison from our results did not show significant difference between those two types of diluent buffer.
3. This is used as a positive control for the system.
4. New control lots must be validated by running them 5–10 times as unknowns. A representative mean and standard deviation for the new lot will be calculated for future use.
5. This Technique uses simple "dilute and shoot" approach, and the HPLC columns can become fouled relatively quickly. Use of a pre-column filter and especially a guard column is recommended. And other sample cleaning methods such as using solid phase extraction can also be applied.
6. Mass spectrometry cannot separate compounds with the same $m/z$ ratios which can potentially cause interference and inaccurate quantification. Hydrocortisone is used as a medicine which is the same as cortisol. Therefore, mass spectrometry cannot separate hydrocortisone from cortisol, and presence of hydrocortisone gives falsely high cortisol results. The present

technique picks up no detectable interferences from other drugs commonly seen in patients.

7. The limit of quantitation for salivary cortisol was determined to be 50 ng/dL. Values less than 50 should be reported as <50 ng/dL. Linearity has been demonstrated to 100,000 ng/dL for this assay; samples that exceed the highest point of the calibration curve (10,000 ng/dL) should be re-tested on dilution from the original tube, using PBS as diluent.
8. No carry-over was observed up to a spiked-cortisol level of 10 μg/mL (or 1,000,000 ng/dL).
9. Detected cortisol can be falsely elevated in the presence of prednisolone. Since this interference is reflected in the calculated peak ratio values, significant prednisolone interference will cause a rejection of the analytical data (because the ratios are out of range) rather than the reporting of an incorrect value.

## References


1. van Eck M, Berkhof H, Nicolson N, Sulon J (1996) The effects of perceived stress, traits, mood states, and stressful daily events on salivary cortisol. Psychosom Med 58:447–458
2. Jacobson L, Sapolsky R (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12:118–134
3. Giubilei F, Patacchioli FR, Antonini G, Sepe Monti M, Tisei P, Bastianello S, Monnazzi P, Angelucci L (2001) Altered circadian cortisol secretion in Alzheimer's disease: clinical and neuroradiological aspects. J Neurosci Res 66:262–265
4. Cleare AJ, Bearn J, Allain T, McGregor A, Wessely S, Murray RM, O'Keane V (1995) Contrasting neuroendocrine responses in depression and chronic fatigue syndrome. J Affect Disord 34:283–289
5. Taylor RL, Machacek D, Singh RJ (2002) Validation of a high-throughput liquid chromatography-tandem mass spectrometry method for urinary cortisol and cortisone. Clin Chem 48:1511–1519
6. Woolston JL, Gianfredi S, Gertner JM, Paugus JA, Mason JW (1983) Salivary cortisol: a nontraumatic sampling technique for assaying cortisol dynamics. J Am Acad Child Psychiatry 22:474–476
7. Vining RF, McGinley RA, Maksvytis JJ, Ho KY (1983) Salivary cortisol: a better measure of adrenal cortical function than serum cortisol. Ann Clin Biochem 20(Pt 6):329–335
8. Hansen AM, Garde AH, Persson R (2008) Sources of biological and methodological variation in salivary cortisol and their impact on measurement among healthy adults: a review. Scand J Clin Lab Invest 68:448–458
9. Laudat MH, Cerdas S, Fournier C, Guiban D, Guilhaume B, Luton JP (1988) Salivary cortisol measurement: a practical approach to assess pituitary-adrenal function. J Clin Endocrinol Metab 66:343–348
10. Martinelli CE Jr, Sader SL, Oliveira EB, Daneluzzi JC, Moreira AC (1999) Salivary cortisol for screening of Cushing's syndrome in children. Clin Endocrinol (Oxf) 51:67–71
11. Putignano P, Toja P, Dubini A, Pecori Giraldi F, Corsello SM, Cavagnini F (2003) Midnight salivary cortisol versus urinary free and midnight serum cortisol as screening tests for Cushing's syndrome. J Clin Endocrinol Metab 88:4153–4157
12. Raff H, Raff JL, Findling JW (1998) Late-night salivary cortisol as a screening test for Cushing's syndrome. J Clin Endocrinol Metab 83:2681–2686
13. Yaneva M, Mosnier-Pudar H, Dugue MA, Grabar S, Fulla Y, Bertagna X (2004) Midnight salivary cortisol for the initial diagnosis of Cushing's syndrome of various causes. J Clin Endocrinol Metab 89:3345–3351
14. Nieman LK, Biller BM, Findling JW, Newell-Price J, Savage MO, Stewart PM, Montori VM (2008) The diagnosis of Cushing's syndrome: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 93: 1526–1540
15. Gatti R, Antonelli G, Prearo M, Spinella P, Cappellin E, De Palo EF (2009) Cortisol assays

and diagnostic laboratory procedures in human biological fluids. Clin Biochem 42:1205–1217

16. Miller R, Plessow F, Rauh M, Groschl M, Kirschbaum C (2013) Comparison of salivary cortisol as measured by different immunoassays and tandem mass spectrometry. Psychoneuroendocrinology 38:50–57
17. Kataoka H, Matsuura E, Mitani K (2007) Determination of cortisol in human saliva by automated in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. J Pharm Biomed Anal 44:160–165
18. De Palo EF, Antonelli G, Benetazzo A, Prearo M, Gatti R (2009) Human saliva cortisone and cortisol simultaneous analysis using reverse phase HPLC technique. Clin Chim Acta 405:60–65
19. Perogamvros I, Owen LJ, Newell-Price J, Ray DW, Trainer PJ, Keevil BG (2009) Simultaneous measurement of cortisol and cortisone in human saliva using liquid chromatography-tandem mass spectrometry: application in basal and stimulated conditions. J Chromatogr B Analyt Technol Biomed Life Sci 877:3771–3775
20. Rauh M (2009) Steroid measurement with LC-MS/MS in pediatric endocrinology. Mol Cell Endocrinol 301:272–281
21. Shackleton C (2010) Clinical steroid mass spectrometry: a 45-year history culminating in HPLC-MS/MS becoming an essential tool for patient diagnosis. J Steroid Biochem Mol Biol 121:481–490
22. Rege J, Nakamura Y, Satoh F, Morimoto R, Kennedy MR, Layman LC, Honma S, Sasano H, Rainey WE (2013) Liquid chromatography-tandem mass spectrometry analysis of human adrenal vein 19-carbon steroids before and after ACTH stimulation. J Clin Endocrinol Metab 98:1182–1188
23. Jones RL, Owen LJ, Adaway JE, Keevil BG (2012) Simultaneous analysis of cortisol and cortisone in saliva using XLC-MS/MS for fully automated online solid phase extraction. J Chromatogr B Analyt Technol Biomed Life Sci 881–882:42–48

# Chapter 10

# Quantification of Dihydroxyacetone Phosphate (DHAP) in Human Red Blood Cells by HPLC-TripleTOF 5600™ Mass Spectrometer

**Shuang Deng, David Scott, Douglas Myers, and Uttam Garg**

## Abstract

Triosephosphate isomerase (TPI) is a glycolytic enzyme which catalyzes the interconversion between glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). TPI deficiency results in accumulation of DHAP in human red blood cells and other tissues. The disease is characterized by congenital hemolytic anemia, and progressive neuromuscular dysfunction. The laboratory diagnosis is generally made by measurement of TPI activity in RBCs. Measurement of DHAP can be useful in further confirmation and follow-up of the disease. We developed HPLC/TOF-MS method for quantitation of DHAP in RBCs. The method involves simple protein precipitation, reverse phase C8 column chromatography, ion pairing with tributylamine, and long run time of 50 min to separate the two isomers (G3P and DHAP).

**Key words** Red blood cells, Dihydroxyacetone phosphate, Tributylamine (ion pair reagent), Production ion

## 1 Introduction

Dihydroxyacetone phosphate (DHAP) is a glycolytic metabolite and interconverts with glyceraldehyde-3-phosphate (G-3-P) by a glycolytic enzyme, triosephosphate isomerase (TPI). TPI deficiency is a rare autosomal recessive disease that presents with various clinical symptoms including hemolytic anemia and neuromuscular dysfunction [1–5]. Only <100 cases have been described in the scientific literature.

In healthy individuals, DHAP is maintained at low concentrations. However, in TPI deficiency, DHAP accumulates in RBCs and other tissues. The laboratory diagnosis is generally made by measurement of TPI activity in RBCs. Measurement of DHAP can be useful in further confirmation and follow-up of the disease. DHAP can be measured using enzymatic or chromatographic methods [6–8]. Enzymatic methods need specific enzymes and

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_10, 

reagents. Since TPI is a rare disorder, keeping enzymatic method ready could be challenging. Mass spectrometric methods are preferred since they do not need special reagents and are more specific as compared to enzymatic methods. We describe a HPLC/TOF-MS (time of flight mass spectrometry) method for the assay of DHAP in RBCs. The method involves simple protein precipitation, reverse phase C8 column chromatography, and ion pairing with tributylamine. The run time is long (50 min) to separate the two isomers (G3P and DHAP). Mass spectrometer, Triple TOF™ 5600 was used in a high sensitivity and product ion scan negative mode. The method was used in the measurement of RBCs DHAP levels in a patient diagnosed with TPI deficiency.

## 2 Materials

### 2.1 Samples

1 ml blood in sodium heparin (green top no gel) tube. Centrifuge blood at 1200 × *g* for 7 min in a refrigerated centrifuge. Discard the plasma and dilute packed RBCs in HPLC grade water (1:1). Sonicate the mixture for 2 min to break the RBC cell membranes. Store the hemolysate at −70 °C until analysis.

### 2.2 Solvents and Reagents

1. 0.3 N Zinc Sulfate (Sigma).
2. Tributylamine (Sigma).
3. Dihydroxyacetone phosphate lithium salt (Sigma).
4. Mobile phase A: 10 mM tributylamine, 15 mM acetic acid in HPLC grade water, pH 5.
5. Mobile phase B: Methanol.
6. Protein precipitation reagent: Methanol : 0.3 N $ZnSO_4$ (7:3).

### 2.3 Standards and Quality Controls

1. Red blood cell matrix for preparation of calibrators and controls: This is prepared using left-over blood submitted for immunosuppressant testing. Centrifuge blood at 1200 × *g* for 7 min in a refrigerated centrifuge. Discard the supernatant and dilute packed RBCs in HPLC grade water (1:1). Sonicate the mixture for 2 min to break the RBC cell membranes. Store the hemolysate at −70 °C until analysis.
2. Primary standard 1° (100 mM): Weigh 17 mg DHAP powder and dissolve into 1 ml HPLC grade water. Stable for 6 month when stored at −70 °C.
3. Secondary standard 2° (10 mM): Pipette 500 μl of primary standard and Q.S. to 5 ml with HPLC grade water. Stable for 6 month when stored at −70 °C.
4. Tertiary standard 3° (1 mM): Pipette 500 μl of secondary standard and Q.S. to 5 ml with HPLC grade water. Stable for 6 month when stored at −70 °C.

**Table 1**
**Preparation of calibrators**

| | 2°(µl) | 3° (µl) | RBC matrix | Final conc. (µM) |
|---|---|---|---|---|
| Cal 1 | | 100 | q.s. to 10 ml | 10 |
| Cal 2 | 100 | | q.s. to 10 ml | 100 |
| Cal 3 | 200 | | q.s. to 10 ml | 200 |
| Cal 4 | 500 | | q.s. to 10 ml | 500 |

RBC matrix was prepared using left-over blood submitted for immunosuppressant testing. DHAP concentration in this matrix was <10 µM

**Table 2**
**Preparation of quality controls**

| | 2°(µl) | 3° (µl) | RBC matrix | Final conc. (µM) |
|---|---|---|---|---|
| QC 1 | | 200 | q.s. to 10 ml | 20 |
| QC 2 | 150 | | q.s. to 10 ml | 150 |
| QC 3 | 400 | | q.s. to 10 ml | 400 |

RBC matrix was prepared using left-over blood submitted for immunosuppressant testing. DHAP concentration in this matrix was <10 µM

5. Calibrators are made according to Table 1 using standards.
6. Quality controls (QCs) are made according to Table 2 using standards (*see* **Note 1**).

### *2.4 Analytical Equipment and Supplies*

1. Triple TOF™5600 (AB Sciex).
2. Acuity UPLC (Waters).
3. Column: Agilent Eclipse XDB, C8, 3.5 µM, 4.6×150 mm.

## 3 Methods

### *3.1 Stepwise Procedure*

1. Pipette 100 µl calibrators, patient sample hemolysate or controls to 1.5 ml microcentrifuge tubes.
2. Add 200 µl protein precipitation reagent and vortex for 1 min.
3. Centrifuge for 10 min at 12,000×*g*.
4. Transfer 100 µl supernatants to autosampler vials.
5. Inject into 10 µl into the HPLC/TOF-MS.

### *3.2 Instrument Operating Conditions*

*See* Tables 3 and 4 for HPLC and mass spectrometer conditions (*see* **Notes 2** and **3**).

**Table 3**
**HPLC gradient and parameters**

| Time (min) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|
| Initial | 100 | 0 |
| 15 | 100 | 0 |
| 25 | 80 | 20 |
| 36 | 80 | 20 |
| 36.5 | 0 | 100 |
| 38 | 0 | 100 |
| 38.1 | 100 | 0 |
| 50 | 100 | 0 |

Column temperature –25 °C. Flow rate—0.2 ml/min

**Table 4**
**Mass spectrometry parameters**

| | |
|---|---|
| Ion Source Gas 1 (GS1) | 60 psi |
| Ion Source Gas 2 (GS2) | 60 psi |
| Curtain gas (CUR) | 30 psi |
| Temperature (TEM) | 450 °C |
| IonSpray voltage floating (ISVF) | –4000 V |

### *3.3 Data Analysis*

1. TOF-MS is used with electrospray ionization source in negative ion mode. The instrument is calibrated by taurocholic acid (2 ng/ml) before run (*see* **Note 4**). Data are collected using Analyst TF 1.6 software and quantified using MultiQuant software version 3.0 (AB Sciex).
2. Standard curves are generated based on linear regression of the analyte response ($y$) versus analyte concentration ($x$) using $m/z$ 97 as quantifying ion. Other ions are listed in Table 5 (*see* **Note 5**).
3. A typical calibration curve has a correlation ($r^2$) > 0.99.
4. Quality control samples are evaluated with each run. The acceptable results are within +/– 20 % of target values.
5. Typical chromatograms are shown in (Fig. 1).

**Table 5**
**DHAP specific parameters**

| Precursor ion | Product ion 1 | Product ion 2 | DP (V) | CE (eV) |
|---|---|---|---|---|
| 169 | 97 | 79 | −35 | −14 |

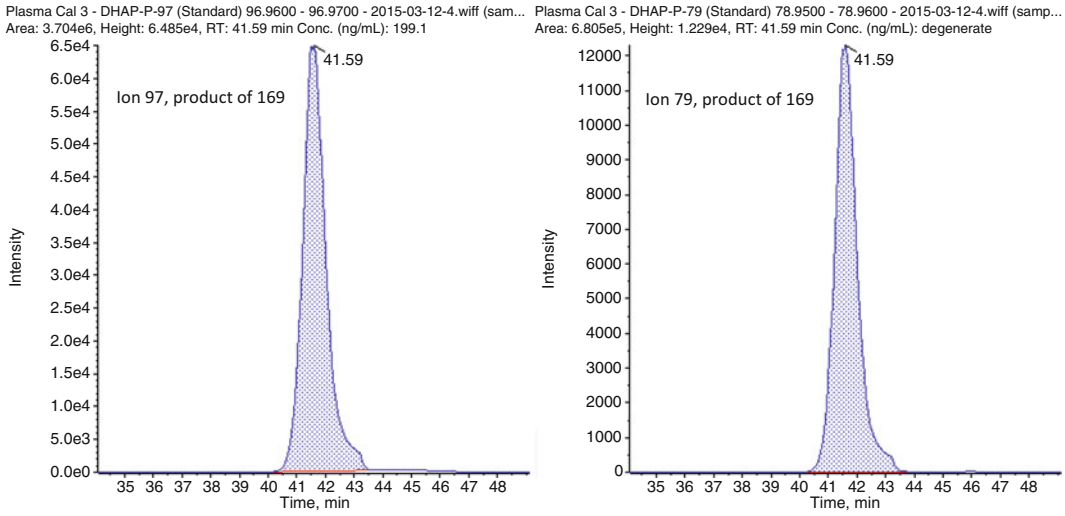



**Fig. 1** TOF-MS ion chromatograms for DHAP

## 4 Notes

1. Calibrators and quality controls are prepared separately using different normal RBC matrices. Of note, normal RBC matrices have DHAP concentrations <10 μM.
2. This is a time-consuming method and each run is 50 min. This is required to separate DHAP and its isomer glyceraldehyde-3 phosphate. Both the compounds have same precursor and product ions.
3. The use of ion pairing reagent TBA may contaminate the HPLC system and mass spectrometer. This may affect instrument sensitivity and calibration. Post-run cleaning may be needed to maintain instrument performance.
4. Instrument calibrant and calibration method may vary significantly among different instruments.
5. Results are expressed as μmol/l of hemolysate.

## References

1. Aissa K, Kamoun F, Sfaihi L, Ghedira ES, Aloulou H, Kamoun T, Pissard S, Hachicha M (2014) Hemolytic anemia and progressive neurologic impairment: think about triosephosphate isomerase deficiency. Fetal Pediatr Pathol 33:234–238
2. Clay SA, Shore NA, Landing BH (1982) Triosephosphate isomerase deficiency: a case report with neuropathological findings. Am J Dis Child 136:800–802
3. Olah J, Orosz F, Keseru GM, Kovari Z, Kovacs J, Hollan S, Ovadi J (2002) Triosephosphate isomerase deficiency: a neurodegenerative misfolding disease. Biochem Soc Trans 30: 30–38
4. Poll-The BT, Aicardi J, Girot R, Rosa R (1985) Neurological findings in triosephosphate isomerase deficiency. Ann Neurol 17:439–443
5. Schneider AS, Valentine WN, Hattori M, Heins HL Jr (1965) Hereditary hemolytic anemia with triosephosphate isomerase deficiency. N Engl J Med 272:229–235
6. Arias-Mendoza F, Pina E (1991) A sensitive multienzymatic assay for the measurement of pyruvate, dihydroxyacetone phosphate, oxaloacetate, and acetoacetate in clear extracts from biological samples. Prep Biochem 21:211–214
7. Huck JH, Struys EA, Verhoeven NM, Jakobs C, van der Knaap MS (2003) Profiling of pentose phosphate pathway intermediates in blood spots by tandem mass spectrometry: application to transaldolase deficiency. Clin Chem 49: 1375–1380
8. Luo B, Groenke K, Takors R, Wandrey C, Oldiges M (2007) Simultaneous determination of multiple intracellular metabolites in glycolysis, pentose phosphate pathway and tricarboxylic acid cycle by liquid chromatography-mass spectrometry. J Chromatogr A 1147: 153–164

# Chapter 11

# Simultaneous Quantitation of Estradiol and Estrone in Serum Using Liquid Chromatography Mass Spectrometry

**Catherine P. Riley, Richard E. Mathieu Jr., and Carmen Wiley**

## Abstract

Accurate measurement of the endogenous estrogens, estrone (E1) and estradiol (E2), is important in the clinical diagnosis and monitoring of multiple disorders. Typically, given the efficacy and low cost, radioimmunoassays (RIA) and enzyme-linked immunoassays (EIA) are used to quantify these hormones in biological samples. Unfortunately, at low levels these assays lack the necessary sensitivity and specificity for diagnosis of certain disorders in adult and pediatric endocrinology and oncology. In response to this need, we developed a fast and sensitive high performance liquid chromatography negative electrospray ionization tandem mass spectrometry (LC-MS/MS) method to measure serum estrone (E1) and estradiol (E2) without chemical derivatization. Samples are spiked with a stable isotopic carbon thirteen ($^{13}C$) labeled internal standard and the estrogens are isolated by liquid–liquid extraction (LLE) with hexane:Methyl-tert-butyl ether (MTBE) (9:1). Following centrifugation and dry down samples are reconstituted with deionized water, and separated on a C18 reverse phase column. The analytes are quantified using a six point calibration curve with a linearity of 2.6–625 pg/ml and with a variability of less than 8 % across analytical range.

**Key words** Estrogen, Estradiol, Estrone, Mass spectrometry, Reproductive hormones

## 1 Introduction

Female secondary sex characteristics and reproductive function are developed and maintained by the estrogen hormones. Estrogens are also responsible for regulation of the menstrual cycle, germ cells maturation, and sustentation of pregnancy [1–4]. In addition, estrogens play an important role in gender-specific processes, including nervous system maturation, bone growth and metabolism, and endothelial responsiveness [5–10]. In nonpregnant humans estrone (E1) and estradiol (E2) are the major biologically active estrogens. Estriol (E3), a third bioactive estrogen, plays a major role in sustaining pregnancy yet has no significant role in nonpregnant women or men [11].

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_11, 

Estradiol (E2) concentrations are widely utilized in the evaluation of reproductive function in females, including assessment of infertility, hypogonadism, amenorrhea, oligomenorrhea, menopausal status, as well as monitoring ovulation in preparation for in vitro fertilization. Simultaneous measurement of E1 and E2 can be used to help in diagnosis of inborn errors of sex steroid metabolism, precocious puberty, estrogen deficiency in men, fracture risk assessment in menopausal women, and polycystic ovarian syndrome [1–3]. Measured concentrations of E1 and E2 are also being used more frequently for therapeutic drug monitoring, in the context of either low-dose female hormone replacement therapy or antiestrogen treatment [12]. Furthermore, studies have shown a correlation between low estrogen levels and osteoporosis as well as cardiovascular and neurologic diseases [5–10].

Commonly, immunoassays are used in the clinic to measure the concentration of serum estrogens in biological samples. In some cases the traditional immunoassay approach to measuring estrogens is adequate; however, other clinical situations require extra sensitivity. Given their modest sensitivity, immunoassays are ineffective when attempting to measure low physiologic concentrations of estrogen as seen in men, children, and postmenopausal women. Moreover, immunoassays are unable to reproducibly elucidate the estrogens from interfering endogenous substances, resulting in poor specificity particularly at lower concentrations [13, 14]. The following chapter describes a simple, robust, highly sensitive method for the rapid evaluation of serum estradiol and estrone without chemical derivatization. Two hundred and fifty microliters of serum is spiked with a stable isotopic internal standard, in the form of estradiol 2,3,4-$^{13}C_3$ and the sample is extracted, dried down, and reconstituted. The concentrated sample is then separated on a XB-C18 50×2.1 mm; 2.6 µm 100 Å column followed by negative electrospray ionization multiple reaction monitoring mass spectrometry.

## 2 Materials

### 2.1 Samples

Serum or plasma. Separate serum or plasma from cells within 1 h of collection. If using SST tube, serum must be separated from gel within 24 h of draw.

### 2.2 Solvents and Reagents

1. Methanol, LC/MS Grade >99.9 %.
2. n-Hexane, HPLC Grade >95 %.
3. Methyl-*tert*-butyl ether (MTBE) HPLC 99.9 %.
4. Mobile Phase "A" Solution, 30 % Methanol (v/v): Add 300 ml of methanol to 1 l volumetric flask and bring to volume with deionized water, mix, and sonicate. Stable at room temperature up to 1 month.

5. Mobile Phase "B" Solution, 100 % Methanol (v/v): Add 1000 ml of methanol to 1 l volumetric flask and sonicate. Stable at room temperature up to 1 month.
6. Extraction Reagent 90 % Hexane & 10 % MTBE (v/v): The Extraction Reagent is prepared by adding 225 ml of Hexane (HPLC Grade) to 25 ml of Methyl-tert-butyl ether (MTBE). This reagent needs to be prepared in an approved fume hood due to volatility of the reagents. Stable for 3 months when stored at 2–8 °C.

### 2.3 Internal Standards, Calibration, and Control Material

1. Stock internal standard: Estradiol 2,3,4-$^{13}C_3$ (100 µg/ml) (Cerilliant, Round Rock, Texas) (*see* **Note 1**).
2. Intermediate internal standard: (100 ng/ml). The intermediate stock solution is prepared by adding 10 µl of labeled stock to 9.99 µl of acetonitrile (1:1000).
3. Working internal standard: (2.5 ng/ml). The working internal standard is prepared by adding 1.25 ml intermediate stock estradiol 2,3,4-$^{13}C_3$ to 48.75 ml of methanol.
4. Stock estradiol calibration material:Estradiol (1.0 mg/ml) (Cerilliant, Round Rock, Texas).
5. Intermediate estradiol calibration material: Estradiol (1.0 µg/ml). The stock material needs to be sonicated briefly before preparing the intermediate calibration material. The intermediate stock is prepared by diluting the Stock calibration material 1:1000 in acetonitrile (10 µl of stock is added to 9.99 ml acetonitrile). The intermediate Stock Solution should be considered stable for up to 1 year if it is stored at <–60 °C.
6. Stock estrone calibration material: Estrone (1.0 mg/ml) (Cerilliant, Round Rock, Texas).
7. Intermediate estrone calibration material: Estrone, (1.0 µg/ml). The intermediate stock is prepared by diluting the Stock Calibration material 1:1000 in methanol (10 µl of original stock is added to 9.99 ml methanol). The intermediate Stock Solution should be considered stable for up to 1 year if it is stored at <–60 °C.
8. Working estradiol and estrone calibration material: Estradiol (1.0 ng/ml) and Estrone (1.0 ng/ml). The working stock solution is prepared at a concentration of 1.0 ng/ml of both estradiol and estrone. The working stock is prepared by carefully pipetting out 10 µl of estradiol intermediate stock solution (1.0 µg/ml) and 10 µl of estrone intermediate stock solution (1.0 µg/ml) and adding it to 9.98 ml of deionized water (1:1000). Mix thoroughly by gentle inversion prior to storage or use. The working stock solution should be considered stable for one use only.

9. Stock Estrone control material: Estrone (100 µg/ml) (IsoScience, King of Prussia, PA). Once opened the Stock solution should be considered stable for up to 12 months when stored at <–65 ° C.
10. Intermediate Estrone control material: Estrone(100 ng/ml). The intermediate stock is prepared by carefully pipetting out adding 10 µl of estrone stock solution (100 µg/ml) and adding it to 9.99 ml of methanol (1:1000). Mix thoroughly by gentle inversion prior to storage or use. The intermediate stock solution should be considered stable for up to 1 year if it is stored at <–65 °C.
11. Working Estrone control material: (1.0 ng/ml). The working stock solution is prepared at a concentration of 1.0 ng/ml. The working stock is prepared by carefully pipetting out adding 100 µl of estrone intermediate stock solution (100 ng/ml) and adding it to 9.90 ml of deionized water (1:100). Mix thoroughly by gentle inversion prior to storage or use. The working stock solution should be considered stable for one use only.
12. Stock estradiol control material: Lyphochek Immunoassay Plus Control Level 1–3 (Bio-Rad, Hercules, CA). Estradiol levels will vary depending on the lot of Bio-Rad material. See package insert for expected estradiol values (*see* **Note 2**).

### *2.4 Calibrators and Control Preparation and Operating Parameters*

1. Calibrators: Calibrators 1–6 are prepared by serial dilution of the working stock calibration material as describe in Table 1. For each dilution step add the appropriate volume of both the previous calibration material and the deionized water to a 13×100 ml glass tubes. Thorough mixing should occur between the transfers of the diluted calibrator. Discard the final 2.0 ml from calibrator 1 and aliquot into 300 µl aliquots and store at –70 °C. Stable for up to 30 days (*see* **Note 3**).
2. Controls: Bio-Rad Lyphochek Immunoassay Plus Control Level 1–3: To prepare controls 2–4 add appropriate volume of working

**Table 1**
**Preparation of calibrators**

| Calibrator number | Volume of previous calibration material (ml) | Volume of deionized water (ml) | Final concentration (pg/ml) |
|---|---|---|---|
| 6 | 3.75 (working stock) | 2.25 | 625 |
| 5 | 2.0 (Cal #6) | 4.0 | 208 |
| 4 | 2.0 (Cal #5) | 4.0 | 69 |
| 3 | 2.0 (Cal #4) | 4.0 | 23 |
| 2 | 2.0 (Cal #3) | 4.0 | 7.7 |
| 1 | 2.0 (Cal #2) | 4.0 | 2.6 |

**Table 2**
**Preparation of controls**

| Control | Volume of control estrone material (µl) | Volume of deionized water (ml) | Final concentration of estrone (pg/ml) |
|---|---|---|---|
| 2 | 500 | 4.5 | 100 |
| 3 | 1000 | 4.0 | 200 |
| 4 | 2000 | 3.0 | 400 |

**Table 3**
**HPLC operating conditions**

| | | | |
|---|---|---|---|
| *Column temp* | | 45 °C | |
| *Flow rate* | | 0.45 ml/min | |
| *Equilibration time* | | 0.5 min | |
| *Gradient* | *Step* | *Time* (min) | *Parameter* (%) |
| | 1 | 0.3 | 45 |
| | 2 | 0.5 | 45 |
| | 3 | 2.00 | 80 |
| | 4 | 2.20 | 80 |
| | 5 | 2.50 | 90 |
| | 6 | 2.80 | 90 |
| | 7 | 3.00 | 45 |
| | 8 | 3.50 | Stop |

estrone control material and deionized water to each vial of lyphochek for a total volume of 5.0 ml (see below, Table 2.) and allow to stand for 15 min prior to use. Mix by swirling. Control 1 will be prepared by diluting control 2 1:10 with deionized water during each run. Aliquot into 300 µl aliquots and store at <−20 °C. Stable for up to 30 days (*see* **Note 3**).

3. Operating parameters: Set instrument parameters according to Tables 3 and 4. Parameters are optimized specifically for an API 5500 with Shimadzu 20A Prominence HPLC System; therefore, tune setting may vary slightly between instruments.

### 2.5 Equipment and Supplies

1. 2.0 ml 9 mm Short-cap Screw Thread Vial (Restek, Belle fonte, PA).
2. 2.0 ml 9 mm Short-cap Screw Thread Vial Closure (Restek, Bellefonte, PA).
3. Vial Inserts, 100 µl, tri-spring (Phenomenex, Foster City CA).

**Table 4**
**Mass spectrometry operating conditions**

| | |
|---|---|
| Mode | MRM |
| Polarity | Negative |
| Curtain gas | 15.0 psi |
| Ion source gas 1 | 70.0 psi |
| Ion source gas 2 | 75.0 psi |
| Nebulizing current | –5.00 |
| Collision cell exit potential | –15.00 |
| Entrance potential | –13.0 |
| TEMP | 700.0 |

4. 13 × 100 ml Aliquot Tube.
5. Eppendorf Pipette Tips EP 2–200 µl.
6. Eppendorf Tips, 2.5 ml.
7. Repeater pipet tips 50 ml.
8. Centrifuge: Capable of Speeds of 3267 × *g* (RCF).
9. TurboVap® IV Evaporator (Zymark Corporation, Hopkinton, MA).
10. Instrumentation: API 5500 with Shimadzu 20A Prominence HPLC System (AB Sciex, Foster City CA).
11. Software: Analyst 1.6 quantitative software and Multiquant Software Version 2.1 (AB Sciex, Foster City CA).
12. Analytical Column: Kinetex XB-C18, 2.6 µl, 50 × 2.10 mm 100 Å (Phenomenex, Torrance, California).

## 3 Methods

### *3.1 Stepwise Procedure*

1. Label an appropriate number of 13 × 100 ml glass tubes: Cal 1–6, Blank (Negative/Carry-Over), QC1, QC2, QC3, & QC4.
2. For QC1 Add 25 µl of Bio-Rad control 1 (QC2) to QC1 tube plus 225 µl of deionized water. For all other controls and calibrators, add 250 µl of control or calibration material to the appropriate tubes, to the Blank and Negative/Carry-Over control add 250 µl of deionized water.
3. Using an appropriate pipette transfer 250 µl of patient sample to its corresponding tube.
4. Add 20 µl of Internal Standard to each tube.

5. Let samples stand at room temperature for approximately 30 s to let internal standard equilibrated.
6. Add 5 ml extraction reagent to each tube. Following the addition of the extraction reagent, vortex each tube for 10 min (quickly eyeball samples to be sure all samples are mixing well) to insure that the internal standard, extraction reagent, and sample are uniformly suspended into the solution (*see* **Note 4**).
7. Centrifuge the samples between 2600 and 3267 × *g* for 5–10 min to assist with separation of the organic layer from the aqueous layer.
8. Place the samples in the −70 °C freezer for a minimum of 20 min. This step serves to solidify and freeze the aqueous portion of the solution.
9. Verify that the aqueous portion is frozen then collect the organic layer by pouring into fresh tube.
10. Dry samples down with nitrogen at a pressure between 10 and 15 psi for 10–15 min (temperature is not critical for the dry down, ambient to 43 °C is fine).
11. Insure that the tubes are dry and none of the organic solvent remains; reconstitute samples with 125 µl of deionized water and vortex tubes for 5 s.
12. Transfer reconstituted sample from the tube to appropriately labeled vials containing spring bottom inserts, cap vials.
13. Load samples onto the LC-MS/MS and inject 50 µl of sample for analysis. Representative ion chromatographs for the estradiol internal standard, estradiol, and estrone are shown in Fig. 1 (*see* **Note 5**).

### 3.2 Analysis

#### 3.2.1 Calibration Acceptability

1. Instrument Settings and Operating Conditions are provided in Subheading 2 and Tables 3, 4, and 5.
2. Data Analysis in performed using Multiquant software version 2.1 (ABSciex).
3. Curves are linear via 1/X for the regression calculation and are generated based on the analyte/internal standard peak area ratio using the qualifying ions from Table 5.
4. The calibrator accuracy should be ±10 % of the expected value.
5. Ion ratios between transitions are generated using the peak area ratio of the quantifying/qualifying transition and should be within acceptable limits, ± 35 % of the known ratio for the calibrators (Table 5).
6. All peaks must be symmetrical in shape, and have no significant peak leading, tailing, or splitting.

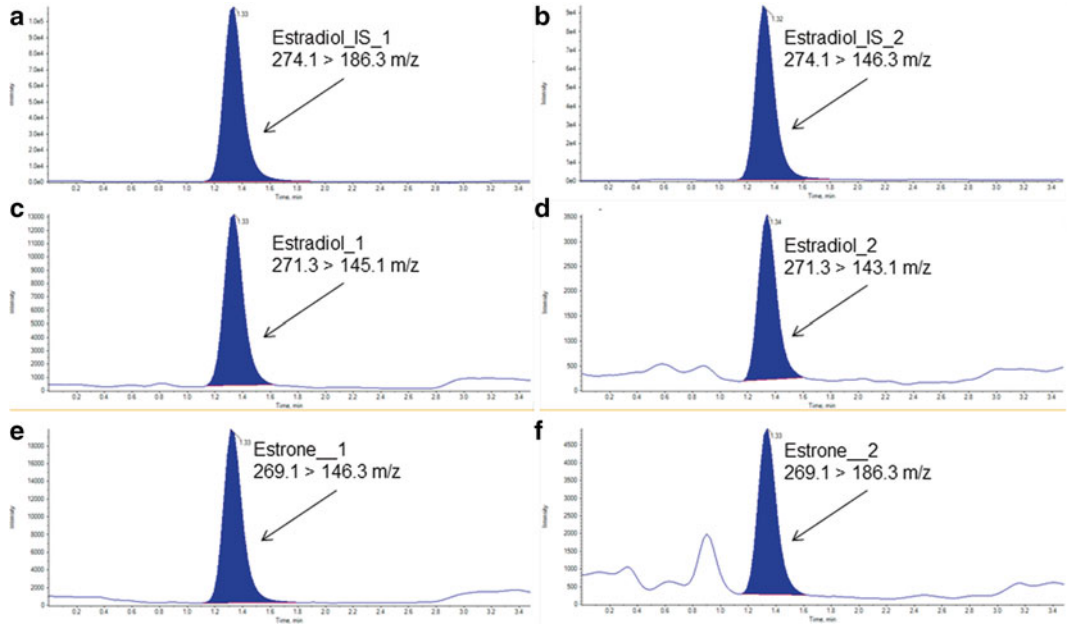



**Fig. 1** Extracted ion chromatographs of 23 pg/ml estradiol, estrone, and the associated internal standard. (**a**) Estradiol internal standard quantifying peak. (**b**) Estradiol internal standard qualifying peak. (**c**) Estradiol quantifying peak. (**d**) Estradiol qualifying peak. (**e**) Estrone quantifying peak. (**f**) Estrone qualifying peak

7. The correlation coefficient ($r$) for the curves generated must be ≥0.99 (*see* **Notes 6–8**).
8. Expected retention time is 1.3 for all analytes and should be within ±0.1 min (*see* **Note 9**).
9. The linearity/limit of quantitation of the method is 2.6–625 pg/ml. Samples that exceed the upper limit of quantitation should be diluted with deionized water retested.

### *3.3 Quality Control Acceptability*

1. The negative control must demonstrate the presence of internal standard and have a concentration less than that of the limit of quantification (2.6 pg/ml).
2. The positive controls must have acceptable chromatography, retention time, ion pattern, and ratios (*see* **Note 9**).
3. Control values must fall within ±2SD of the established target values.

### *3.4 Specimen Acceptability*

1. All peaks for both analytes and internal standard must be symmetrical in shape, and have no significant peak leading, tailing, or splitting.
2. Ion ratios between transitions are generated using the peak area ratio of the quantifying/qualifying transition and should be within acceptable limits, + 35 % of the known ratio for all calibrators, controls, and patient samples. Table 5.

**Table 5**
**Parent and precursor ions and associated energies**

| Compound | Parent ion (M/Z) | Product ion (M/Z) | Declustering potential (DP) | Collision energy (CE) |
|---|---|---|---|---|
| Estradiol_IS_1 | 274.3 | 186.3 | −120.0 | −56.0 |
| Estradiol_IS_2 | 274.3 | 146.3 | −130.0 | −76.0 |
| Estradiol_1 | 271.3 | 145.1 | −110.0 | −57.0 |
| Estradiol_2 | 271.3 | 143.1 | −110.0 | −57.0 |
| Estrone_1 | 269.1 | 146.1 | −125.0 | −46.0 |
| Estrone_2 | 269.1 | 186.1 | −125.0 | −36.0 |

3. There must be no interfering peaks in extracted ion chromatograms and the chromatography must show good separation and peak resolution.
4. The retention time is consistent with the calibrators and quality control within the run (*see* **Note 9**).

## 4 Notes

1. Estradiol 2,3,4-$^{13}C_3$ is used as the internal standard for both estradiol and estrone.
2. Estrone is not present in the Bio-Rad estradiol control materials estrone and therefore requires estrone spiking.
3. This assay is a multiplex and monitors both the estradiol and estrone values followed by a calculation to determine total estrogen values. The calculation is a simple summing of the estradiol and estrone values. Calibration material contains both estradiol and the estrone at a starting concentration of 625 pg/ml. Control material also contains both analytes, the estradiol control values are lot to lot specific and can be found in the package insert supplied by Bio-Rad. The estrone control values are listed in Table 2.
4. It is imperative that the samples get mixed thoroughly for the full 10 min. If this is not done, it will result in a reduction in the recovery of the estrogens and poor overall peak quality.
5. Each analyte has two transitions (ions); the transitions numbered 1 for each analyte are the quantifying transition. The transitions numbered 2 for each analyte are the qualifying transition and are only used for quality control.
6. A unique standard curve (Calibrators 1–6) is extracted and generated with each analytical run.

7. To confirm the integrity of the calibration material overlay the new calibration curves against an established reference curves in the Multiquant software. Also closely monitor QC data for trends or bias in the material that could indicate a breakdown of the calibration material.
8. If any one point does not lie on the curve, it can be removed and the run reprocessed. If the absence of this point means that the curve has a shape dissimilar to that of the generally accepted calibration curve, or more than one point appears to be anomalous, then the assay should be repeated. Points dropped from the upper or lower end of the run must be evaluated to insure that the deletion of that point does not jeopardize the upper or lower range of linearity for the analyte.
9. Retention times may vary slightly with minor changes to the instrumentation such as length of tubing, and the addition of switching values. Small retention time shifts can also occur between batches of mobile phase.
10. Interference studies were performed using commonly encountered sample conditions (hemolysis, lipemia, and icterus), anticoagulants (EDTA and Heparin), and other endogenous steroids (testosterone). Ion suppression studies were performed using a sample infusion method. No significant interference or suppression was observed.

## Acknowledgment

We acknowledge Dawn Goertz for her expert editing.

### References

1. Kushnir MM, Rockwood AL, Bergquist J, Varshavsky M, Roberts WL, Yue B, Bunker AM, Meikle AW (2008) High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. Am J Clin Pathol 129(4):530–539
2. Naessen T, Rodriguez-Macias K (2006) Menopausal estrogen therapy counteracts normal aging effects on intima thickness, media thickness and intima/media ratio in carotid and femoral arteries: an investigation using noninvasive high-frequency ultrasound. Atherosclerosis 189:387–392
3. Iughetti L, Predieri B, Ferrari M et al (2000) Diagnosis of central precocious puberty: endocrine assessment. J Pediatr Endocrinol Metab 13:709–715
4. Bidlingmaier F, Wagner-Barnack M, Butenandt O, Knorr D (1973) Plasma estrogens in childhood and puberty under physiologic and pathologic conditions. Pediatr Res 7(11): 901–907
5. Napoli N, Donepudi S, Sheikh S et al (2005) Increased 2-hydroxylation of estrogen in women with a family history of osteoporosis. J Clin Endocrinol Metab 90:2035–2041
6. Singh M, Dykens JA, Simpkins JW (2006) Novel mechanisms for estrogen-induced neuroprotection. Exp Biol Med 231: 514–521
7. Green PS, Gordon K, Simpkins JW (1997) Phenolic A ring requirement for the neuroprotective effects of steroids. J Steroid BiochemMol Biol 63:229–235
8. Green PS, Simpkins JW (2000) Neuroprotective effects of estrogens: potential mechanisms of action. Int J Dev Neurosci 18:347–358

9. Orwoll ES (1998) Osteoporosis in men. Endocrinol Metab Clin North Am 27:349–367
10. Dubey RK, Jackson EK (2001) Genome and hormones: gender differences in physiology: invited review: cardiovascular protective effects of 17β-estradiol metabolites. J Appl Physiol 91:1868–1883
11. Shutt DA, Smith ID, Shearman RP (1974) Oestrone, oestradiol-17beta and oestriol levels in human foetal plasma during gestation and at term. J Endocrinol 60(2):333–341
12. Davis R, Batur P, Thacker HL (2014) Risks and effectiveness of compounded bioidentical hormone therapy: a case series. J Women Health (Larchmt) 8:642–8. doi:10.1089/jwh.2014.4770
13. Hsing AW, Stanczyk FZ, Bélanger A, Schroeder P, Chang L, Falk RT, Fears TR (2007) Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry. Cancer Epidemiol Biomarkers Prev 16(5):1004–8
14. Dorgan JF, Fears TR, McMahon RP, Aronson Friedman L, Patterson BH, Greenhut SF (2002) Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry. Steroids 67(3–4):151–8

# Chapter 12

# Direct Measurement of Free Estradiol in Human Serum and Plasma by Equilibrium Dialysis-Liquid Chromatography-Tandem Mass Spectrometry

**Julie A. Ray, Mark M. Kushnir, Alan L. Rockwood, and A. Wayne Meikle**

## Abstract

We describe a direct method of measurement of free estradiol using equilibrium dialysis followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Serum aliquots and internal standards are extracted by liquid-liquid extraction using methyl-tert-butyl ether (MTBE) followed by derivatization with dansyl chloride. An API 5500 mass spectrometer operated in positive electrospray mode is used for detection.

**Key words** Equilibrium dialysis, Free estradiol, Derivatization, LC-MS/MS, SHBG

## 1 Introduction

Estrogens play an important role in female reproductive functions while simultaneously contributing to the preservation of bone health and protection of the cardiovascular system. High levels of non-metabolized estrogens can lead to breast [1–3], ovarian [4, 5], and thyroid cancers [6, 7] in women and gynecomastia in men [8]. Estradiol, the most potent of estrogens, is produced in varying concentrations in the ovaries during the menstrual cycle and exists in two states: bound to proteins such as albumin and SHBG (sex hormone binding globulin) and the free or unbound form [9, 10]. 90 % or more of the hormone exists in the bound form. The free hormone hypothesis suggests the incapability of the large protein-bound estradiol to cross capillary barriers in order to reach target tissues and the significant dependence of total hormone levels on varying binding protein concentrations. Hence measurement of the free hormone provides a better assessment of estradiol present in circulation [11–16].

Free estradiol can be separated from biological samples by ultrafiltration [17, 18], steady-state gel filtration, as well as

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_12, 

equilibrium dialysis. Subsequently it can be measured by indirect means such as calculation of the concentration of free estradiol based on total estradiol concentration and the non-SHBG bound fraction of labeled estradiol and thereby indirectly predicting the concentration of the free hormone [19, 20]. Very few published reports directly measure free estradiol using equilibrium dialysis followed by LC-MS/MS which is independent of the measurement of total estradiol or SHBG and albumin concentrations. We have used this method to establish clinically relevant concentrations and reference intervals of free estradiol in women [21]. The dansyl chloride derivatization method used in this assay has been reported earlier by our laboratory for the measurement of total estradiol [22].

## 2 Materials

### 2.1 Samples

Both serum and plasma samples are acceptable sample types. Free estradiol is stable at room temperature for the time required for analysis and is fairly stable at 4 °C (for 7 days) and −20 °C (for 30 days) without degradation. No evidence of degradation is observed after three freeze-thaw cycles of samples.

### 2.2 Reagents and Buffers

1. 17β-estradiol, 17β-estradiol-16, 16, 17-d3 (Sigma Aldrich, St Louis, MO).
2. 1 g/L dansyl chloride: (Sigma Aldrich, St Louis, MO). Prepare in high purity acetonitrile. Stable for 6 months at −70 °C.
3. 1 g/L sodium carbonate: Prepare in deionized water. Stable for 3 months at 4 °C.
4. Derivatizing agent: Prepare fresh derivatizing solution by mixing solution of dansyl chloride (1 g/L) with equal volume of sodium carbonate solution (1 g/L).
5. Equilibrium dialysis buffer: NaCl (5.19 g), $Na_3PO_4.12H_2O$ (0.5 g), KCl (0.323 g), $MgSO4 \cdot 7H_2O$ (0.246 g), HEPES (free acid 37.72 g), urea (0.3 g), $CaCl_2$. $2H_2O$ (0.275 g), and $NaN_3$ (0.52 g). Add NaCl to 800 mL deionized water while stirring followed by the addition of the remaining salts in the order provided above with proper stirring. Transfer the solution to a pH meter and use 1M NaOH to adjust the solution to a pH of $7.28 \pm 0.01$. QS the solution to 1 L with deionized water and transfer to a 1 L glass bottle. Store between 2 and 8 °C for 1 year.
6. Phosphate buffered saline (PBS): Prepare by adding NaCl (8.77 g) to 800 mL deionized water while stirring. Add to this sodium phosphate (21.3 g) and mix until completely dissolved. Adjust the pH to 7.2 using NaOH or $H_3PO_4$. QS the solution

to 1 L with deionized water and transfer into a glass bottle. Stable at room temperature for 1 year.

7. Estrogen free bovine serum albumin (BSA Equitech-Bio Inc., Kerrville, TX) 0.05 % in PBS. Stable at −70 °C for 2 years.
8. 10 mM formic acid in water.
9. 10 mM formic acid in acetonitrile.
10. 10 mM formic acid in methanol.
11. Autosampler syringe wash solution A: 45 % 2-propanol, 45 % acetonitrile, 10 % acetone, 0.3 % trifluoroacetic acid.
12. Autosampler syringe wash solution B: 50 % water, 50 % methanol, 0.1 % formic acid.
13. Autosampler injection valve wash solution A: 45 % 2-propanol, 45 % acetonitrile, 10 % acetone, 0.3 % trifluoroacetic acid.
14. Autosampler injection valve wash solution B: 50 % water, 50 % methanol, 0.1 % formic acid.

### *2.3 Standards, Calibrators, and Quality Controls*

1. Stock standards (1 mg/mL in methanol): Estradiol (stable for 3 years at −70 °C) and the deuterated estradiol internal standard (IS) (stable for 5 years at −70 °C).
2. Calibration standard of estradiol (20 pg/mL, 0.7 pmol/L): It is prepared in water and methanol (1:1) according to Table 1.
3. Calibration standard of d3-estradiol (50 pg/mL, 183.5 pmol/L): It is prepared in water and methanol (1:1) according to Table 2.

**Table 1**
**Dilutions for preparation of estradiol working calibration standard**

| Standard dilutions | Standard estradiol, ng/mL | Volume of standard added, µL | Volume of solution, mL | Concentration, ng/mL |
|---|---|---|---|---|
| Dilution 1 | 10,00,000 | 40 | 10 | 4000 |
| Dilution 2 | 4000 | 50 | 10 | 20 |
| Calibration standard | 20 | 25 | 25 | 0.02 |

**Table 2**
**Dilutions for preparation of d3-estradiol working internal standard**

| Standard dilutions | Standard d3-estradiol, ng/mL | Volume of standard added, µL | Volume of solution, mL | Concentration, ng/mL |
|---|---|---|---|---|
| Dilution 1 | 10,00,000 | 50 | 10 | 5000 |
| Dilution 2 | 5000 | 300 | 10 | 150 |
| Working internal standard | 150 | 8.3 | 25 | 0.05 |

**Table 3**
**Preparation of calibration curve**

| Concentration (pg/mL) | Working calibration standard (μL) | BSA (μL) |
|---|---|---|
| 0.0 | 0 | 200 |
| 0.5 | 5 | 200 |
| 1.0 | 10 | 200 |
| 2.5 | 25 | 200 |
| 5.0 | 50 | 200 |
| 10.0 | 100 | 200 |

4. Calibrators are prepared in 0.05 % BSA in PBS and freshly spiked with calibration standard of estradiol at concentrations of 0.5, 1.0, 2.5, 5.0, and 10 pg/mL (1.8, 3.7, 9.2, 18.4, 36.7 pmol/L) according to Table 3.
5. Quality control samples:
   (a) Negative control: 0.05 % BSA in PBS. Stable for 2 years at −70 °C.
   (b) Quality control level 1: Prepare from a human serum pool containing low total estradiol by spiking 100 times in excess (to account for estradiol binding to proteins such as SHBG) to give a concentration of 1.5 pg/mL. Stable for 6 months at −70 °C.
   (c) Quality control level 2: Prepare from a human serum pool containing low total estradiol by spiking 100 times in excess (to account for estradiol binding to proteins such as SHBG) to give a concentration of 6.0 pg/mL. Stable for 6 months at −70 °C.
   (d) Quality control level 3: Prepare from a human serum pool containing low total estradiol by spiking 100 times in excess (to account for estradiol binding to proteins such as SHBG) to give a concentration of 23.0 pg/mL. Stable for 6 months at −70 °C.

### 2.4 Equipment

1. A triple quadrupole mass spectrometer API5500 with TurboV ion source (AB SCIEX, Foster City, CA).
2. Software: Analyst 1.6.2.
3. Two binary HPLC pumps series 1200 SL (Agilent Technologies, Santa Clara, CA), vacuum degasser, 6-port switching valve, autosampler CTC PAL (Carrboro, NC) equipped with fast wash station.
4. Vortex with adaptor for microcentrifuge tubes.
5. Evaporator for 96-well plates.

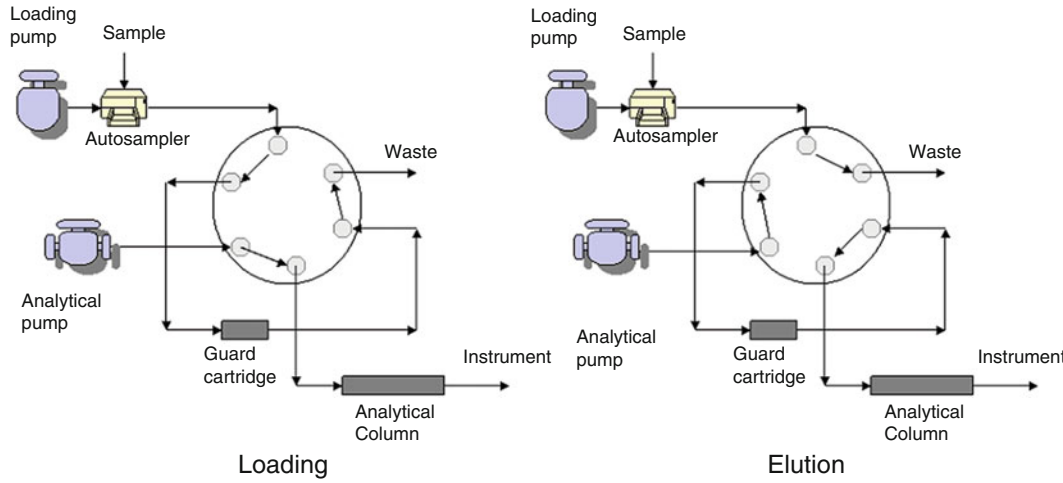



**Fig. 1** Switching valve setup for chromatographic separation

6. Centrifuge for 2 mL microcentrifuge tubes.
7. Centrifuge with buckets for 96-well plates.

### 2.5 Supplies

1. Eppendorf microcentrifuge tubes (2 mL).
2. 96-well plates (deep plates with square wells, 2 mL) with sealing mats (Phenomenex).
3. Trap cartridge for first dimension separation: guard cartridge C1 (Cat # AJO 4299) in Security Guard™ cartridge holder (both Phenomenex, CA) maintained at ambient temperature.
4. HPLC column for analytical separation: Gemini C6 phenyl TMS end capping column (Cat # 00D-4443-B0) with Security Guard™ cartridge holder (Cat # AJO 7914) (both Phenomenex, CA) maintained at 30 °C.
5. The trap and HPLC column are connected to the six port/two position switching valve (VICI VALCO Instruments Inc., Houston, TX) (Fig. 1).
6. Harvard Equilibrium Dialysis plate (Cat # 74-2330 Harvard Apparatus).

## 3 Methods

### 3.1 Stepwise Procedure

1. 250 µL of patient samples and controls are aliquoted into the clear side of the Harvard Equilibrium Dialysis plate and an equal volume of dialysis buffer added to the orange side of the plate. The wells are numbered corresponding to the samples. The unused wells of the plate are covered with caps to avoid contamination. The calibrators do not undergo dialysis.
2. The plate is allowed to spin at 37 °C for 22 ± 1 h.

3. A print layout of the 96-well plate with sample IDs listed in corresponding wells of the map is prepared.
4. At the end of dialysis remove the plate from the chamber and remove 200 µL of buffer from the buffer side of the plate and aliquot into the 96-well plate.
5. Label 2.0 mL microcentrifuge tubes with sample ID.
6. Organize the run to include calibrators. Prepare the calibration curve by adding 200 µL of 0.05 % BSA to each tube and a negative control. Aliquot appropriate volumes of working calibration standard to the respective tubes as indicated in Table 3.
7. Add 20 µL of internal standard (50 pg/mL) to all the tubes.
8. Add 1.3 mL MTBE to the tubes and cap them.
9. Vortex tubes at low speed (setting 2–3) for 10 min (vortexing at high speed may result in formation of emulsion).
10. Centrifuge tubes at –4 °C at 14,000 × *g* for 10 min.
11. Set tubes in –70 °C freezer for 10 min (or until bottom aqueous solution is frozen).
12. Transfer the organic layer to the numbered wells of the 96-well plate taking care not to transfer any aqueous portion.
13. Evaporate the organic layer in a 96-well plate evaporator at 50 °C and a nitrogen flow rate of 50 psi.
14. Take the remaining aqueous layer and repeat the extraction process.
15. Add 1 mL MTBE to all tubes containing the aqueous layer.
16. Vortex for 5 min at low speed.
17. Centrifuge tubes at –4 °C at 14,000 × *g* for 10 min.
18. Set tubes in –70 °C freezer for 10 min (or until bottom aqueous solution is frozen).
19. Carefully transfer the organic layer into the corresponding wells of the 96-well plate into the corresponding wells by covering all rows except the row in which samples will be immediately transferred with a parafilm.
20. Evaporate the organic layer as before.

### 3.2 Derivatization

1. Using an 8-channel pipette add 50 µL of the derivatizing solution into each well.
2. Cover plate with mat (make sure that labels on the mat correspond to the wells of the plate), vortex for 1 min, and incubate the plate in an oven at 70 °C for 10 min.
3. Remove the mat from the plate and using an 8-channel pipette add 50 µL of a mixture of water/acetonitrile (1:1) into each well of the plate. Cover the plate with a mat and vortex the plate for 1 min.

**Table 4**
**Chromatographic gradient**

| *Loading pump* | | | | | |
|---|---|---|---|---|---|
| Step | Time (min) | Flow (mL/min) | %A | %B | Wash A |
| 1 | 0.0 | 1.0 | 90 | 10 | |
| 2 | 0.1 | 1.0 | 90 | 10 | |
| 3 | 0.2 | 1.0 | 30 | 70 | |
| 4 | 0.5 | 1.0 | 30 | 70 | |
| 5 | 1.0 | 1.0 | 2 | 98 | |
| 6 | 1.5 | 1.0 | 2 | 98 | |
| 7 | 3.3 | 2.0 | | | 100 |
| 8 | 4.8 | 2.0 | | | 100 |
| 9 | 4.9 | 1.0 | 90 | 10 | |
| 10 | 8.77 | 1.0 | 90 | 10 | |
| *Eluting pump* | | | | | |
| Step | Time (min) | Flow (ml/min) | %A | %B | |
| 1 | 0 | 0.7 | 50 | 50 | |
| 2 | 1.1 | 0.7 | 50 | 50 | |
| 3 | 1.5 | 0.7 | 49 | 51 | |
| 4 | 3.3 | 0.7 | 34.2 | 65.8 | |
| 5 | 4.8 | 0.7 | 23 | 77 | |
| 6 | 4.9 | 0.7 | 22 | 78 | |
| 7 | 8.0 | 0.7 | 5 | 95 | |
| 8 | 8.6 | 0.7 | 5 | 95 | |

4. Centrifuge plate for 2 min at 4000 × *g*.
5. Analyze the samples.

### 3.3 Chromatographic Conditions

1. Chromatographic gradient used in the method is listed in Table 4.
2. Mobile phase for sample trapping (first dimension separation) A: methanol with 10 mM Formic acid, B: Water with 10 mM formic acid.
3. Mobile phase for analytical separation (second dimension separation) B: Acetonitrile with 10 mM Formic acid, B: Water with 10 mM formic acid.
4. Injection volume: 75 μL.
5. Syringe cleaning: two washes each with wash solutions A, B.
6. Injection valve cleaning: two washes each with wash solutions A, B.

### 3.4 Mass Spectrometer Conditions

1. Mass transitions for dansylated free estradiol and d3-estradiol are listed in Table 5.
2. Optimized mass spectrometric voltages and gas flow rates are as follows:
   (a) Curtain Gas: 30.0.
   (b) Ion spray voltage: 5000 V.

**Table 5**
**Mass transitions and voltages for dansylated free estradiol and d3-estradiol**

| Q1 mass (Da) | Q3 mass (Da) | Dwell (ms) | Collison energy, V | Transition ID |
|---|---|---|---|---|
| 506.2 | 171.1 | 100 | 45 | Estradiol primary |
| 506.2 | 156.1 | 100 | 75 | Estradiol secondary |
| 509.2 | 171.1 | 100 | 45 | d3-estradiol primary |
| 509.2 | 156.1 | 100 | 75 | d3-estradiol secondary |

(c) Gas 1 and 2: both set at 50.0.
(d) Collision gas: 10.0.
(e) Declustering potential: 210 V.
(f) Entrance potential: 10.0.
(g) Collision exit potential: 13.0.

3. Mass analyzer Q1 is tuned for unit resolution (0.7 Da at 50 % height) and Q3 tuned for low resolution (1.0 Da at 50 % height).

### *3.5 Data Analysis*

1. Data analysis is performed on Analysis 1.6.2 (Applied Biosystems SCIEX, Foster City, CA).
2. Calibration curve for free estradiol is prepared with every batch of samples in 0.05 % BSA. The regression is linear forced through zero with no weighting applied.
3. Concentrations are determined from the quantitative transitions of estradiol.
4. Specificity of the analysis is evaluated using ratios of concentrations determined from the primary and the secondary mass transitions. If in patient samples the ratio of concentrations determined from the primary (506.2/171.1) and the secondary mass transitions (506.2/156.1) would be outside of the acceptability range i.e., outside of ±30 %, interference may be expected and the sample may be retested only after dialyzing again.
5. Each chromatogram should be evaluated for acceptable peak shape and peak area (Fig. 2).

## 4 Comments

1. The method does not allow more than one injection per sample.
2. The method does not allow dilution.

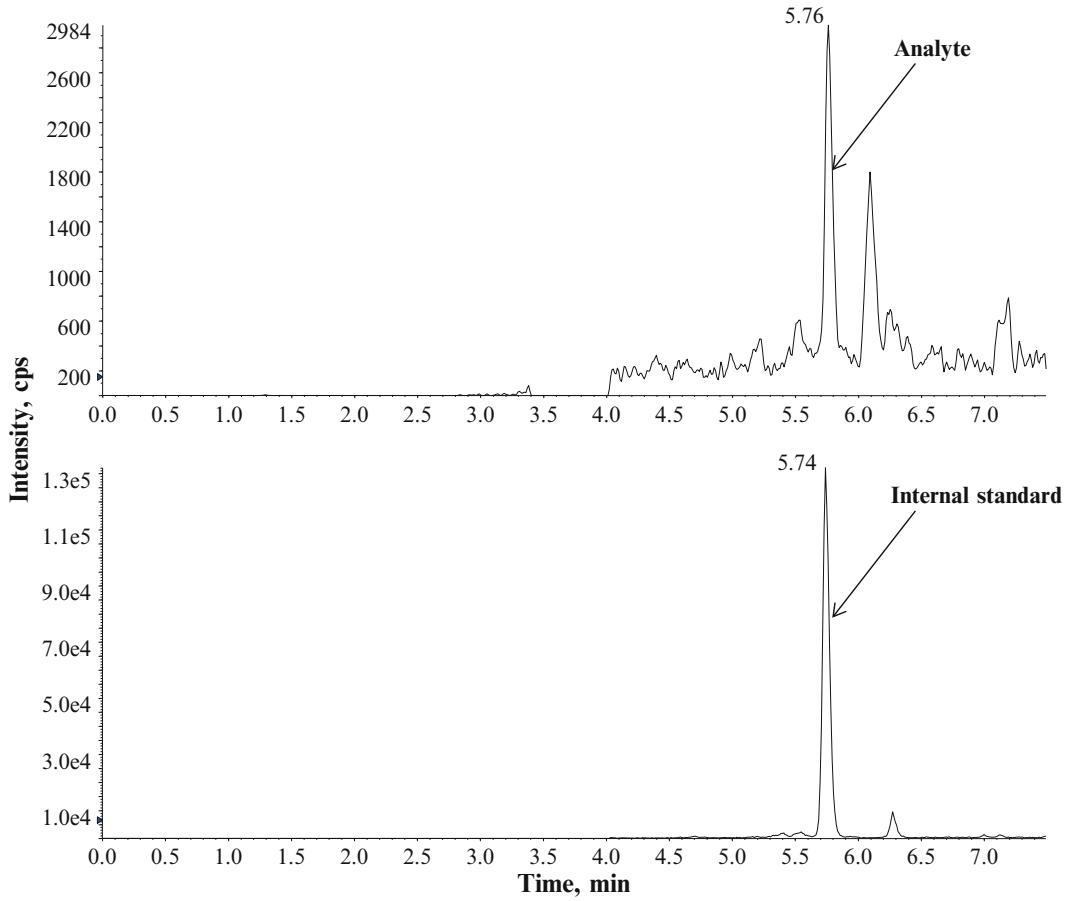



**Fig. 2** Chromatograms of primary mass transitions of free estradiol (m/z) 506.2/171.1 and internal standard (m/z) 509.2/171.1 in a patient serum sample with concentration 0.59 pg/mL (2.2 pmol/L)

3. Derivatized samples degrade if stored beyond 5–6 h at –4 °C. Therefore it is not recommended to run more than 70 samples (including controls and calibrators) per run.
4. Concentration of free estradiol in the negative control should be below the limit of quantitation of the method (0.5 pg/mL).
5. If a run contains a sample with concentration of free estradiol greater than 60 pg/mL, the following sample should be evaluated for carryover. To evaluate for carryover the sample should be re-extracted.

## Acknowledgement

We thank ARUP Institute for Clinical and Experimental Pathology® for supporting this project.

## References


1. Nikolov IT, Dimitrov OA (1990) Estradiol binding by serum proteins in the postmenopause of women with breast cancer. Probl Endokrinol 36:40–43
2. Levin E, Tomchinsky S, Lopez S (1990) Displacement by tamoxifen of the estradiol-estrogen receptor binding: a functional assay for breast cancer studies. J Steroid Biochem Mol Biol 37:681–686
3. Maynadier M, Nirde P, Ramirez JM, Cathiard AM, Platet N, Chambon M, Garcia M (2008) Role of estrogens and their receptors in adhesion and invasiveness of breast cancer cells. Adv Exp Med Biol 617:485–491
4. Seeger H, Mueck AO (2006) The effect of estradiol metabolites and progestogens on the proliferation of human ovarian cancer cells. Panminerva Med 48:13–17
5. Song J, Fadiel A, Edusa V, Chen Z, So J, Sakamoto H, Fishman DA, Naftolin F (2005) Estradiol-induced ezrin overexpression in ovarian cancer: a new signaling domain for estrogen. Cancer Lett 220:57–65
6. Kumar A, Klinge CM, Goldstein RE (2010) Estradiol-induced proliferation of papillary and follicular thyroid cancer cells is mediated by estrogen receptors alpha and beta. Int J Oncol 36:1067–1080
7. Banu KS, Govindarajulu P, Aruldhas MM (2001) Testosterone and estradiol have specific differential modulatory effect on the proliferation of human thyroid papillary and follicular carcinoma cell lines independent of TSH action. Endocr Pathol 12:315–327
8. Eren E, Edgunlu T, Korkmaz HA, Cakir ED, Demir K, Cetin ES, Celik SK (2014) Genetic variants of estrogen beta and leptin receptors may cause gynecomastia in adolescent. Gene 541:101–106
9. Knochenhauer ES, Boots LR, Potter HD, Azziz R (1998) Differential binding of estradiol and testosterone to SHBG. Relation to circulating estradiol levels. Journal Reprod Med 43:665–670
10. Fortunati N, Catalano MG, Boccuzzi G, Frairia R (2010) Sex hormone-binding globulin (SHBG), estradiol and breast cancer. Mol Cell Endocrinol 316:86–92
11. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M (2014) Vitamin D and DBP: the free hormone hypothesis revisited. J Steroid Biochem Mol Biol 144 Pt A:132–137
12. Ekins R (1992) The free hormone hypothesis and measurement of free hormones. Clin Chem 38:1289–1293
13. Adams JS (2005) "Bound" to work: the free hormone hypothesis revisited. Cell 122: 647–649
14. Mendel CM (1992) The free hormone hypothesis. Distinction from the free hormone transport hypothesis. J Androl 13:107–116
15. Yue B, Rockwood AL, Sandrock T, La'ulu SL, Kushnir MM, Meikle AW (2008) Free thyroid hormones in serum by direct equilibrium dialysis and online solid-phase extraction—liquid chromatography/tandem mass spectrometry. Clin Chem 54:642–651
16. Kushnir MM, Rockwood AL, Yue B, Meikle AW (2010) High sensitivity measurement of estrone and estradiol in serum and plasma using LC-MS/MS. Methods Mol Biol 603:219–228
17. MacMahon W, Stallings J, Sgoutas D (1983) A simple ultrafiltration method for determining unbound estradiol in serum. Clin Biochem 16:240–243
18. Lin YT, Yoshida N, Sekiba K (1987) Determination by ultrafiltration of the fraction of unbound estradiol and its variation in peritoneal fluid during the menstrual cycle. Acta Med Okayama 41:1–9
19. Sodergard R, Backstrom T, Shanbhag V, Carstensen H (1982) Calculation of free and bound fractions of testosterone and estradiol-17 beta to human plasma proteins at body temperature. J Steroid Biochem 16:801–810
20. Mazer NA (2009) A novel spreadsheet method for calculating the free serum concentrations of testosterone, dihydrotestosterone, estradiol, estrone and cortisol: with illustrative examples from male and female populations. Steroids 74:512–519
21. Ray JA, Kushnir MM, Bunker A, Rockwood AL, Meikle AW (2012) Direct measurement of free estradiol in human serum by equilibrium dialysis-liquid chromatography-tandem mass spectrometry and reference intervals of free estradiol in women. Clin Chim Acta 413:1008–1014
22. Kushnir MM, Rockwood AL, Bergquist J, Varshavsky M, Roberts WL, Yue B, Bunker AM, Meikle AW (2008) High-sensitivity tandem mass spectrometry assay for serum estrone and estradiol. Am J Clin Pathol 129:530–539

# Chapter 13

# Quantification of γ-Aminobutyric Acid in Cerebrospinal Fluid Using Liquid Chromatography-Electrospray Tandem Mass Spectrometry

**Erland Arning and Teodoro Bottiglieri**

## Abstract

We describe a simple stable isotope dilution method for accurate and precise measurement of γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter in human cerebrospinal fluid (CSF) as a clinical diagnostic test. Determination of GABA in CSF (50 μL) was performed utilizing high performance liquid chromatography coupled with electrospray positive ionization tandem mass spectrometry (HPLC-ESI-MS/MS). Analysis of free and total GABA requires two individual sample preparations and mass spectrometry analyses. Free GABA in CSF is determined by a 1:2 dilution with internal standard (GABA-$D_2$) and injected directly onto the HPLC-ESI-MS/MS system. Determination of total GABA in CSF requires additional sample preparation in order to hydrolyze all the bound GABA in the sample to the free form. This requires hydrolyzing the sample by boiling in acidic conditions (hydrochloric acid) for 4 h. The sample is then further diluted 1:10 with a 90 % acetonitrile/0.1 % formic acid solution and injected into the HPLC-ESI-MS/MS system. Each assay is quantified using a five-point standard curve and is linear from 6 nM to 1000 nM and 0.63 μM to 80 μM for free and total GABA, respectively.

**Key words** GABA, Cerebrospinal fluid, Seizures, Mass spectrometry

## 1 Introduction

γ-aminobutyric acid (GABA), a primary inhibitory neurotransmitter in the brain, is synthesized from glutamate by the pyridoxine-dependent enzyme glutamic acid decarboxylase (GAD). The first step in the catabolism of GABA involves degradation to succinic semialdehyde in a reaction catalyzed by GABA-transaminase (GABA-T). Succinic acid is then converted to succinic semialdehyde by the enzyme succinic semialdehyde dehydrogenase (SSADH). GABA is also present in non-neuronal tissues and may exist in peripheral and central tissues in the form of a dipeptide with histidine, as a compound known as homocarnosine [1]. Several inherited disorders of GABA metabolism have been identified; these are SSADH deficiency, GABA-T deficiency, and homocarnosinosis [2].

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_13, 

The latter two are extremely rare and require CSF determination of both free and total GABA. The clinical presentation of these cases may vary considerably and include psychomotor retardation, convulsions, ataxia, hypotonia, hyperreflexia, and delayed speech development. In addition an autosomal recessive disorder has been characterized in a group of patients with pyridoxine-dependent seizures [3]. In these cases the presumed abnormality is due to reduced binding of pyridoxal-5-phosphate to GAD. Elevation of CSF free and total GABA is characteristic of GABA-T and SSADH deficiency, whereas low CSF GABA has been reported in pyridoxal-5-phosphate-dependent seizures.

Several methods for determination of free and total GABA in CSF have been published utilizing HPLC with pre- or post-column derivatization. A variety of detection methods for determining CSF GABA have been developed ranging from HPLC-fluorescence [4], HPLC-electrochemical detection [5], GC-MS [6], CE-MS [7], and LC-MS [8]. Many of these methods employ lengthy and labor-intensive sample preparation and derivatization prior to analysis. We have developed a method which involves a simple sample preparation for both free and total GABA which does not require prior sample cleanup or derivatization. Analysis of both free and total GABA requires two individual sample preparations and analyses. Free GABA is performed by simple stable isotope dilution (1:2) followed by analysis by HPLC-ESI-MS/MS. Extra care must be taken to ensure accurate determination of free GABA in CSF caused by delayed freezing and/or repeated freeze/thaw cycles will result in an artifactual increase in free GABA resulting from the breakdown of bound GABA. Analysis of total GABA requires boiling the CSF sample in the presence of 6 N hydrochloric acid (HCl) for 4 h to ensure complete hydrolysis of all bound GABA. Following boiling step, sample is diluted 1:10 with a 90 % acetonitrile/0.1 % formic acid solution and analyzed by HPLC-ESI-MS/MS.

## 2 Materials

### 2.1 Samples

Human lumbar cerebrospinal fluid—Specimen drawn any time during the day will be acceptable. No patient preparation is required. If the spinal fluid is clear, the sample should be immediately frozen at the bedside on dry ice. If blood contaminated, the sample should be placed on wet ice, centrifuged within 5 min, and the clear CSF transferred to another vial and frozen on dry ice (*see* **Note 1** for more information regarding sample stability).

### 2.2 Solvents and Reagents

1. All the reagents should be ACS or HPLC grade.
2. Mobile Phase A (0.5 % formic acid/0.25 % heptafluorobutyric acid in water): In a hood, add 5 mL of formic acid and 2.5 mL of heptafluorobutyric acid to a 1 L volumetric flask, bring to

volume with water, and mix. Stable at room temperature, 18–24 °C, up to 3 months.

3. Mobile Phase B (0.5 % formic acid/0.25 % heptafluorobutyric acid in acetonitrile): In a hood, add 5 mL of formic acid and 2.5 mL of heptafluorobutyric acid to a 1 L volumetric flask, bring to volume with acetonitrile, and mix. Stable at room temperature, 18–24 °C, up to 3 months.
4. Deproteinizing solution (90 % acetonitrile/0.1 % formic acid in water): Add 45 mL of acetonitrile and 50 μL formic acid to a 50 mL volumetric flask. Bring to volume with water and mix. Stable at room temperature, 18–24 °C, up to 3 months.
5. 10× Artificial CSF (aCSF): 1450 mM NaCl, 27 mM KCl, 10 mM $MgCl_2$, 12 mM $CaCl_2$, 20 mM $Na_2HPO_4$.
   (a) Weigh the following and combine in 100 mL volumetric flask containing 50 mL water: 8.474 g NaCl, 0.201 g KCl, 0.203 g $MgCl_2$, 0.176 g $CaCl_2$, 0.284 g $Na_2HPO_4$.
   (b) Bring to volume with water.
   (c) Add small magnetic stir bar and mix on magnetic stirrer until dissolved.
   (d) Adjust pH to 7.4 with 85 % phosphoric acid.
   (e) Store at 2–8 °C for up to 1 year.
6. 1× aCSF: Add 1 mL 10× aCSF to a 10 mL volumetric flask and bring to volume with water. 1× aCSF is stable for up to 8 h at 2–8 °C and must be made fresh daily.

### 2.3 Internal Standards and Standards

1. Primary standard: GABA (γ-aminobutyric acid) (Sigma-Aldrich).
2. Primary internal standard (I.S.): GABA-$D_2$ ($^2H_2$-γ-aminobutyric acid) (CDN Isotopes).
3. GABA Standard Stock Solution (1 mM): Add 10.3 mg GABA to 100 mL volumetric flask, bring to volume with 0.1 M HCl, and mix. Store in 200 μL aliquots at −80 °C for up to 4 years (*see* **Note 2**).
4. GABA-$D_2$ I.S. Stock Solution (1 mM): Add 10.5 mg GABA-$D_2$ to 100 mL volumetric flask, bring to volume with 0.1 M HCl, and mix. Store in 200 μL aliquots at −80 °C for up to 4 years (*see* **Note 2**).
5. I.S. Working Solution (Free GABA-2 μM GABA-$D_2$ in 1× aCSF and Total GABA 40 μM GABA-$D_2$ in 1× aCSF).
   (a) Free GABA I.S. Working Solution: Combine 2 μL 1 mM GABA-$D_2$ with 998 μL of 1× aCSF and mix well by vortex. Stable at 2–8 °C for up to 8 h.
   (b) Total GABA I.S. Working Solution: Combine 40 μL 1 mM GABA-$D_2$ with 960 μL of water and mix well by vortex. Stable at 2–8 °C for up to 8 h.

### 2.4 Calibrators and Controls

1. Calibrators: Free GABA Working Standard Curve, dilute stock solutions of GABA as follows:
   (a) Dilution A (100 μM): Add 100 μL of GABA 1 mM stock solution to 900 μl of 1× aCSF and mix well by vortex.
   (b) Dilution B (10 μM): Add 100 μL Dilution A to 900 μL of 1× aCSF and mix well by vortex.
   (c) Dilution C (1 μM): Add 100 μL Dilution B to 900 μL of 1× aCSF and mix well by vortex.
   (d) Working Standard Curve (25–400 nM): Add 400 μl of Dilution C to 600 μl of 1× aCSF and mix well by vortex. Perform four additional serial dilutions by adding 500 μl of previous standard to 500 μl of 1× aCSF. This will provide a calibration curve of (400, 200, 100, 50, 25 nM). Working standard curve may be stored in the refrigerator at 4 °C for up to 8 h (*see* **Note 3**).
2. Calibrators: Total GABA Working Standard Curve, dilute stock solutions of GABA as follows:
   Working Standard Curve (2.5–40 μM): Add 40 μL of GABA 1 mM stock solution to 960 μl of 1× aCSF and mix well by vortex. Perform four additional serial dilutions by adding 500 μl of previous standard to 500 μl of 1× aCSF and mix well by vortex. This will provide a calibration curve of (40, 20, 10, 5, 2.5 μM). Working standard curve may be stored in the refrigerator at 4 °C for up to 8 h (*see* **Note 3**).
3. Control: Free GABA Low Control (60–100 nM target value):
   (a) Prepare 10 mL pooled CSF.
   (b) Assay pooled CSF (10 mL) to quantitate the native concentration of free GABA.
   (c) Spike or dilute pooled CSF with diluted stock standard or water to obtain a final concentration of 60–100 nM free GABA. Store in 80 μL aliquots at −80 °C for up to 4 years (*see* **Notes 1** and **3**).

   *Example: Pooled CSF free GABA = 30 nM; add 300 μL 1 μM GABA to 9.7 mL of the pooled CSF.
4. Control: Free GABA High Control (300–400 nM target value):
   (a) Prepare 10 mL pooled CSF.
   (b) Assay pooled CSF (10 mL) to quantitate the native concentration of free GABA.
   (c) Spike or dilute pooled CSF with diluted stock standard or water to obtain a final concentration of 300–400 nM free GABA. Store in 80 μL aliquots at −80 °C for up to 4 years (*see* **Notes 1** and **3**).

   *Example: Pooled CSF free GABA = 50 nM; add 300 μL 10 μM GABA to 9.7 mL of the pooled CSF.

5. Control: Total GABA Low Control (4–8 μM target value):
   (a) Prepare 10 mL pooled CSF.
   (b) Assay pooled CSF (10 mL) to quantitate the native concentration of total GABA.
   (c) Spike or dilute pooled CSF with diluted stock standard or water to obtain a final concentration of 4–8 μM total GABA. Store in 80 μL aliquots at −80 °C for up to 4 years (*see* **Note 2**).

   *Example: Pooled CSF total GABA = 6 μM; no additional dilution necessary. To obtain target value pooled CSF may need to be either diluted with phosphate buffered saline or spiked with diluted standard.
6. Control: Total GABA High Control (30–40 μM target value):
   (a) Prepare 10 mL pooled CSF.
   (b) Assay pooled CSF (10 mL) to quantitate the native concentration of total GABA.
   (c) Spike or dilute pooled CSF with diluted stock standard or water to obtain a final concentration of 30–40 μM total GABA. Store in 80 μL aliquots at −80 °C for up to 4 years (*see* **Note 2**).

   *Example: Pooled CSF total GABA = 30 μM; add 300 μL of 1 mM GABA to 9.7 mL of the pooled CSF.

### 2.5 Analytical Equipment and Supplies

1. Shimadzu Prominence liquid chromatograph system with AB Sciex 4000QTRAP® with Analyst software.
2. Analytical Column: Phenomenex EZfaast, 3 μm, 250 × 2 mm.
3. Guard Column: Phenomenex Security Guard, 5 μm, 4 × 3 mm.
4. Electric skillet.
5. Metal 1.5 mL screw-top rack.
6. 1.5 mL microcentrifuge tubes.

## 3 Methods

### 3.1 Sample Preparation (Free GABA)

1. To labeled 1.5 mL microcentrifuge tubes, pipette 50 μL CSF (calibrators, controls, patient CSF).
2. Add 50 μL of Free GABA I.S. Working Solution.
3. Cap and vortex mix tubes at maximum speed for 3 s.
4. Centrifuge for 5 min at 14,000 × *g*.
5. Transfer 90 μL supernatant into corresponding work list position in 96-well microtiter plate and cover with silicone cover.
6. Place completed 96-well microtiter plate onto refrigerated autosampler (4 °C).

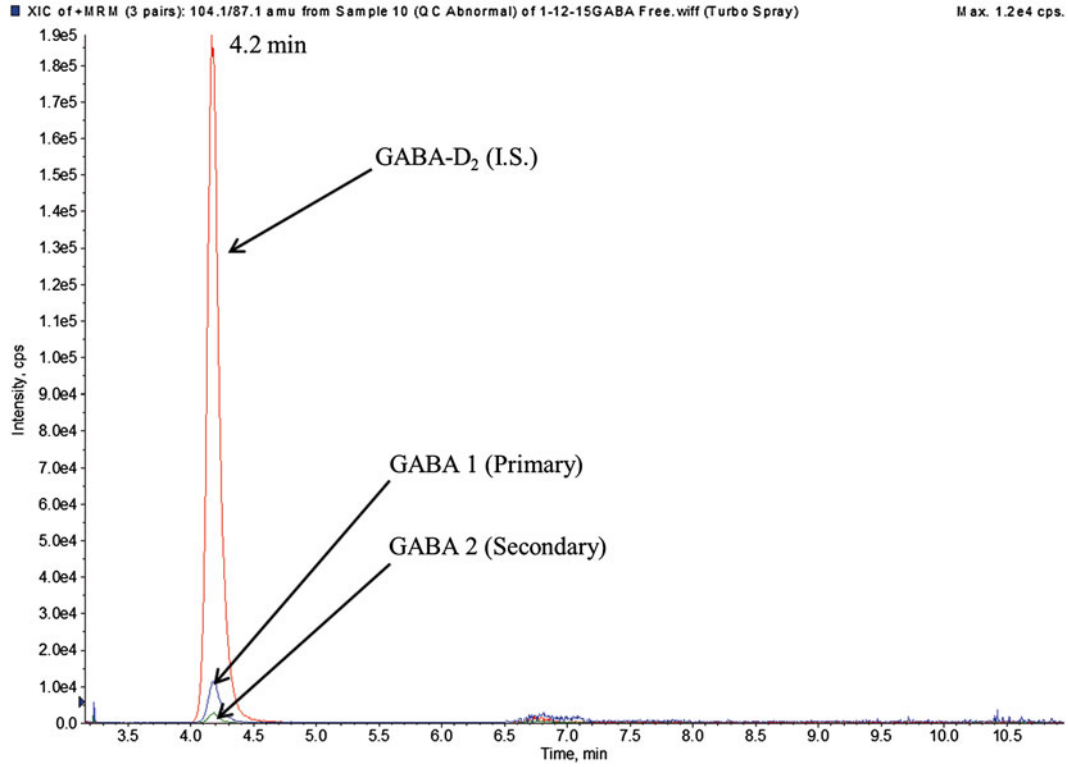



**Fig. 1** HPLC-ESI-MS/MS ion chromatogram of GABA 1 (m/z 104.1 > 87.1), GABA 2 (m/z 104.1 > 69.1), GABA-$D_2$ (m/z 106.0 > 89.1). Concentration of GABA shown is 169 nM

7. Inject 10 µL of sample onto HPLC-ESI-MS/MS. Representative HPLC-ESI-MS/MS ion chromatograms for free GABA and I.S. are shown in Fig. 1 (*see* **Notes 4** and **5**).

### *3.2 Sample Preparation (Total GABA)*

1. To labeled 1.5 mL microcentrifuge tubes, pipette 50 µL CSF (calibrators, controls, patient CSF).
2. Add 50 µL of Total GABA I.S. Working Solution.
3. Add 200 µL 6 N HCl.
4. Cap and vortex mix tubes at maximum speed for 3 s.
5. Place tubes in metal rack with screw-top rack.
6. Place metal rack with tubes in electric skillet filled set at 300 °C filled with water.
7. Boil samples for 4 h.
8. After boiling, remove samples and allow to reach room temperature.
9. Centrifuge for 1 min at 14,000 × *g*.
10. To new labeled 1.5 mL microcentrifuge tubes, add 180 µL deproteinizing solution.

11. Transfer 20 μL of hydrolyzed (boiled) sample to 1.5 mL tube and mix well by vortex.
12. Centrifuge for 5 min at 14,000 × *g*.
13. Transfer 160 μL supernatant into corresponding work list position in 96-well microtiter plate and cover with silicone cover.
14. Place completed 96-well microtiter plate onto the autosampler.
15. Inject 10 μL of sample onto HPLC-ESI-MS/MS. Representative HPLC-ESI-MS/MS ion chromatograms for free GABA and I.S. are shown in Fig. 2 (*see* **Notes 4** and **5**).

### 3.3 Data Analysis

1. Instrumental operating parameters are given in Table 1 A, B, and C.
2. Data are analyzed using Analyst software (AB Sciex).
3. Standard curves are generated based on linear regression of the analyte/I.S. peak-area ratio (y) versus analyte concentration (x) using the primary ions indicated in Table 2.
4. Acceptability of each run is confirmed if the calculated control concentrations fall within two standard deviations of the target values. Inter-day precision was evaluated by repeated analysis

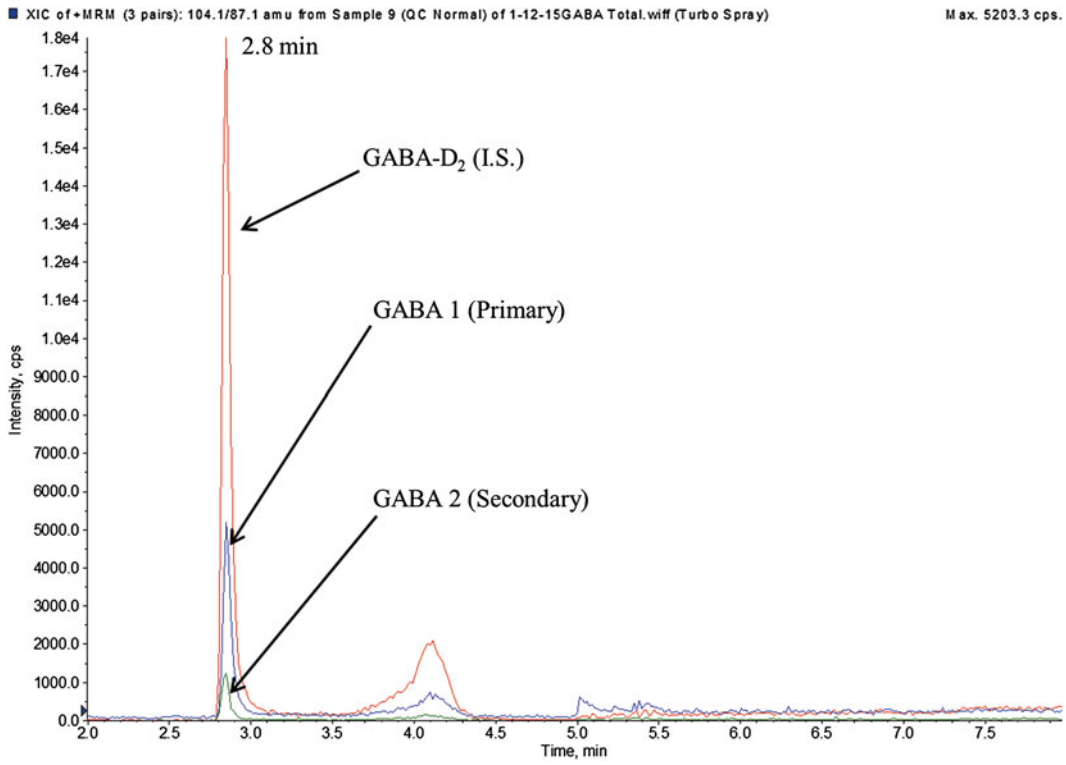



**Fig. 2** HPLC-ESI-MS/MS ion chromatogram of GABA 1 (m/z 104.1 > 87.1), GABA 2 (m/z 104.1 > 69.1), GABA-$D_2$ (m/z 106.0 > 89.1). Concentration of GABA shown is 8.8 μM

**Table 1**
**HPLC-ESI-MS/MS operating conditions**

| | | |
|---|---|---|
| *A. HPLC (Free GABA)*[a] | | |
| Column temp. | 40 °C | |
| Flow rate | 0.230 mL/min | |
| Gradient | Time (min) | Mobile phase A (%) |
| | 0 | 90 |
| | 5 | 25 |
| | 5.1 | 0 |
| | 6 | 0 |
| | 6.1 | 90 |
| | 10 | Stop |
| *B. HPLC (Total GABA)*[a] | | |
| Column temp. | 40 °C | |
| Flow rate | 0.230 mL/min | |
| Gradient | Time (min) | Mobile phase A (%) |
| | 0 | 90 |
| | 4 | 40 |
| | 4.1 | 90 |
| | 8 | Stop |
| *C. MS/MS tune settings*[b] | | |
| Entrance potential (V) | 10 | |
| Curtain gas (psi) | 10 | |
| CAD gas | Medium | |
| Ion spray (V) | 5500 | |
| Temp. (°C) | 600 | |
| GS1 (psi) | 40 | |
| GS2 (psi) | 40 | |
| Resolution Q1 and Q3 | Unit | |

[a]Optimized for Shimadzu Prominence liquid chromatography system equipped with Phenomenex EZfasst, 3 μm, 250 × 2 mm analytical column; Mobile phase A: 0.5 % formic acid-0.25 % heptafluorobutyric acid in water; Mobile phase B: 0.5 % formic acid-0.25 % heptafluorobutyric acid in methanol

[b]Optimized for AB Sciex 4000QTRAP®. Tune settings may vary slightly between instruments

**Table 2**
**HPLC-ESI-MS/MS operating conditions**

| Compound | Q1 (m/z) | Q3 (m/z) | Dwell time (ms) | DP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|
| GABA 1 | 104.1[a] | 87.1[a,b] | 300 | 36 | 15 | 6 |
| GABA 2 | 104.1[a] | 69.1[a,c] | 300 | 36 | 23 | 4 |
| GABA-$D_2$ | 106[a] | 89.1[a,b] | 300 | 26 | 15 | 6 |

[a]Optimized m/z may change based on tuning parameters and instrument used
[b]Primary ions for GABA quantification
[c]Secondary ion used for MRM ratio

**Table 3**
**Age-specific reference range for CSF GABA (total and free)**

| Age | Free GABA (nmol/L) | Total GABA (μmol/L) |
|---|---|---|
| <2 years | 17–67 | 4.2–13.4 |
| >2 years | 32–170 | 3.3–12.2 |

*See* ref. [6]

of bi-level QC material analyzed in duplicate over a period of 20 different days.

5. Limits for the ratio of primary to secondary product ion areas are used to increase the specificity of the assay for GABA and are established for each assay from the calibration curve. Calculated MRM ratio ranges from 20 to 24 %.
6. Liquid chromatography retention time window limits for GABA and GABA-D2 are set at [Free] 4.2 and 4.2 (±0.2) min; [Total] 2.8 and 2.8 (±0.2) min, respectively.
7. The assays have a lower limit of quantitation of 6 nM and 0.63 μM for free and total GABA, respectively, with precisions of <6 % over the entire range. *See* **Note 6** for information regarding ion suppression studies. *See* Table 3 for age-specific reference range [6].

## 4 Notes

1. Approximately 98 % of GABA in CSF is present in the bound form as homocarnosine, homoanserine, GAB-lysine, GABA-cystathionine, and possibly other unknown GABA conjugates [9, 10]. Efforts should be made to prevent controls and patient samples from sitting at room temperature, on wet ice, or at 4 °C for any extended period of time to prevent artifactual increases in free GABA due to hydrolysis of bound GABA.

2. Individual sets of GABA Standard Stock Solution, GABA-D2 I.S. Stock Solution, controls can be pre-aliquoted and frozen until use in each analytical run. For each set pipette 80 µL of stock standard/control solution into 1.5 mL microfuge tubes and freeze at −80 °C until use. Thaw completely before use. Stable for 4 years at −80 °C.
3. A new standard curve (free and total GABA) should be prepared with each analytical run to optimize method performance.
4. Analysis of free GABA should be started immediately upon finishing sample preparation. Assay for free GABA will have to be re-extracted if there is delayed testing (>2 h) due to equipment failure or other delays.
5. The controls are analyzed at the beginning of analysis, every five unknowns, and at the end of the assay as analysis verification.
6. Ion suppression effects were evaluated by sample infusion method. No significant interferences or ion suppression was identified.

## References


1. Perry TL, Kish SJ, Sjaastad O et al (1979) Homocarnosinosis: increased content of homocarnosine and deficiency of homocarnosinase in brain. J Neurochem 32(6): 1637–1640
2. Gibson K, Jakobs C (2001) Disorders of β- and γ-amino acids in free and peptide linked forms. In: Scriver CR, Beaudet AL, Valle D, Sly WS (eds) The metabolic and molecular bases of inherited disease, 8th Edition. McGraw-Hill, New York, pp 2079–2105
3. Goto T, Matsuo N, Takahashi T (2001) CSF glutamate/GABA concentrations in pyridoxine-dependent seizures: etiology of pyridoxine-dependent seizures and the mechanisms of pyridoxine action in seizure control. Brain Dev 23:24–29
4. Goldsmith RF, Earl JW, Cunningham AM (1987) Determination of γ-aminobutyric acid and other amino acids in cerebrospinal fluid by reversed-phase liquid chromatography. Clin Chem 33(10):1736–1740
5. Naini AB, Vontzalidou E, Cote LJ (1993) Isocratic HPLC assay with electrochemical detection of free γ-aminobutyric acid in cerebrospinal fluid. Clin Chem 39(2):247–250
6. Struys EA, Guérand WS, ten Brink HJ, Jakobs C (1999) Combined method for the determination of gamma-aminobutyric and beta-alanine in cerebrospinal fluid by stable isotope dilution mass spectrometry. J Chromatogr Biomed Sci Appl 732:245–249
7. Song Y, Shenwu M, Dhossche DM, Liu Y (2005) A capillary liquid chromatographic/tandem mass spectrometric method for the quantification of γ-aminobutyric acid in human plasma and cerebrospinal fluid. J Chromatogr B Analyt Technol Biomed Life Sci 814:295–302
8. Eckstein JA, Ammerman GM, Reveles JM, Ackermann BL (2008) Analysis of glutamine, pyroglutamate, and GABA in cerebrospinal fluid using ion pairing HPLC with positive electrospray LC/MS/MS. J Neurosci Methods 171:190–196
9. Baxter CF (1976) Some recent advances in studies of GABA metabolism and compartmentation. In: Roberts E, Chase TN, Tower DB (eds) GABA in nervous system function, Vol. 5, Raven Press, New York, p 61–87
10. Perry TL, Hanson S, Kennedy J (1975) CSF amino acids and plasma-CSF amino acid ratios in adults. J Neurochem 24:587–589

# Chapter 14

# Quantitation of Insulin Analogues in Serum Using Immunoaffinity Extraction, Liquid Chromatography, and Tandem Mass Spectrometry

**J. Grace Van Der Gugten, Sophia Wong, and Daniel T. Holmes**

## Abstract

Insulin analysis is used in combination with glucose, C-peptide, beta-hydroxybutyrate, and proinsulin determination for the investigation of adult hypoglycemia. The most common cause is the administration of too much insulin or insulin secretagogue to a diabetic patient or inadequate caloric intake after administration of either. Occasionally there is a question as to whether hypoglycemia has been caused by an exogenous insulin—whether by accident, intent, or even malicious intent. While traditionally this was confirmed by a low or undetectable C-peptide in a hypoglycemic specimen, this finding is not entirely specific and would also be expected in the context of impaired counter-regulatory response, fatty acid oxidation defects, and liver failure—though beta-hydroxybutyrate levels can lend diagnostic clarity. For this reason, insulin is often requested. However, popular automated chemiluminescent immunoassays for insulin have distinctly heterogeneous performance in detecting analogue synthetic insulins with cross-reactivities ranging from near 0 % to greater than 100 %. The ability to detect synthetic insulins is vendor-specific and varies between insulin products. Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS) offers a means to circumvent these analytical issues and both quantify synthetic insulins and identify the specific type. We present an immunoaffinity extraction and LC-MS/MS method capable of independent identification and quantitation of native sequence insulins (endogenous, Insulin Regular, Insulin NPH), and analogues Glargine, Lispro, Detemir, and Aspart with an analytical sensitivity for endogenous insulin of between 1 and 2 μU/mL in patient serum samples.

**Key words** Hypoglycemia, Insulin, C-peptide, Tandem mass spectrometry, Lispro, Glargine, Detemir, Aspart, Humulin, Lantus, Humalog, Levemir, Novorapid

## 1 Introduction

Spontaneous hypoglycemia is a clinical scenario for which endocrinologists and internists occasionally require specialized analysis and consultation with Clinical Chemists and Pathologists. While the most common cause of hypoglycemia is unintentional over-administration of exogenous insulin or insulin secretagogue to a diabetic patient, there are many other medications reported to

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_14, 

cause hypoglycemia in the general patient population [1]. Adult spontaneous hypoglycemia due to endogenous hyperinsulinism has a number of more diagnostically challenging etiologies. In particular, it can be caused by insulin secreting tumors of the pancreas ("insulinomas"), the insulin autoantibody syndrome ("Hirata Disease"), and activating antibodies to the insulin receptor. Insulinoma is generally identified by the development of physiologically inappropriate insulin and C-peptide levels in the context of hypoglycemia during a monitored 72 h fast. Very rarely hypoglycemia can be caused by the production of insulin-like growth factor II which presents as a paraneoplastic syndrome associated with large tumors of mesenchymal or epithelial origin [2].

Occasionally, the question arises as to whether a patient has accidentally or intentionally received or self-administered insulin or an insulin secretagogue. For insulin secretagogues, serum and urine obtained at or as near as possible to the time of the hypoglycemic event can be screened for the relevant drugs by liquid chromatography and tandem mass spectrometry (LC-MS/MS) or by other related approaches [1]. The matter of detection of synthetically manufactured insulin is more challenging.

Exogenous insulin comes in six formulations in North America: Insulin Regular (Humulin® R), Insulin NPH (Novolin®), Insulin Glargine (Lantus®), Insulin Lispro (Humalog®), Insulin Detemir (Levemir)®, Insulin Aspart (Novolog®/Novorapid®), and Insulin Glulisine (Apidra®). Insulin Regular and Insulin NPH have the same amino acid sequence as endogenous human insulin but are synthetically manufactured. The remainder of these insulins have one or more amino acid substitutions or additions, with the exception of Insulin Detemir, which has myristic acid attached lysine at amino acid B29, i.e., the 29th amino acid of the insulin B chain.

Popular commercial automated chemiluminescent immunoassays IAs for insulin have widely variable cross-reactivity for the synthetic insulins with the exception of Insulin Regular and Insulin NPH, which will be detected by any commercial IA. The cross-reactivity profile of the commercial IAs has been characterized by various studies [3–5]. For example, the Roche Cobas methods detects only natural-sequence insulins, whereas the Siemens Centaur has excellent cross-reactivity with all the synthetic insulins mentioned except for Insulin Detemir for which cross-reactivity is moderate (~30 %) and Insulin Glulisine, for which it is poor (2–8 %) [5]. In the setting of insulin-mediated hypoglycemia, measurement of synthetic insulin is only of value if the assay happens to detect the analogue of interest to a meaningful extent. Reliable quantitation of analogue insulins will obviously be problematic by any IA. Generally, a hypoglycemic specimen showing a low or undetectable C-peptide without evidence of an appropriate ketone response indicates exogenous insulin use [1]. However, there are circumstances where it may be desirable to (1) accurately quantify

synthetic insulin and/or (2) positively identify the specific analogue insulin. These situations include accidental administration (medication error), intention to harm, intention to self-harm, Munchausen Syndrome, and Munchausen Syndrome by Proxy, for doping control purposes in sport and forensic analysis.

Significant efforts have been made to establish for insulin using LC-MS/MS. Initial approaches were qualitative and involved immunoaffinity extraction and LC-MS/MS [6]. Although methods have generally focused on serum or plasma, analysis of urine [7] and vitreous humor [8] is reported and methods have also been extended to insulin degradation products [9].

In addition to immunoaffinity extraction with paramagnetic beads, quantitative methods using mass spectrometric immunoassay (MSIA) have been described wherein insulin is extracted using a "disposable automated research [pipette] tip" (DART) filled with antibody-coated beads, eluted and analyzed using liquid chromatography and high resolution mass spectrometry [10, 11]. An antibody-free method for endogenous insulin employing, reduction of disulfide bonds, solid phase extraction and LC-MS/MS analysis of the B chain is also reported [12]. Finally, SPE followed by 2-dimensional chromatography has also been successfully applied to insulin analog testing [13].

We report a protocol for insulin using immunopurification with antibody-coated beads, followed by elution and high-flow 1-dimensional LC-MS/MS analysis on the ABSCIEX API-5500 system operating in positive ion mode. The method uses 500 μL of human serum spiked with Bovine Insulin as internal standard and simultaneously detects and quantifies Insulin Regular/NPH/endogenous Insulin, and Insulins Glargine, Lispro, Detemir, and Aspart. Observed recoveries are 91–113 % in spiking experiments into a single sample at concentrations ranging from 2.5 to 100 μU/mL.

## 2 Materials

### 2.1 Samples

Serum from gel-free red-top tubes has been evaluated. EDTA plasma and heparin plasma collected in gel-free tubes are expected to be suitable samples. No evaluation of gel-separator tubes has been undertaken.

### 2.2 Solvents and Reagents

1. Bovine Serum Albumin, Fraction V, Omnipur® (EMD Millipore. Billerica, MA).
2. 100 mg/mL BSA: Add 100 mL of DI water to 10 g of BSA. Mix and/or sonicate until dissolved.
3. Phosphate buffered saline (PBS) tablets (Sigma-Aldrich, St. Louis, MO).

4. PBS buffer: dissolve 1 tablet in 200 mL of deionized (DI) water. Store at 2–8 °C. Expected stability: up to 6 months.
5. Dextran Sulfate, Sodium Salt (Fisher BioReagents, Waltham, MA).
6. Magnesium Chloride, anhydrous >98 % (Sigma-Aldrich, St. Louis, MO).
7. Delipidation Reagent (5 g/L Dextran Sulfate and 0.5 M Magnesium Chloride): Dissolve 0.05 g Dextran Sulfate and 0.565 g Magnesium Chloride in 10 mL of DI Water. Expected stability: 6 months at 2–8 °C.
8. Mobile Phase A (0.1 % acetic acid in water): Add 1 mL of acetic acid to 1 L of DI water. Mix well. Expected stability: 3 months at room temperature.
9. Mobile Phase B (0.1 % acetic acid in 75:25 Acetonitrile: Isopropanol): Mix 750 mL of acetonitrile with 250 mL of isopropanol. Add 1 mL of acetic acid. Mix well. Expected stability: 3 months at room temperature.
10. 2 % acetic acid in water: Add 2.0 mL of acetic acid to 98 mL of DI water. Mix well. Expected stability: 3 months at room temperature.
11. 1 % acetic acid in water: Add 1.0 mL of acetic acid to 99 mL of DI water. Mix well. Expected stability: 3 months at room temperature.
12. Steroid Free Serum (SFS), DC Mass Spect Gold (Golden West Biologicals, Temecula, CA, cat #MSG-4000) (*see* **Note 1**).
13. Pooled human serum: pool discarded, anonymized pooled human serum samples that show undetectable endogenous insulin by a sensitive immunoassay. Store at −70 °C (*see* **Note 2**).
14. An appropriately prepared antibody-coated paramagnetic bead suspension (*see* **Note 3**).

### 2.3 Internal Standards and Standards

1. Insulin analogues can be obtained from a hospital or outpatient pharmacy or from the respective companies, all at 100 U/mL in solution:
   (a) Lantus®—Insulin Glargine, rDNA origin (Sanofi Aventis).
   (b) Humalog®—Insulin Lispro, rDNA Origin (Lilly).
   (c) Humulin® R—human biosynthetic, rDNA origin regular (Lilly).
   (d) NovoRapid®—Insulin Aspart (NovoNordisk Canada).
   (e) Levemir®—Insulin Detemir (NovoNordisk Canada).
2. Prepare 1 U/mL stock solutions of each insulin by adding 10 µL of the 100 U/mL solutions to 990 µL of 2 % acetic acid (*see* **Note 4**). Prepare and store all solutions in Eppendorf LoBind tubes. Store at −70 °C.

3. Bovine Insulin (internal standard), ≥27 U/mg (HPLC) powder (Sigma-Aldrich, St. Louis, MO).
4. Bovine Insulin stock solution (~54 U/mL) in 2 % acetic acid: Prepare a solution of 2 mg/mL Bovine Insulin in 2 % acetic acid, noting that 2 mg/mL x ≥ 27 U/mg = ≥ 54 U/mL. Prepare in Eppendorf LoBind tubes. Store at −70 °C.
5. Prepare working solutions of the Bovine Insulin:
   (a) ~1.08 U/mL in 2 % acetic acid: Aliquot 20 µL of the 54 U/mL stock solution in 0.98 mL of 2 % acetic acid. Mix well. Aliquot to Eppendorf LoBind tubes. Store at −70 °C.
   (b) ~10.8 mU/mL in 2 % acetic acid: Aliquot 10 µL of the ~1.08 U/mL working solution to 990 µL of 2 % acetic acid. Mix well. Aliquot to Eppendorf LoBind tubes. Store at −70 °C.
6. Prepare the Bovine Insulin internal standard spiking solution (~540 µU/mL) in 20 % acetonitrile: Dilute the ~10.8 mU/mL Bovine Insulin working solution 20-fold into 20 % acetonitrile in DI water. Aliquot to Eppendorf LoBind tubes. Store at −70 °C.

### 2.4 Calibrators and Controls

1. Working solutions of the insulins using the stock solutions prepared in Subheading 2.3:
   (a) Prepare 10 mU/mL mixed working solution containing Glargine, Lispro, Aspart, and Detemir: Add 10 µL of each insulin Glargine, Lispro, Aspart, and Detemir stock at 1 U/mL to 960 µL of 20 % acetonitrile solution. Mix well. Aliquot 100 µL to 10 labeled Eppendorf LoBind tubes and store immediately at −70 °C.
   (b) Prepare 10 mU/mL working solution containing Insulin Regular only: Add 10 µL of the Insulin Regular stock at 1 U/mL to 990 µL of 20 % acetonitrile. Mix well. Aliquot 100 µL to 10 labeled Eppendorf LoBind tubes and store immediately at −70 °C (*see* **Note 5**).
2. Calibrators:
   (a) Mixed insulin calibrator in pooled human serum: Add 20 µL of 10 mU/mL mixed insulin working solution to 1.98 mL of pooled human serum to prepare the 100 µU/mL (high) mixed insulin calibrator. Mix well. Dilute directly into the appropriate wells of a BSA-treated 96-well plate as shown in Table 1 to prepare 6 calibrators (2.5–100 µU/mL).
   (b) Insulin Regular calibrator in SFS: Add 20 µL of 10 mU/mL Insulin Regular working solution to 1.98 mL of steroid free serum to prepare the 100 µU/mL (high) Insulin Regular calibrator. Mix well. Dilute as shown in Table 2 to prepare calibrators.

**Table 1**
**Dilution scheme for insulin analogue calibrators**

| Std level (μU/mL) | Volume of 100 μU/mL mixed calibrator (μL) | Volume of serum pool (μL) |
|---|---|---|
| 2.5 | 12.5 | 487.5 |
| 5 | 25 | 475 |
| 10 | 50 | 450 |
| 25 | 125 | 375 |
| 50 | 250 | 250 |

**Table 2**
**Dilution scheme for insulin regular calibrators**

| Std level (μU/mL) | Volume of 100 μU/mL calibrator (μL) | Volume of SFS (μL) |
|---|---|---|
| 2.5 | 12.5 | 487.5 |
| 5 | 25 | 475 |
| 10 | 50 | 450 |
| 25 | 125 | 375 |
| 50 | 250 | 250 |

### *2.5 Analytical Equipment and Supplies*

1. Eppendorf LoBind tubes, 2.0 mL (Eppendorf, Mississauga, ON).
2. 2 mL Nunc® 96 DeepWell™ round-bottom well plates (Thermo Scientific, Waltham, MA).
3. BSA-treated 2 mL 96 deep well plates (*see* **Note 6**):
   (a) Add 0.5 mL of 100 mg/mL BSA to each well of a 96-well plate. Seal with capmat.
   (b) Attach on rotator or rocker to and rotate/rock for 2 h.
   (c) Remove from rocker or rotator and let sit at room temperature for an additional 22 h.
   (d) After 24 h, discard BSA from plate(s).
   (e) Add ~1 mL PBS to each well of plate(s), mix, and discard.
   (f) Repeat PBS wash two times, for a total of three PBS washes.
   (g) Centrifuge plates upside-down at 1100 × *g* for 10 min to remove all residual PBS from the plate.
4. Vacuum manifold or positive pressure manifold, installed in robotic liquid handler or manually controlled.

5. Acroprep Advance 2 mL 1 μm glass fiber filter plates (PALL Life Sciences, Ville St Laurent, Quebec).
6. Nunc® capmats for round bottom plates (Thermo Scientific, Waltham, MA).
7. AB Sciex5500 QTRAP® triple quadrupole mass spectrometer (AB SCIEX, Concord, ON) or other mass spectrometer capable of reaching the required limit of detection, equipped with appropriate software (e.g., Analyst®).
8. Shimadzu 20 AC LC System with pumps, column oven, degasser, autosampler.
9. Analytical column: ACE C18-300, 50 × 2.1 mm, 5 μm (ACE HPLC Columns, Aberdeen, Scotland).
10. Guard cartridge: C18 4 × 3.0 mm ID (Phenomenex, Torrance, CA).

## 3 Methods

### 3.1 Incubation Procedure

1. Aliquot 500 μL of standards and samples to BSA-treated 96-well plate.
2. Add 25 μL of 540 μU/mL Bovine Insulin (Internal Standard) to each standard and sample.
3. Add 5 μL of delipidation reagent.
4. Seal plate with capmat, and vortex mix at high speed for approximately 20 s.
5. Remove capmat, and add appropriate volume of antibody-coated paramagnetic bead slurry. In our case, this has been determined to be 250 μL (*see* **Note 3**).
6. Seal the plate with a capmat, swirl plate manually until beads are evenly distributed in all wells.
7. Let sit at room temperature for 1 h.

### 3.2 Extraction Procedure

1. Tape off unused positions of filter plate for future use if desired.
2. Remove capmat from incubation plate, and transfer all standards and sample to filter plate.
3. Apply a weak vacuum—eluent goes to waste.
4. Wash each well with 3 × 1 mL of PBS, applying weak vacuum after each addition to elute the PBS wash to waste.
5. After the last PBS wash, apply vacuum for 1 min to remove all wash from plate. Remove filter plate, tap on paper towel to remove residual wash drips from the filter plate.
6. Place a BSA-treated 96-well plate in the collection position of the vacuum manifold.
7. Replace filter plate on vacuum manifold.

8. Add 75 µL of 1 % acetic acid to standard and sample positions in the filter plate.
9. Let sit for 2 min.
10. Apply vacuum to elute acetic acid to the collection plate.
11. Add an additional 75 µL of 1 % acetic acid to the filter plate, and let sit for 1 min.
12. Apply vacuum to elute acetic acid to the collection plate.
13. The collection plate is ready to inject on the LC-MS/MS.

### 3.3 Analysis

1. HPLC gradient is provided in Table 3 and Fig. 1.
2. Instrument operating parameters are given in Table 4.
3. Data are analyzed using Analyst® Software (AB SCIEX, Concord, ON).
4. Standard curves are generated based on linear regression with 1/x weighting of the analyte/IS peak area ratio (y) versus analyte concentration (x) using the quantifying ions indicated in Table 4.
5. Typical extracted ion chromatograms and elution times for all insulins are shown in Fig. 2a–f.

## 4 Notes

1. The absence of insulin immunoreactivity in this product should be confirmed by immunoassay before use.
2. This material is used for preparing the calibrators of the four synthetic insulins which lack a natural amino acid sequence.

**Table 3**
**HPLC gradient optimized for Shimadzu LC20AD**

| *Column temp.* | 60 °C | |
|---|---|---|
| *Flow rate* | 0.55 mL/min | |
| *Gradient* | *Time* | *Mobile phase A* (%) |
| | 0.00 | 78 |
| | 0.50 | 78 |
| | 1.00 | 66 |
| | 3.00 | 63 |
| | 4.00 | 2 |
| | 6.25 | 2 |
| | 6.35 | 78 |
| | 8.5 | 78 |

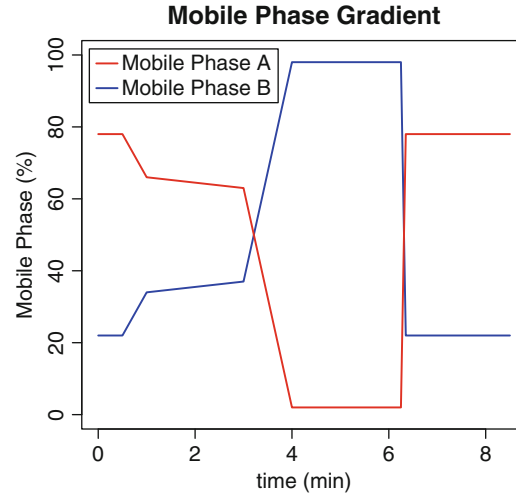



**Fig. 1** HPLC gradient

If some low-level immunoreactivity is detected in the pool, it can be left to sit for 24 h or longer at room temperature to allow any low-level endogenous insulin present to degrade, recognizing that there is a risk of bacterial growth. Confirmed insulin-free commercial serum could be substituted for a human insulin-free pool.

3. It is necessary for the user to identify a suitable monoclonal or polyclonal antibody which results in non-selective immunoaffinity extraction of all the insulins desired. Antibodies directed at the N-terminal of the B chain should detect all insulins listed but will be expected to have problems capturing Insulin Glulisine which has an amino acid substitution of lysine for asparagine at position B3. In cooperation with our laboratory, the University of Victoria Proteomics Centre has systematically examined a number of monoclonal and polyclonal commercial antibodies. Of those examined: (1) Santa Cruz monoclonal Insulin B Antibody (C-12) (cat #sc-377071) (2) Santa Cruz polyclonal Insulin B Antibody (N-20) (cat #sc-7838), and (3) Abcam Monoclonal Anti-Insulin + Proinsulin antibody [D6C4] (cat #ab8304), it was found that the monoclonal product from Santa Cruz was most suitable. The sc-377071 antibody can be coupled to Dynabeads® Protein G from Life Technologies (cat #10003D, 10004D, or 10009D) according to the manufacturer's protocols and used for the immunopurification step. The required amount of bead slurry is a matter of trial and error depending on the desired specimen volume and analytical range. As an alternative, it is possible and even less expensive to use pre-prepared paramagnetic beads from the reagent kits of certain automated chemiluminescent IA analyzers.

**Table 4**
**Optimized instrument settings for the AB SCIEX API-5500 QTRAP®**

| **Source parameters** | | | | | |
|---|---|---|---|---|---|
| Curtain gas: | 40 µL/min | | | | |
| Source temperature: | 600 °C | | | | |
| Gas1: | 70 µL/min | | | | |
| Gas2: | 50 µL/min | | | | |
| CAD gas: | High | | | | |
| IonSpray voltage: | 5500 V | | | | |
| Entrance potential: | 10 V | | | | |
| *Compound dependant parameters* | | | | | |
| *Compound name* | *Q1 mass* | *Q3 mass* | *Dwell time* (ms) | *Declustering potential* (V) | *Collision energy* (V) |
| Humulin | 1162.4 | 345.2 | 35 | 100 | 60 |
| Humulin (quantifier) | 1162.4 | 226.1 | 35 | 100 | 62 |
| Humulin | 1162.3 | 652.0 | 35 | 100 | 47 |
| Detemir (quantifier) | 1184.0 | 454.4 | 18 | 90 | 35 |
| Detemir | 1184.0 | 357.4 | 18 | 90 | 60 |
| Detemir | 987.0 | 454.4 | 18 | 90 | 41 |
| Aspart | 1166.0 | 219.0 | 22 | 100 | 50 |
| Aspart | 971.7 | 226.0 | 22 | 100 | 60 |
| Aspart (quantifier) | 971.7 | 136.0 | 22 | 100 | 100 |
| Lispro | 1162.4 | 217.0 | 22 | 100 | 56 |
| Lispro (quantifier) | 968.6 | 217.0 | 22 | 100 | 51 |
| Glargine | 1011.4 | 1164.2 | 40 | 115 | 42 |
| Glargine | 1011.4 | 1179.4 | 40 | 115 | 43 |
| Glargine (quantifier) | 867.2 | 136.0 | 40 | 115 | 100 |
| Bovine insulin (IS) (quantifier) | 956.5 | 136.1 | 18 | 100 | 101 |
| Bovine insulin (IS) | 956.5 | 226.2 | 18 | 100 | 53 |

Interested readers should contact the corresponding author for details.

4. Some insulins, such as Insulin Glargine and Insulin Regular, come prepared as chelates to $Zn^{2+}$. In order to free the insulin from these chelates for LC-MS/MS analysis, it is necessary to acidify them.

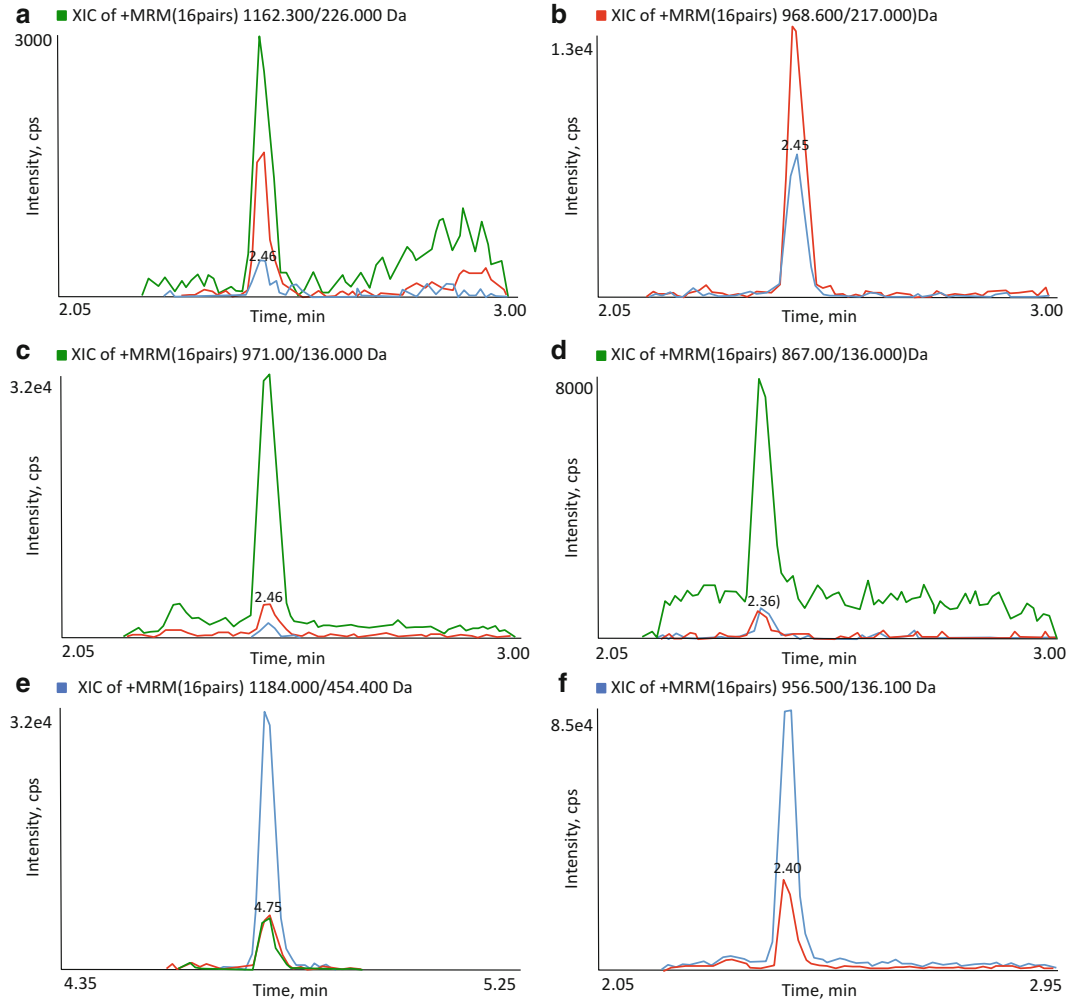



**Fig. 2** Extracted ion chromatograms for various insulins. (**a**) Humulin, (**b**) Lispro, (**c**) Aspart, (**d**) Glargine, (**e**) Detemir, (**f**) Bovine

5. Insulin Regular and Insulin Lispro are identical in molecular weight, and only differ in structure in the order of 2 amino acids (lysine and proline) in the B chain at positions 28 and 29. For this reason, they are difficult to chromatographically resolve, have the same precursor ion $m/z$ ratios, and when Insulin Lispro is fragmented, there are spurious contributions in each of the Insulin Regular MRMs. We have not observed the converse however. For this reason, the Insulin Regular calibrant is prepared in isolation. This phenomenon is easily identified by unexpected ion ratios in the Insulin Regular MRMs.
6. All insulins, particularly Insulin Detemir, are vulnerable to adsorption onto plastic surfaces and can be observed to vanish over the course of minutes from aqueous solution. For this reason, it is necessary to coat 96-well plates with BSA to prevent insulin adsorption and under-recovery.

## References


1. Cryer PE, Axelrod L, Grossman AB, Heller SR, Montori VM, Seaquist ER, Service FJ (2009) Evaluation and management of adult hypoglycemic disorders: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 94(3):709–728
2. Fukuda I, Hizuka N, Ishikawa Y, Yasumoto K, Murakami Y, Sata A, Morita J, Kurimoto M, Okubo Y, Takano K (2006) Clinical features of insulin-like growth factor-II producing non-islet-cell tumor hypoglycemia. Growth Horm IGF Res 16(4):211–216
3. Owen WE, Roberts WL (2004) Cross-reactivity of three recombinant insulin analogs with five commercial insulin immunoassays. Clin Chem 50(1):257–259
4. Heald A, Bhattacharya B, Cooper H, Ullah A, McCulloch A, Smellie S, Wark G (2006) Most commercial insulin assays fail to detect recombinant insulin analogues. Ann Clin Biochem 43(4):306–308
5. Heurtault B, Reix N, Meyer N, Gasser F, Wendling MJ, Ratomponirina C, Jeandidier N, Sapin R, Agin A (2014) Extensive study of human insulin immunoassays: promises and pitfalls for insulin analogue detection and quantification. Clin Chem Lab Med 52(3):355–362
6. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schänzer W (2005) Qualitative determination of synthetic analogues of insulin in human plasma by immunoaffinity purification and liquid chromatography-tandem mass spectrometry for doping control purposes. Anal Chem 77(11):3579–3585
7. Thevis M, Thomas A, Schänzer W, Östman P, Ojanperä I (2012) Measuring insulin in human vitreous humour using lc-ms/ms. Drug Test Anal 4(1):53–56
8. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schänzer W (2006) Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography-tandem mass spectrometry. Anal Chem 78(6):1897–1903
9. Thevis M, Thomas A, Schänzer W (2008) Mass spectrometric determination of insulins and their degradation products in sports drug testing. Mass Spectrom Rev 27(1):35–50
10. Oran PE, Jarvis JW, Borges CR, Sherma ND, Nelson RW (2011) Mass spectrometric immunoassay of intact insulin and related variants for population proteomics studies. Proteomics Clin Appl 5(7–8):454–459
11. Peterman S, Niederkofler EE, Phillips DA, Krastins B, Kiernan UA, Tubbs KA, Nedelkov D, Prakash A, Vogelsang MS, Schoeder T et al (2014) An automated, high-throughput method for targeted quantification of intact insulin and its therapeutic analogs in human serum or plasma coupling mass spectrometric immunoassay with high resolution and accurate mass detection (msia-hr/am). Proteomics 14(12):1445–1456
12. Chen Z, Caulfield MP, McPhaul MJ, Reitz RE, Taylor SW, Clarke NJ (2013) Quantitative insulin analysis using liquid chromatography–tandem mass spectrometry in a high-throughput clinical laboratory. Clin Chem 59(9):1349–1356
13. Chambers EE, Fountain KJ, Smith N, Ashraf L, Karalliedde J, Cowan D, Legido-Quigley C (2013) Multidimensional lc-ms/ms enables simultaneous quantification of intact human insulin and five recombinant analogs in human plasma. Anal Chem 86(1):694–702

# Chapter 15

# Quantitation of Insulin-Like Growth Factor 1 in Serum by Liquid Chromatography High Resolution Accurate-Mass Mass Spectrometry

**Hemamalini Ketha and Ravinder J. Singh**

## Abstract

Insulin-like growth factor 1 (IGF-1) is a 70 amino acid peptide hormone which acts as the principal mediator of the effects of growth hormone (GH). Due to a wide variability in circulating concentration of GH, IGF-1 quantitation is the first step in the diagnosis of GH excess or deficiency. Majority (>95 %) of IGF-1 circulates as a ternary complex along with its principle binding protein insulin-like growth factor 1 binding protein 3 (IGFBP-3) and acid labile subunit. The assay design approach for IGF-1 quantitation has to include a step to dissociate IGF-1 from its ternary complex. Several commercial assays employ a buffer containing acidified ethanol to achieve this. Despite several modifications, commercially available immunoassays have been shown to have challenges with interference from IGFBP-3. Additionally, inter-method comparison between IGF-1 immunoassays has been shown to be suboptimal. Mass spectrometry has been utilized for quantitation of IGF-1. In this chapter a liquid chromatography high resolution accurate-mass mass spectrometry (LC-HRAMS) based method for IGF-1 quantitation has been described.

**Key words** High resolution mass spectrometry, Insulin-like growth factor-1 quantitation, Protein quantitation using mass spectrometry

## 1 Introduction

Growth is a complex process and is a result of an interplay between a number of biochemical processes resulting in cell proliferation and ultimately an increase in size of the organism. Growth hormone (GH)-insulin-like growth factor 1 (IGF-1) axis plays an important role in regulating growth. GH, secreted by the anterior pituitary, stimulates the production of IGF1 in the liver and regulates several biochemical processes leading to an increased cell proliferation and decreased apoptosis [1]. An excess or deficiency in GH production leads to abnormalities in growth-related processes. Acromegaly is a result of excess GH production whereas GH deficiency leads to short stature among other physiological

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_15, 

consequences. IGF-1, a 70 amino acid peptide hormone (MW: 7649 Da), is the principal mediator of physiological effects of growth hormone (GH).

GH measurement in a randomly collected serum sample is not useful for the diagnosis of GH excess or deficiency due to a high degree of variability in serum GH concentration depending on various factors including time of the day, food intake, and exercise. On the other hand, serum IGF-1 circulates at a narrower concentration range relative to GH making it a useful biomarker in the clinical assessment of diseases related to GH deficiency or excess [2, 3]. Majority (~95 %) of IGF-1 circulates as a ternary complex bound to its major carrier protein insulin-like growth factor binding protein-3 (IGFBP-3) and acid labile subunit (ALS). Therefore disruption of the ternary complex prior to quantitation is an important consideration in development and optimization of any IGF-1 assay (*see* **Note 1**). The first radioimmunoassay for quantitation of serum IGF-1 was developed in 1977 [4]. Now several immunoassays on automated platforms are commercially available for use in clinical laboratories. Several currently available assays utilize an extraction step with a buffer containing acidified ethanol which disrupts the IGF-1/IGFBP complex followed by addition of excess IGF-2 to keep IGFBP from complexing with IGF-1 [5, 6]. Despite various sample preparation approaches used, IGF-1 immunoassays are prone to interferences from IGFBPs. Additionally, there is a lack of standardization and poor inter-method agreement amongst IGF-1 immunoassays [5–8]. Mass spectrometry has been successfully employed for quantitation of IGF-1 to circumvent the challenges with IGF-1 immunoassays [9–13].

In this chapter we describe a liquid chromatography high resolution accurate-mass mass spectrometry (LC-HRAMS) method for serum IGF-1 quantitation [12, 14]. The serum sample is treated with a buffer containing acidified ethanol to precipitate large proteins and to disrupt the IGF-1/IGFBP complex followed by centrifugation, neutralization, and a cooling step. Due to a relatively small size IGF-1 remains soluble in the supernatant and is chromatographed on an online solid phase extraction cartridge followed by separation on reverse phase column and quantitation on a high resolution mass spectrometer. The intact, length IGF-1 and oxidized rat IGF-1 (ratIGF-$1_{Ox}$) as the internal standard (IS) are monitored. Quantification is performed using calibrators made from recombinant IGF-1 along with IGF-$1_{Ox}$. A linear calibration standard curve constructed from the peak area ratio (sample/IS) is used to calculate the concentration of the sample. The assay shows cross reactivity towards synthetic IGF-1 (Mecasermin) used as therapeutic agent.

## 2 Materials

### 2.1 Samples

Serum collected in a gel-free (red-top) tube or in serum separator tube is an acceptable sample type for this method. Samples for IGF-1 analysis are acceptable if stored for up to 7 days at ambient temperature or refrigerated. Up to three freeze-thaw cycles are acceptable.

### 2.2 Reagents and Buffers

1. Extraction buffer: (87.5 % ethanol, 12.5 % 1 N HCl): Combine 700 mL of ethanol with 100 mL 1 N HCl and mix thoroughly. Store at ambient temperature. Stable for 1 month at room temperature.
2. 1.5 M Trizma (Sigma Aldrich Chemicals): Dissolve 18 g trizma base in 100 mL water. Store at room temperature. Stable at room temperature for 3 months.
3. Mobile Phase A: 0.2 % formic acid in water. Stable at room temperature for 1 month.
4. Mobile Phase B: 0.2 % formic acid in acetonitrile. Stable at room temperature for 1 month.
5. Pooled human stripped serum from Goldenwest Biologicals (Temecula, CA). For preparation of quality control (QC) samples. Store frozen at –80 °C until use.
6. Standard diluent—3 % bovine serum albumin (BSA). Dissolve 3 g BSA in 100 mL water. Store refrigerated, stable for 1 month.

### 2.3 Calibrators, Internal Standard, and Quality Control Samples

1. Standards and Calibrators: Human IGF-1 protein from Ajinomoto Science (Raleigh, NC). Available as a stock solution, store frozen at –80 °C.

   Rat IGF-1 protein for use as internal standard (IS) in the form of oxidized rat IGF-1 from Cell Sciences (Canton, MA) or Prospec Tany (Rehovot, Israel). Store frozen at –80 °C.
2. A calibrator high concentration stock is prepared from diluting the IGF-1 commercial standard to 10 μg/mL in standard diluent (also to be used as zero standard). Calibrators ranging in concentration from 15 to 2000 ng/mL are prepared by diluting the high calibrator stock into zero standard. Aliquot and store unused high stock and calibrators at –80 °C.
3. Treat a stock of rat IGF1 as IS with 1 % aqueous $H_2O_2$ at room temperature for 30 min followed by addition of 2 mM methylthioethanethiol. Prepare a working IS solution at 10 μg/mL in the standard diluent.
4. Three levels of quality control samples are prepared by spiking stripped serum with 10 μg/mL stock standard. Aliquot and store frozen at –80 °C for 2 years.

### 2.4 Supplies and Equipment

1. Transfer pipettes, vortex and titer plate shaker.
2. Robotic liquid handler.
3. Square 2 mL deep well microtiter plates and EZ PIERCE template film (Chromtech).
4. Online extraction cartridge C12, 4 mm L × 2.0 mm ID guard cartridge, analytical column, Onyx monolithic column (50 mm × 2.1 mm, C18) (Phenomenex, Torrance, CA).
5. MassHunter Quant software (Agilent, Santa Clara, CA).
6. Aria TX-4 automated online chromatography system (ThermoFisher, San Jose, CA).
7. Agilent 6530 qTOF instrument (Santa Clara, CA).

## 3 Method

### 3.1 Sample Preparation

1. Thaw the calibrators, controls, and blanks and vortex well to mix thoroughly.
2. Pipette 100 μL of each of samples calibrators, QC, and blanks into individual well of the 96 deep well plate.
3. Add 10 μL IS (ratIGF-$1_{Ox}$) into each well. Cover the plate with an adhesive plate seal and incubate at ambient temperature on the plate shaker for 10 min with constant shaking.
4. Pipette 400 μL of acid ethanol extraction buffer. Cover the plate with an adhesive plate seal and incubate at ambient temperature on the plate shaker for 30 min with vigorous constant shaking.
5. Add 90 μL trizma base and incubate at ambient temperature with shaking for 1 min.
6. Centrifuge 10 min at 2000 × *g* in a plate centrifuge.
7. Incubate the 96 well plate for at least 30 min at –20 °C.
8. Centrifuge 10 min 2000 × *g* in plate centrifuge.
9. Initiate LC-MS analysis.

### 3.2 HPLC Conditions

1. Mobile phase A: water/0.2 % formic acid.
2. Mobile phase B: acetonitrile/0.2 % formic acid.
3. The sample extract (from **Step 8** from sample preparation method) is injected onto the SPE online extraction cartridge, washed with 85 % solvent A at a flow rate of 0.5 mL/min (*see* **Note 2**).
4. The analytes are then transferred onto the analytical column. Analytical separation of IGF-1 and internal standard was performed using a linear gradient of increasing concentration of solvent B in solvent A (25 % solvent A ramped to 100 % B over 2 min). The analytical column is maintained at room temperature.

**Table 1**
**Mass spectrometry conditions**

| Ion | Mass (amu) | Charge state | Mass accuracy |
|---|---|---|---|
| IGF-1 analyte | 1093.5209 | +7 | 10 ppm |
| | 1093.3475 | +7 | 10 ppm |
| | 1093.6640 | +7 | 10 ppm |
| Rat IGF-$1_{Ox}$ IS | 1098.9622 | +7 | 10 ppm |
| Compound parameters | | | |
| Capillary voltage | 5000 V | | |
| Nozzle voltage | 500 V | | |
| Nebulizer | 50 psi | | |
| Sheath gas temperature | 250 °C | | |
| Sheath gas | 5 L/min | | |
| Scan width (at full scan) | *m/z* 900–1100 | | |

### 3.3 Mass Spectrometer Conditions

1. Mass spectrometry conditions are provided in Table 1 (*see* **Note 3**).

### 3.4 Data Analysis and Quantitation

1. Data is quantitated using the Mass Hunter Quant software (Agilent, Santa Clara, CA).
2. Calibration curves are established for every day of analytical run based on linear fit regression of IS peak area/IS concentration (*x*-axis) vs. analyte concentration/IS peak area (*y* axis). *See* **Note 4**.
3. Three "fingers" in the IGF-1 isotopic envelope (1093.5249, 1093.3475, and 1093.6640) with narrow mass extraction width of 5 ppm are used for quantitation.
4. Calibration curve is acceptable if correlation coefficient is ≥0.99. If one of the calibrators are out of range by greater than ±20 % of expected value, then the curve may be plotted after eliminating the calibrator. The lowest and the highest calibrator may not be removed as it will affect the LOQ and reportable range.
5. QC values are acceptable if found to be with ±2SD of the established mean for the lot.
6. The chromatographic retention times are monitored closely. A ±0.03 min shift in retention time on each LC system is considered acceptable.
7. A representative chromatograph is given in Fig. 1.

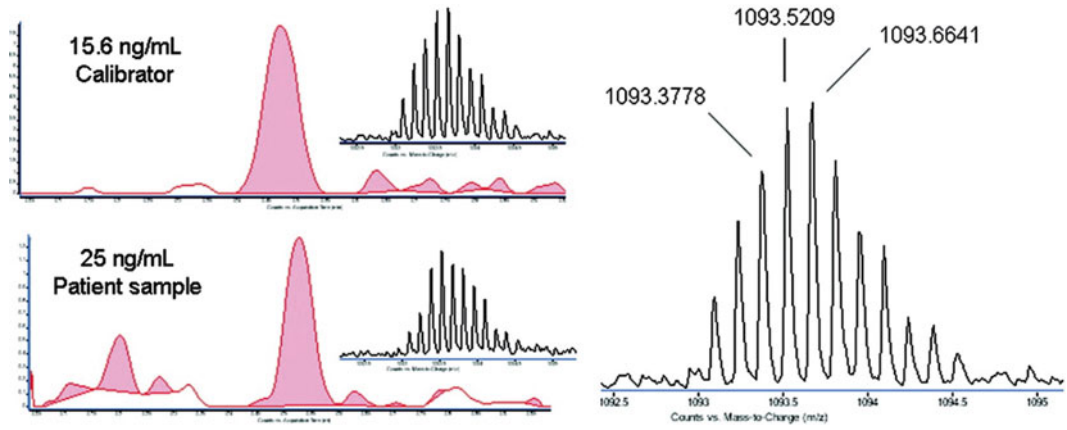



**Fig. 1** Example chromatograms and averaged spectra for IGF-1 in calibrators (15.6 ng/mL) and patient samples (25 ng/mL). The isotopic resolution of the most abundant charge state (7+) for IGF-1 is shown with the quantifier (*m/z* 1093.5209) and two qualifier ions (*m/z* 1093.3778 and 1093.6641) indicated. Published in: Cory E. Bystrom; Shijun Sheng; Nigel J. Clarke; *Anal. Chem*. 2011, 83, 9005–9010. Reprinted (adapted) with permission from (*Anal. Chem*. 2011, 83, 9005–9010). Copyright (2011) American Chemical Society. *Copyright permission obtained*

## 4 Notes

1. Accurate mass of the ion (analyte and IS) has to be optimized on every instrument.
2. The extracts can be stored at –20 °C for up to a week without loss in signal intensity.
3. Tuning of the mass spectrometer should be performed every day to ensure that there is no drift in mass calibration.
4. It is preferable to run calibrators every day of the analytical run.

### References

1. Laron Z (2001) Insulin-like growth factor 1 (IGF-1): a growth hormone. Mol Pathol 54: 311–316
2. Melmed S, Casanueva FF, Cavagnini F, Chanson P, Frohman L, Grossman A, Ho K, Kleinberg D, Lamberts S, Laws E et al (2002) Guidelines for acromegaly management. J Clin Endocrinol Metab 87:4054–4058
3. Giustina A, Barkan A, Chanson P, Grossman A, Hoffman A, Ghigo E, Casanueva F, Colao A, Lamberts S, Sheppard M et al (2008) Guidelines for the treatment of growth hormone excess and growth hormone deficiency in adults. J Endocrinol Invest 31:820–838
4. Furlanetto RW, Underwood LE, Van Wyk JJ, D'Ercole AJ (1977) Estimation of somatomedin-C levels in normals and patients with pituitary disease by radioimmunoassay. J Clin Invest 60:648–657
5. Frystyk J, Freda P, Clemmons DR (2010) The current status of IGF-I assays—a 2009 update. Growth Horm IGF Res 20:8–18

6. Clemmons DR (2007) IGF-I assays: current assay methodologies and their limitations. Pituitary 10:121–128
7. Breier BH, Gallaher BW, Gluckman PD (1991) Radioimmunoassay for insulin-like growth factor-I: solutions to some potential problems and pitfalls. J Endocrinol 128:347–357
8. Krebs A, Wallaschofski H, Spilcke-Liss E, Kohlmann T, Brabant G, Volzke H, Nauck M (2008) Five commercially available insulin-like growth factor I (IGF-I) assays in comparison to the former Nichols Advantage IGF-I in a growth hormone treated population. Clin Chem Lab Med 46:1776–1783
9. Niederkofler EE, Phillips DA, Krastins B, Kulasingam V, Kiernan UA, Tubbs KA, Peterman SM, Prakash A, Diamandis EP, Lopez MF et al (2013) Targeted selected reaction monitoring mass spectrometric immunoassay for insulin-like growth factor 1. PLoS One 8:e81125
10. Kay RG, Barton C, Velloso CP, Brown PR, Bartlett C, Blazevich AJ, Godfrey RJ, Goldspink G, Rees R, Ball GR et al (2009) High-throughput ultra-high-performance liquid chromatography/tandem mass spectrometry quantitation of insulin-like growth factor-I and leucine-rich alpha-2-glycoprotein in serum as biomarkers of recombinant human growth hormone administration. Rapid Commun Mass Spectrom 23:3173–3182
11. Barton C, Kay RG, Gentzer W, Vitzthum F, Pleasance S (2010) Development of high-throughput chemical extraction techniques and quantitative HPLC-MS/MS (SRM) assays for clinically relevant plasma proteins. J Proteome Res 9:333–340
12. Bystrom CE, Sheng S, Clarke NJ (2011) Narrow mass extraction of time-of-flight data for quantitative analysis of proteins: determination of insulin-like growth factor-1. Anal Chem 83:9005–9010
13. Whiteaker JR, Zhao L, Abbatiello SE, Burgess M, Kuhn E, Lin C, Pope ME, Razavi M, Anderson NL, Pearson TW et al (2011) Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. Mol Cell Proteomics 10(M110):005645
14. Bystrom C, Sheng S, Zhang K, Caulfield M, Clarke NJ, Reitz R (2012) Clinical utility of insulin-like growth factor 1 and 2; determination by high resolution mass spectrometry. PLoS One 7:e43457

# Chapter 16

# Quantitation of Free Metanephrines in Plasma by Liquid Chromatography-Tandem Mass Spectrometry

**Courtney Heideloff, Drew Payto, and Sihe Wang**

## Abstract

Plasma metanephrines are measured to aid in the diagnosis of pheochromocytomas. In patients with pheochromocytomas there is excessive production of catecholamines and metanephrines. Measurement of plasma free metanephrines is one of the first-line clinical tests that are used for the diagnosis and follow-up of pheochromocytoma. We describe here a liquid chromatography-tandem mass spectrometry method to measure free metanephrines in plasma. Free metanephrine and normetanephrine are extracted via solid-phase extraction. After extraction and evaporation, the reconstituted supernatant is analyzed by high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). The MS/MS is set to selective reaction monitoring mode ($180.1 \rightarrow 148.1$ $m/z$ for metanephrine, $183.1 \rightarrow 168.1$ for d3-metanephrine, $166.1 \rightarrow 134.1$ $m/z$ for normetanephrine, and $169.1 \rightarrow 137.2$ $m/z$ for d3-normetanephrine) with positive electrospray ionization. Quantitation is based on peak area ratio of the analyte to its respective deuterated internal standard. The assay is linear from 5.9 to 4090.0 pg/mL for metanephrine and 22.0 to 4386.7 pg/mL for normetanephrine with precision of <6 % over the ranges.

**Key words** Metanephrine, Normetanephrine, Mass spectrometry, Liquid chromatography, Plasma, Quantification, Pheochromocytoma

## 1 Introduction

Pheochromocytomas are neuroendocrine tumors typically found in the adrenal gland. They often lead to increased blood pressure due to their production and secretion of catecholamines which are metabolized to metanephrines [1]. Plasma catecholamines are still ordered to detect pheochromocytomas; however their short half-life makes it difficult to distinguish between overproduction due to tumors from a burst of secretion caused by stress of blood sampling [2]. Studies have shown that measurement of free plasma metanephrines provides superior diagnostic sensitivity and specificity to the measurement of the catecholamines for the detection of pheochromocytoma [3].

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_16, 

High performance liquid chromatography with electrochemical detection is commonly used to measure metanephrines; however these assays typically require extensive sample preparation and long chromatography time [4, 5]. High performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods offer shorter chromatography times while yielding high specificity; however it is vital to ensure that isobaric interference does not exist between normetanephrine and epinephrine [6–8]. This chapter describes an accurate and precise LC-MS/MS method to measure free metanephrines in plasma. In this method, free metanephrine and normetanephrine are extracted from plasma via solid-phase extraction along with internal standards in 10 mM ammonium phosphate. After extraction and evaporation, the reconstituted supernatant is analyzed by LC-MS/MS. The MS/MS is set to selective reaction monitoring mode ($180.1 \rightarrow 148.1\ m/z$ for metanephrine, $183.1 \rightarrow 168.1$ for d3-metanephrine, $166.1 \rightarrow 134.1\ m/z$ for normetanephrine, and $169.1 \rightarrow 137.2\ m/z$ for d3-normetanephrine) with positive electrospray. Quantitation is based on peak area ratios of the analytes to their respective deuterated internal standards [9].

## 2 Materials

### 2.1 Samples

EDTA plasma. Transport collected specimens on ice. Centrifuge samples and remove plasma from cells within 1 h of collection. Freeze plasma at < –20 °C immediately after separation from cells. Freeze samples until analysis; avoid freeze-thaw cycles.

Avoid strenuous exercise for 3 days prior to collection. Discontinue epinephrine and epinephrine-like drugs at least 3 days before collection if possible. Allow patient to be seated in a relaxed position for 10–15 min before collection.

### 2.2 Solvents and Reagents

1. 10 mM Ammonium Phosphate, pH 6.5: Weigh 0.575 g ammonium phosphate and place into a 500 mL volumetric flask. Add approximately 495 mL of clinical laboratory reagent water (CLRW) to the flask. Mix until dissolved. Transfer contents to a beaker with stir bar. Adjust the pH to 6.5 using Ammonium Hydroxide (requires only a few drops of concentrated solution, ammonium hydroxide may be diluted with CLRW if necessary). Transfer solution back to volumetric flask and bring to volume with CLRW. Stable for 2 months at 2–8 °C.
2. 2 % Formic Acid in 95:5 Acetonitrile:CLRW (v/v): Using a graduated cylinder, add 931 mL Acetonitrile to a 1 L bottle. Using a graduated cylinder, add 49 mL CLRW to the same bottle. Using a graduated cylinder, add 20 mL formic acid to the bottle. Stable for 3 months at room temperature.

3. 0.2 % Formic Acid in Acetonitrile: Add approximately 800 mL of Acetonitrile to a 1 L volumetric flask. Using a pipette, add 2 mL formic acid. Bring volume to 1 L with Acetonitrile. Stable for 3 months at room temperature.
4. Mobile Phase A (1 mM Ammonium Formate + 0.1 % Formic Acid): Weigh 0.0631 g of ammonium formate and place into a 1 L volumetric flask. Add approximately 800 mL of CLRW. Using a pipette, add 1 mL formic acid to the flask. Bring to volume with CLRW. Stable for at least 1 month at room temperature.
5. Mobile Phase B (1 mM Ammonium Formate in Methanol + 0.1 % Formic Acid): Weigh 0.0631 g of ammonium formate and place into a 1 L volumetric flask. Add approximately 800 mL of Methanol. Using a pipette, add 1 mL of formic acid to the flask. Bring to volume with Methanol. Stable for at least 3 months at room temperature.

### 2.3 Internal Standards and Standards

1. Primary Standard: Catecholamine Mix 2 (Metanephrine and Normetanephrine) (Cerilliant).
2. Primary Internal Standard (IS): $D_3$-Metanephrine and $D_3$-Normetanephrine, 100 µg/mL (Cerilliant).
3. Combined Intermediate IS Solution (10 µg/mL): Using an adjustable pipette, measure 50 µL each of $d_3$M and $d_3$NM (200 µg/mL Substock) into a 1 mL Class A volumetric flask and bring to volume with 1 mM ammonium formate + 0.1 % formic acid. Mix by vortexing. Do not store.
4. Working IS Solution (10 ng/mL): Using an adjustable pipette, measure 25 µL of (10 µg/mL) Intermediate Internal Working Solutions (10 µg/mL) into a 25 mL Class A volumetric flask. Bring to volume with 1 mM ammonium formate + 0.1 % formic acid. Vortex to mix. Stable for 6 months when stored at < −20 °C.

### 2.4 Calibrators and Controls (See Notes 1 and 2)

1. Calibrators: Prepare working solution by adding 20 µL of Catecholamine Mix 2 to 5 mL volumetric flask. Bring to volume with 10 mM Ammonium Phosphate, pH 6.5. Mix well. Prepare calibrators 1–7 by making serial dilutions of the working solution according to Table 1. Mix well after each dilution step.
2. Controls
   (a) Analyte spiking solution: Measure 10 µL of Cerilliant QC Stock (1.0 mg/mL) into 25 mL volumetric flask. Bring to volume with 10 mM Ammonium Phosphate, pH 6.5.
   (b) Level 1: Measure 3 µL of Analyte Spiking Solution and add to a 50 mL volumetric flask. Bring to volume with pooled plasma. Based on past pools, the concentrations may be approximately 60 pg/mL M and ~200 pg/mL NM.

**Table 1**
**Preparation of calibrators**

| To make | Use source solution | Volume of source solution | Volume of 10 mM ammonium phosphate pH 6.5 (mL) | Metanephrine concentration (pg/mL) | Normetanephrine concentration (pg/mL) |
|---|---|---|---|---|---|
| Std7 | Working solution | 100 µL | QS to 100 mL | 4000.00 | 4000.00 |
| Std6 | Std 7 | 50 mL | QS to 100 mL | 2000.00 | 2000.00 |
| Std5 | Std 6 | 25 mL | QS to 100 mL | 500.00 | 500.00 |
| Std4 | Std 5 | 25 mL | QS to 100 mL | 125.00 | 125.00 |
| Std3 | Std 4 | 25 mL | QS to 100 mL | 31.25 | 31.25 |
| Std2 | Std 3 | 50 mL | QS to 100 mL | 15.63 | Exclude |
| Std1 | Std 2 | 25 mL | QS to 50 mL | 7.81 | Exclude |

(c) Level 2: Measure 62.5 µL of Analyte Spiking Solution and add to a 50 mL volumetric flask. Bring to volume with pooled plasma. Based on past pools the concentrations may be approximately 535 pg/mL M and ~675 pg/mL NM.

### 2.5 Analytical Equipment and Supplies

1. Nexera Shimadzu HPLC.
2. AB Sciex 5500 QTRAP.
3. Vacuum manifold.
4. Biotage TurboVap.
5. UltraShield UHPLC pre-column filter.
6. Ultra PFP Propyl analytical column (3 µm, 2.1 × 100 mm).
7. Oasis WCX SPE Cartridges.
8. Injection Vials (12 × 32 mm, 350 µL).
9. Injection Vial Caps (11 mm snap PTFE/SIL).

### 2.6 HPLC Parameters

They are given in Table 2.

### 2.7 MS/MS Tune Parameters

They are given below and in Table 3.

(a) Turbo Spray ESI+.
(b) Spray voltage = 1250 V.
(c) Curtain Gas = 30 U.
(d) Collision Gas = Medium.
(e) Temperature = 700 °C.
(f) Ion Source Gas 1 = 50 U.
(g) Ion Source Gas 2 = 40 U.

**Table 2**
**HPLC parameters[a]**

| | | | |
|---|---|---|---|
| Column temp. | 40 °C | | |
| Flow rate | 0.500 mL/min | | |
| Gradient | Time (min) | Event | Parameter |
| | 0 | Pump B conc. | 1 |
| | 0.25 | Pump B conc. | 1 |
| | 0.26 | Pump B conc. | 40 |
| | 2.25 | Pump B conc. | 60 |
| | 2.26 | Total flow | 0.7 |
| | 2.26 | Pump B conc. | 60 |
| | 2.86 | Pump B conc. | 60 |
| | 2.87 | Pump B conc. | 1 |
| | 2.88 | Total flow | 0.5 |

[a]Optimized for Nexera Shimadzu HPLC with Restek Ultra PFP Propyl analytical column (3 μm, 2.1 × 100 mm). Mobile phase A: 1 mM ammonium formate + 0.1 % formic acid; mobile phase B: 1 mM ammonium formate in methanol + 0.1 % formic acid

**Table 3**
**Optimized MS/MS parameters[a]**

| Compound | Q1 mass | Q3 mass | Dwell time | DP | EP | CE | CXP |
|---|---|---|---|---|---|---|---|
| Metanephrine | 180.1 | 148.1 | 25 | 123 | 7 | 24 | 17 |
| Metanephrine-IS | 183.1 | 168.1 | 25 | 123 | 7 | 24 | 17 |
| Normetanephrine | 166.1 | 134.1 | 50 | 95 | 7 | 24 | 13 |
| Normetanephrine-IS | 169.1 | 137.1 | 50 | 95 | 7 | 24 | 13 |

*DP* declustering potential, *EP* entrance potential, *CE* collision energy, *CXP* exit potential
[a]Optimized for the AB Sciex 5500 QTRAP. Tune settings may vary slightly between instruments

## 3 Methods

### *3.1 Stepwise Procedure*

1. Pipette 0.5 mL of standard, control, and patient samples into their respective labeled tubes (*see* **Note 3**).
2. Pipette 25 μL internal standard into each tube. Vortex briefly.
3. Add 0.5 mL of 10 mM Ammonium Phosphate Buffer, pH 6.5 to each tube. Vortex briefly.
4. Label one 12 × 75 mm tube for each standard, control, and patient. Place in vacuum manifold collection rack and set aside.

5. Insert the WCX SPE cartridges into the vacuum box. Position them in the same order as the collection tubes.
6. Wash each cartridge with 1 mL of methanol.
7. Wash each cartridge with 1 mL 10 mM Ammonium Phosphate, pH 6.5. Turn off vacuum.
8. Pipette the entire sample into respective SPE cartridge.
9. Turn on the vacuum. Allow the samples to slowly flow through the cartridges.
10. Wash each cartridge with 1 mL CLRW.
11. Wash each cartridge with 1 mL Methanol.
12. Wash each cartridge with 1 mL 0.2 % Formic Acid in ACN solution.
13. Remove the manifold box cover and place the collection rack into the box. Return the manifold cover to the vacuum box making sure the proper tubes are directly under the respective cartridges.
14. Add 0.5 mL of 2 % formic acid in 95:5 ACN:CLRW to each cartridge.
15. Turn on vacuum and slowly elute the analytes into the collection tubes in the manifold box.
16. Remove the collection rack and tubes from the manifold box.
17. Evaporate the eluent to dryness. TurboVap settings: under nitrogen at 35 °C for 20 min.
18. Reconstitute the dried extract with 100 µL of 0.1 % formic acid in 1 mM Ammonium Formate solution. Vortex tubes.
19. Transfer the contents of each tube to an injection vial and load into autosampler tray.
20. Inject 20 µL of sample onto LC-MS/MS. Sample ion chromatograms for plasma metanephrine and normetanephrine are shown in Figs. 1 and 2.

### 3.2 Data Analysis

1. Data is analyzed using Analyst Software (AB Sciex).
2. Standard curves are generated based on analyte/IS peak area ratio. A 7 and 5 point calibration curve, for metanephrine and normetanephrine respectively, was weighted using a factor of $1/X^2$ and not forced through zero.
3. The assay is linear from 5.9 to 4090 pg/mL and 22 to 4387 pg/mL for metanephrine and normetanephrine respectively. Precision was determined to have a %CV < 6.8 % for both analytes.

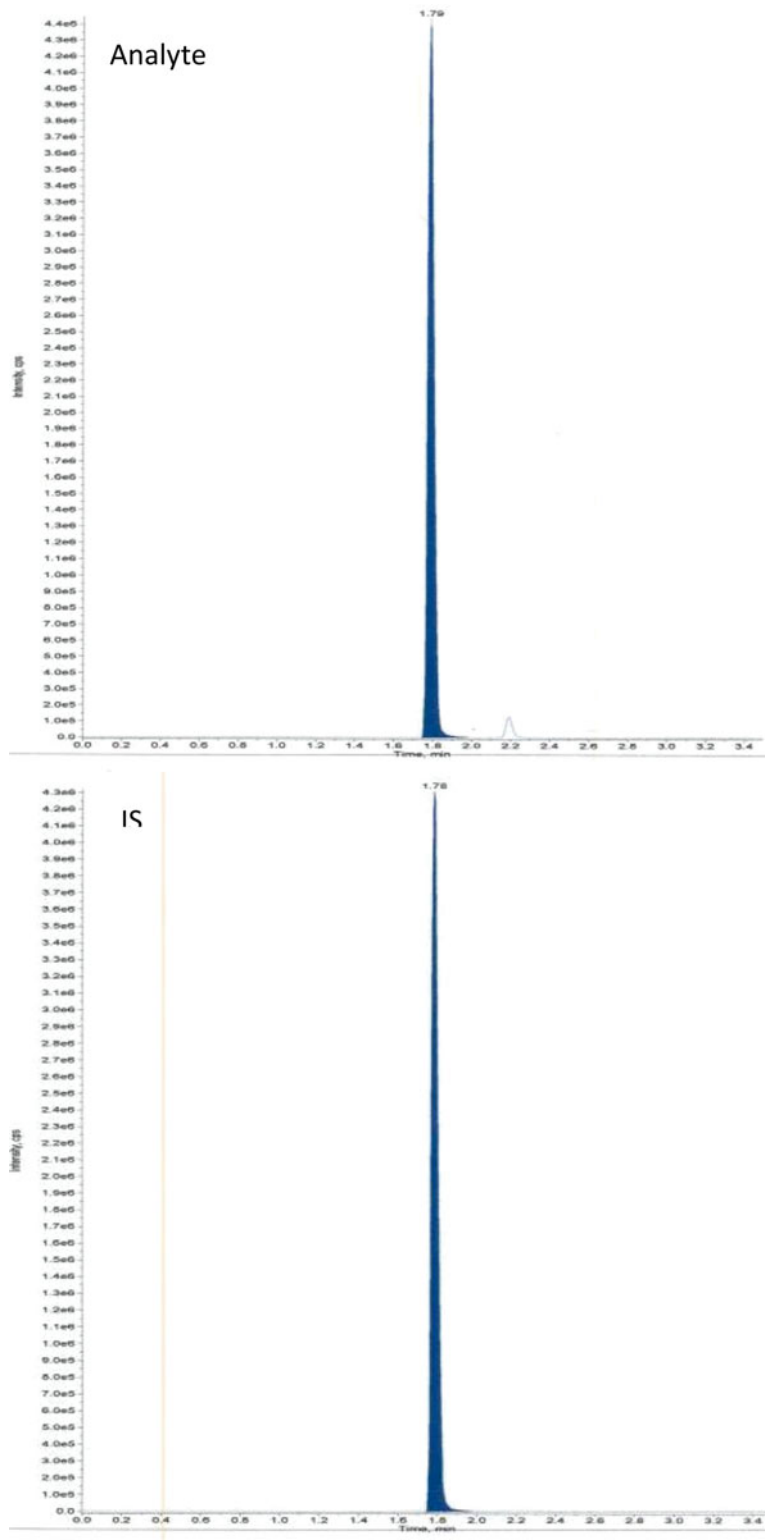



**Fig. 1** Unextracted standard for metanephrine.

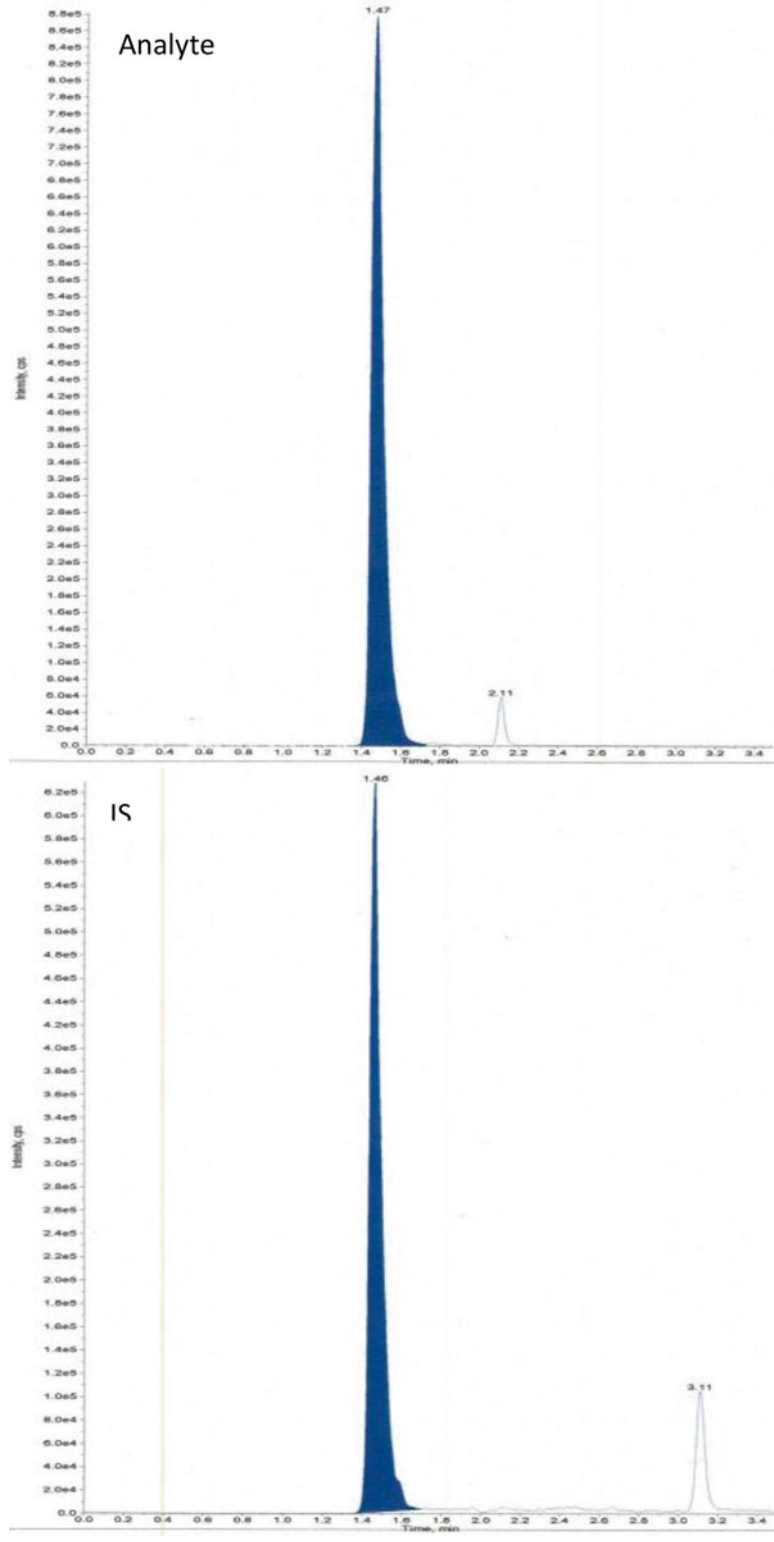



**Fig. 2** Unextracted standard for normetanephrine

## 4 Notes

1. Two separate stock solutions must be used for the preparation of calibrators and quality controls.
2. Calibrators and quality controls should be prepared and then aliquoted into single-use vials. Stable for 6 months when stored at < −60 °C.
3. A calibration curve needs to be extracted and run with every analytical run.

### References


1. Lenders JW, Pacak K, Walther MM, Linehan WM, Mannelli M, Friberg P, Keiser HR, Goldstein DS, Eisenhofer G (2002) Biochemical diagnosis of pheochromocytoma: which test is best? JAMA 287:1427–1434
2. Grouzmann E, Drouard-Troalen L, Baudin E, Plouin PF, Muller B, Grand D, Buclin T (2010) Diagnostic accuracy of free and total metanephrines in plasma and fractionated metanephrines in urine of patients with pheochromocytoma. Eur J Endocrinol 162: 951–960
3. Pacak K, Eisenhofer G, Ahlman H, Bornstein SR, Gimenez-Roqueplo AP, Grossman AB, Kimura N, Mannelli M, McNicol AM, Tischler AS (2007) International symposium on pheochromocytoma. Pheochromocytoma: recommendations for clinical practice from the First International Symposium October 2005. Nat Clin Pract Endocrinol Metab 3:92–102
4. Pillai D, Callen S (2010) Pilot quality assurance programme for plasma metanephrines. Ann Clin Biochem 47:137–142
5. Singh RJ, Eisenhofer G (2007) High-throughput, automated, and accurate biochemical screening for pheochromocytoma: are we there yet? Clin Chem 53:1565–1567
6. Peaston RT, Graham KS, Chambers E, van der Molen JC, Ball S (2010) Performance of plasma free metanephrines measured by liquid chromatography-tandem mass spectrometry in the diagnosis of pheochromocytoma. Clin Chim Acta 411:546–552
7. de Jong WH, Graham KS, van der Molen JC, Links TP, Morris MR, Ross HA, de Vries EG, Kema IP (2007) Plasma free metanephrine measurement using automated online solid-phase extraction HPLC tandem mass spectrometry. Clin Chem 53:1684–1693
8. Lagerstedt SA, O'Kane DJ, Singh RJ (2004) Measurement of plasma free metanephrine and normetanephrine by liquid chromatography-tandem mass spectrometry for diagnosis of pheochromocytoma. Clin Chem 50:603–611
9. Gabler J, Yuan C, Woroniecki W, Liu H, Wang S (2012) A Sensitive and Interference-Free Liquid Chromatography Tandem Mass Spectrometry Method for Measuring Metanephrines in Plasma. J Chromatograph Separat Techniq 4:195. doi:10.4172/2157-7064.1000195

# Chapter 17

# Quantification of Metanephrine and Normetanephrine in Urine Using Liquid Chromatography-Tandem Mass Spectrometry

**Jessica Gabler and Sihe Wang**

## Abstract

Measuring urinary metanephrines aides in the diagnosis of pheochromocytomas–catecholamine producing tumors. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) allows for greater sensitivity and simpler sample preparation as compared with other techniques. Here we describe a simple LC-MS/MS method for measuring metanephrines in urine. Each urine sample was treated with diphenylboronic acid to create boronate complexes, and then applied to a Bond-Elut Plexa cartridge. After solid phase extraction, samples were concentrated and analyzed on an Atlantis T3 column with chromatographic run time totaling 8.5 min. MS/MS was set in positive electrospray ionization mode with multiple reaction monitoring for data collection. The assay was linear from 0.2 to 27.4 μmol/L and 0.3 to 14.6 μmol/L for metanephrine and normetanephrine, respectively. Intra-assay and total precision at three concentration levels over 10 days were <5 % for metanephrine and <10 % for normetanephrine.

**Key words** Liquid chromatography, Mass spectrometry, Pheochromocytoma, Metanephrine, Normetanephrine, Urine

## 1 Introduction

Pheochromocytomas are tumors, primarily of the adrenal medulla, that cause secretion of catecholamines. Catecholamines (epinephrine and norepinephrine) are rapidly converted to their metabolites, metanephrine (M) and normetanephrine (NM), respectively. Compared to patients with no catecholamine-secreting tumors percent increase of metanephrines is much greater than catecholamines when pheochromocytomas present, making measurement of metanephrines a more sensitive indicator of pheochromocytomas [1, 2]. In addition, metanephrines are more stable than catecholamines after sample collection. Therefore, measurement of urinary fractionated metanephrines has been recommended as one of the foremost screening tests for unexplained hypertension to help exclude or diagnose pheochromocytoma [1–7].

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_17, 

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been the preferred technology for measuring urinary metanephrines because it provides less laborious sample preparations, increased sensitivity, and decreased chromatographic run times versus LC methods coupled with electrochemical detection [8, 9]. In this chapter, we present an LC-MS/MS method with simple sample preparation, modified from previous publications [9, 10] to measure urinary metanephrines using boronate complexes. Samples are hydrolyzed prior to solid phase extraction, and after evaporating to dryness, samples are reconstituted in 100 mM ammonium formate + 2 % formic acid. Supernatant (15 µL) is injected onto the LC-MS/MS system for analysis [11].

## 2 Materials

### 2.1 Samples

24 h or random urine collected in a plain urine collection container. Specimens should be stored refrigerated (2–8 °C) throughout collection and transport. Upon specimen receipt, adjust urine pH to <3 using hydrochloric acid and store refrigerated prior to analysis.

### 2.2 Solvents and Reagents

1. Acetonitrile, HPLC grade.
2. Methanol, HPLC grade.
3. Acetone, HPLC grade.
4. 2-Propanol, HPLC grade.
5. Formic Acid, ACS grade.
6. Ammonium formate, MS grade.
7. Hydrochloric Acid, ACS plus grade.
8. Sodium Metabisulfite, BioRad.
9. EDTA, Fisher Scientific.
10. 2-aminoethyl diphenylborinate, Sigma Aldrich.
11. Ammonium Chloride, Molecular Biology grade.
12. Ammonium Hydroxide, ACS grade.
13. Clinical Laboratory Reagent Water (CLRW)—HPLC grade.
14. 5 M Ammonium Chloride ($NH_4Cl$) pH 8.5
    Weigh 267.44 g of $NH_4Cl$ and put into a large beaker (*see* **Note 1**). Add approximately 900 mL of CLRW and a stir bar to the beaker and cover with parafilm. Allow the solution to mix overnight or completely dissolved. Once dissolved, adjust the pH to 8.5 with concentrated ammonium hydroxide (*see* **Note 2**). Pour the solution, minus the stir bar, into a 1000 mL volumetric flask and dilute to volume with CLRW. Stable for 3 months when stored at room temperature (*see* **Note 3**).
15. 0.5 M Ammonium Chloride ($NH_4Cl$)

Using a graduated cylinder, measure 200 mL of 5 M $NH_4Cl$ pH 8.5 and pour into a 2000 mL bottle, add 1800 mL CLRW to the bottle. Stable for 3 months when stored at room temperature.

16. Complexing reagent
    Weigh 5.0 g of EDTA and 2.0 g of 2-aminoethyl diphenylborinate. Place in a 1000 mL Class A volumetric flask. Dilute to volume with 5 M $NH_4Cl$. Place a stir bar into the flask and cap. Mix overnight to dissolve (*see* **Note 4**). Stable for 3 months when stored at room temperature.
17. 20 % Methanol in 0.5 M Ammonium Chloride ($NH_4Cl$)
    Using a graduated cylinder, add 200 mL of methanol, 80 mL of 5 M $NH_4Cl$, and 720 mL CLRW into a bottle. Stable for 3 months when stored at room temperature.
18. 100 mM Ammonium Formate, 2 % Formic Acid
    Weigh 6.303 g of ammonium formate into a 1000 mL Class A volumetric flask. Add approximately 800 mL of CLRW. Using a graduated cylinder, add 20 mL formic acid to the flask. Swirl to dissolve. Dilute to volume with CLRW. Stable for 3 months when stored at room temperature.
19. Mobile Phase Eluting A: 12 mM Ammonium Formate, 1.2 % Formic Acid
    Weigh 0.758 g of ammonium formate into a 1000 mL volumetric flask. Add approximately 800 mL of CLRW. Using a graduated cylinder, add 12 mL of formic acid to the flask. Dilute to volume with CLRW. Stable for 1 month when stored at room temperature.
20. Mobile Phase Eluting B: 100 % Methanol.
21. 3.6 M HCl
    Using a graduated cylinder, add 35 mL of CLRW to a bottle. Using a graduated cylinder, add 15 mL of concentrated HCl. Stable for 1 year when stored at room temperature.
22. 0.1 % Sodium Metabisulfite in 0.1 M HCl
    Weigh 100 mg of sodium metabisulfite into a 100 mL volumetric flask. Add 80 mL of CLRW, and 835 μL of HCl and invert to mix. Dilute to volume with CLRW and invert to mix. Transfer to a bottle. Stable for 1 year when stored at room temperature.

### 2.3 Standards and Internal Standards

1. Standard Stock—Catecholamine Mix 2, including metanephrine and normetanephrine, 1 mg/mL (Cerilliant).
2. Internal Standard (Metanephrine)—dl-Metanephrine-d3 (α-d2,β-d1) HCl, 5 mg (Medical Isotopes).
3. Internal Standard (Normetanephrine)—dl-Normetanephrine-d3 (α-d1,β-d2) HCl, 5 mg (Medical Isotopes).

4. Internal Standard Stocks—separate (1000 μg/mL)
   Add 1 mL 0.1 % sodium metabisulfite in 0.1 M HCl to the metanephrine internal standard container. Allow to dissolve then quantitatively transfer the contents into a 5 mL Class A volumetric flask. Repeat 3 times to ensure complete transfer of material. Dilute to volume with 0.1 % sodium metabisulfite in 0.1 M HCl. Repeat for normetanephrine internal standard using a separate flask. Store separate 1.5 mL aliquots of Metanephrine and Normetanephrine internal standard stocks in vials. Stable for 6 months when stored at <−60 °C.
5. Internal Standard Substock—separate (200 μg/mL)
   Using a volumetric pipette add 4 mL methanol to a 16 × 100 glass screw cap tube. Using a volumetric pipette add 1 mL of metanephrine internal standard stock solution (1000 μg/mL) to tube. Mix well.

   Repeat for normetanephrine internal standard stock solution using separate tube. Stable for 6 months when stored at <−60 °C.
6. Working Internal Standard—combined (1650 ng/mL)
   Pipette 825 μL of each Internal Standard Substock into the same 100 mL Class A volumetric flask. Bring to volume with 100 mM Ammonium Formate, 2 % Formic Acid. Stable for 3 months when stored at 2–8 °C.

### 2.4 Calibrators and Controls

1. Calibrators 1–5
   Prepare calibrator 5 by adding 160 μL of Standard Stock to a 100 mL volumetric flask. Bring to volume with CLRW. Mix well.

   Calibrator 5 is serially diluted to prepare subsequent calibrators as shown in Table 1.
2. Controls—Lyphochek Quantitative Urine Control, Level I and II (BioRad)
   Prepare control levels 1 and 2 by adding 10 mL of CLRW to each vial. Allow the controls to stand for at least 15 min, swirling occasionally.
3. Testmix—Pipette 10 μL of Metanephrine and Normetanephrine standard stock (1 mg/mL) and 10 μL each of Metanephrine and Normetanephrine internal standard stocks (1 mg/mL) into a 50 mL Class A volumetric flask. Dilute to volume with 100 mM Ammonium Formate + 2 % Formic Acid. Stable for 1 year when stored at <−60 °C.

### 2.5 Analytical Equipment and Supplies

1. Thermo Fisher Scientific Quantum Access Tandem Mass Spectrometer.
2. Thermo Fisher Scientific CTC-PAL Autosampler and Aria TLX2 HPLC.
3. Atlantis T3 analytical column (3 μm, 3.0 × 100 mm).

**Table 1**
**Preparation of calibrators**

| To make: | Use source solution | Volume of source solution (mL) | Volume of CLRW | Metanephrine concentration (ng/mL)[a] | Normetanephrine concentration (ng/mL)[a] |
|---|---|---|---|---|---|
| Std 5 | Spiked from Stock | 160 µL | QS to 100 mL | 1600.0 | 1600.0 |
| Std4 | Std 5 | 50 | QS to 100 mL | 800.0 | 800.0 |
| Std3 | Std 4 | 50 | QS to 100 mL | 400.0 | 400.0 |
| Std2 | Std 3 | 50 | QS to 100 mL | 200.0 | 200.0 |
| Std1 | Std 2 | 25 | QS to 100 mL | 50.0 | 50.0 |

[a] *See* **Note 5**

4. T3 guard column cartridge (3 µm, 2.1 × 10 mm).
5. T3 guard column holder.
6. Varian Plexa SPE cartridges (30 mg, 3 mL).
7. Injection Vials with caps (12 × 32 mm A/S Crimp, 350 µL).
8. Pipette Tips (up to 1000 µL capacity).
9. Pipette Tips (10–100 µL capacity).
10. Eppendorf Combitips (25 mL total capacity).
11. 16 × 100 mm disposable borosilicate tubes.
12. 13 × 100 mm disposable borosilicate tubes.
13. pH meter.
14. Vortex Genie Mixer.
15. Boiling water bath.
16. Vacuum manifold.
17. 50 °C water bath with nitrogen supply.

## 3 Methods

### *3.1 Stepwise Procedure*

1. Turn on 56 °C and 100 °C water baths.
2. Bring calibrators, controls, and patient samples to room temperature and mix thoroughly.
3. To labeled 16 × 100 mm tubes, pipette 1.0 mL of calibrator, control, or patient sample.

4. Add 100 µL internal standard to each tube.
5. Using a plastic transfer pipette, add two drops of 3.6 M HCl to each tube. Vortex briefly.
6. Place tubes in a metal rack and cover the top of each tube with a 16 × 125 mm Teflon-lined test tube cap to prevent evaporation. Place the covered tubes into a boiling water bath (100 °C) for 30 min to hydrolyze the samples.
7. Transfer the tubes to a cool tap water bath. Allow the samples to cool to room temperature.
8. Add 2 mL of complexing reagent to each tube. Vortex briefly (*see* **Note 6**).
9. Insert one labeled Plexa SPE cartridge for each sample into the vacuum box.
10. Wash each cartridge with 1 mL of methanol. Repeat.
11. Wash each cartridge with 1 mL 0.5 M $NH_4Cl$. Repeat.
12. Pour the entire hydrolyzed sample into respective Plexa SPE cartridge.
13. Allow the samples to slowly flow through the cartridges.
14. Wash each cartridge with 1 mL 0.5 M $NH_4Cl$. Repeat.
15. Wash each cartridge with 1 mL 20 % Methanol in 0.5 M $NH_4Cl$. Repeat.
16. Remove the manifold box cover and rinse the needles with methanol and wipe needles with a kimwipe (*see* **Note 7**).
17. Place the collection rack containing labeled 13 × 10 mm tubes into the box, making sure the tubes are directly under the respective cartridges.
18. Add 1 mL 100 mM ammonium formate, 2 % formic acid into each cartridge.
19. Slowly allow the eluent to flow through the cartridge. Turn on the vacuum to ensure complete transfer into the collection tubes.
20. Vortex collection tubes briefly.
21. Place tubes in a 50–60 °C evaporation water bath. Evaporate to complete dryness with nitrogen.
22. Reconstitute with 100 µL of 100 mM ammonium formate + 2 % formic acid. Vortex tubes.
23. Transfer the samples to labeled orange top injection vials. Cap vials.
24. Inject 15 µL of sample on HPLC-MS/MS (*see* **Note 8**).

### 3.2 Analysis

1. HPLC instrument operating parameters are given in Table 2.
2. MS instrument operating parameters are given in Table 3.
3. Data is analyzed using LCQuan Software (ThermoFisher Scientific).

**Table 2**
**HPLC gradient conditions**

| Time | Duration (s) | Flow rate (mL/min) | Gradient | %A | %B |
|---|---|---|---|---|---|
| 0.00 | 45.0 | 0.4 | Step | 100.0 | 0.0 |
| 0.75 | 180.0 | 0.4 | Step | 82.0 | 18.0 |
| 3.75 | 60.0 | 0.4 | Step | 50.0 | 50.0 |
| 4.75 | 315.0 | 0.4 | Step | 100.0 | 0.0 |

**Table 3**
**MS source operating parameters**

| Polarity | Positive |
|---|---|
| Spray voltage | 5000 |
| Vaporizer temperature | 0 |
| Sheath gas pressure | 55 |
| Ion sweep gas pressure | 3.5 |
| Aux gas pressure | 55 |
| Capillary temperature | 280 |
| Skimmer offset | 0 |

4. An example chromatogram from a patient urine sample is shown in Fig. 1.
5. Standard curves are generated based on linear regression of the analyte/IS peak area ratio ($y$) versus analyte concentration ($x$) using $1/X^2$ weighting. Analyte and IS quantifying ions are listed in Table 4.
6. Acceptable runs are determined if calculated control concentrations are within ±2 standard deviations of target values (*see* **Note 9**).

## 4 Notes

1. In order to expedite dissolution, crush any large clumps of $NH_4Cl$ to powder before adding to beaker.
2. It will take approximately 12 mL of Ammonium Hydroxide to get to pH 8.5.

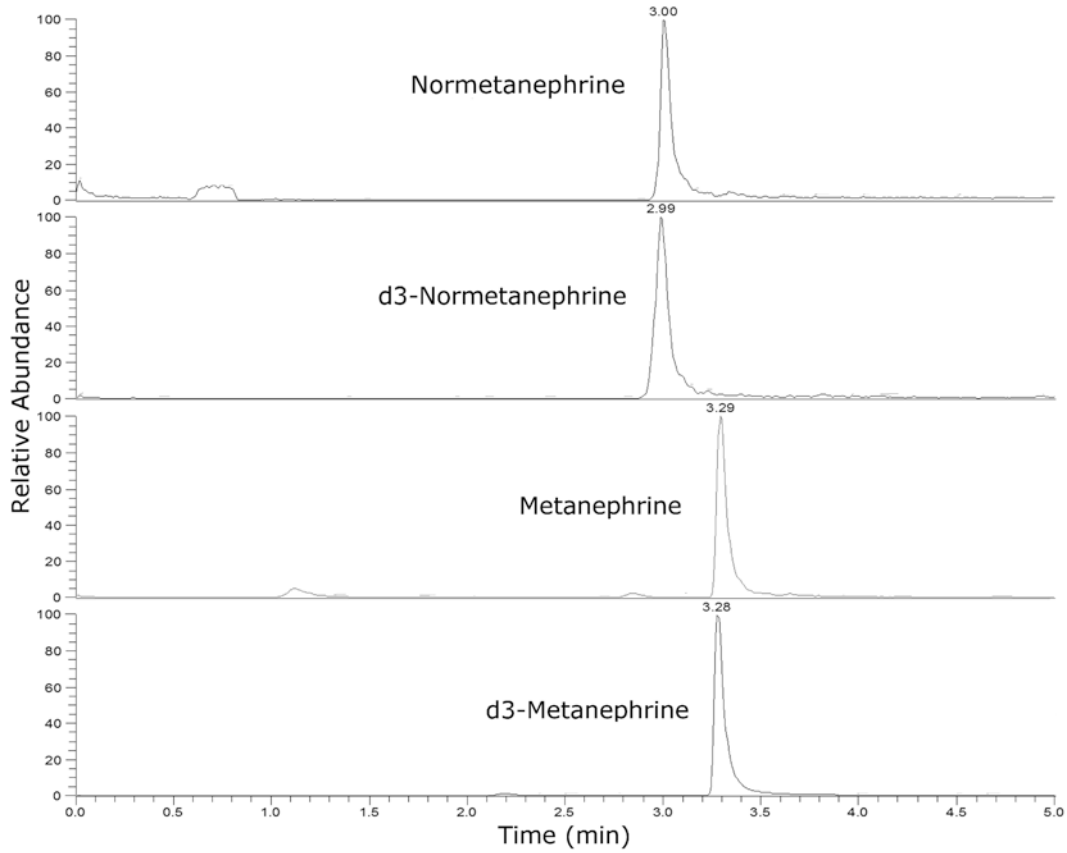



**Fig. 1** Chromatogram of a patient urine specimen

**Table 4**
**MRM transitions**

| Analyte | Q1 | Q3 | Collision energy |
|---|---|---|---|
| Normetanephrine | 166.1 | 134.2 | 13.0 |
| Normetanephrine IS | 169.1 | 137.2 | 17.0 |
| Metanephrine | 198.2 | 180.2 | 11.0 |
| Metanephrine IS | 201.1 | 183.2 | 11.0 |

3. If making Complexing Reagent, 0.5 M Ammonium Chloride and 20 % methanol in 0.5 M Ammonium Chloride on the same day, you will need to prepare double the volume (2000 mL) of 5 M Ammonium Chloride.
4. Prepare Complexing Reagent at least 1 day before it is needed as it will need an extended period of time to completely dissolve.

5. Certificate of Analysis certified concentration for Catecholamine Mix 2 should be used to calculate accurate concentration levels of calibrators. Concentrations listed in Table 1 are estimates based on a CoA concentration of 1 mg/mL.
6. Check to see if samples are cloudy. If samples are cloudy add one drop of $NH_4OH$ and vortex. If sample is still cloudy add additional drop of $NH_4OH$. If more than two drops of $NH_4OH$ are needed consult with supervisory personnel.
7. We have found this cleaning step helps to decrease chances for contamination and improves sample purity.
8. A Testmix sample is injected prior to each run to determine acceptability of instrument and assay performance. Acceptable results are determined if all analytes' peak areas are within 50 % of a rolling mean (determined from in-house assay of testmix sample over time).
9. Target values are determined through in-house assay of controls averaging at least 10 data points covering multiple days.

## References


1. Barron J (2010) Phaeochromocytoma: diagnostic challenges for biochemical screening and diagnosis. J Clin Pathol 63(8):669–674
2. Eisenhofer G et al (2008) Current progress and future challenges in the biochemical diagnosis and treatment of pheochromocytomas and paragangliomas. Horm Metab Res 40(5):329–337
3. Grouzmann E et al (2010) Diagnostic accuracy of free and total metanephrines in plasma and fractionated metanephrines in urine of patients with pheochromocytoma. Eur J Endocrinol 162(5):951–960
4. Lenders JW (2009) Biochemical diagnosis of pheochromocytoma and paraganglioma. Ann Endocrinol (Paris) 70(3):161–165
5. Lenders JW et al (2005) Phaeochromocytoma. Lancet 366(9486):665–675
6. Lenders JW, Pacak K, Eisenhofer G (2002) New advances in the biochemical diagnosis of pheochromocytoma: moving beyond catecholamines. Ann N Y Acad Sci 970:29–40
7. Lenders JW et al (2002) Biochemical diagnosis of pheochromocytoma: which test is best? JAMA 287(11):1427–1434
8. Taylor RL, Singh RJ (2002) Validation of liquid chromatography-tandem mass spectrometry method for analysis of urinary conjugated metanephrine and normetanephrine for screening of pheochromocytoma. Clin Chem 48(3): 533–539
9. Whiting MJ (2009) Simultaneous measurement of urinary metanephrines and catecholamines by liquid chromatography with tandem mass spectrometric detection. Ann Clin Biochem 46(Pt 2):129–136
10. Talwar D et al (2002) Extraction and separation of urinary catecholamines as their diphenyl boronate complexes using C18 solid-phase extraction sorbent and high-performance liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 769(2):341–349
11. Gabler J, Miller A, Wang S (2011) A simple liquid chromatography-tandem mass spectrometry method for measuring metanephrine and normetanephrine in urine. Clin Chem Lab Med 49(7):1213–1216

# Chapter 18

# High-Throughput Analysis of Methylmalonic Acid in Serum, Plasma, and Urine by LC-MS/MS. Method for Analyzing Isomers Without Chromatographic Separation

**Mark M. Kushnir, Gordon J. Nelson, Elizabeth L. Frank, and Alan L. Rockwood**

## Abstract

Measurement of methylmalonic acid (MMA) plays an important role in the diagnosis of vitamin $B_{12}$ deficiency. Vitamin $B_{12}$ is an essential cofactor for the enzymatic carbon rearrangement of methylmalonyl-CoA (MMA-CoA) to succinyl-CoA (SA-CoA), and the lack of vitamin $B_{12}$ leads to elevated concentrations of MMA. Presence of succinic acid (SA) complicates the analysis because mass spectra of MMA and SA are indistinguishable, when analyzed in negative ion mode and the peaks are difficult to resolve chromatographically. We developed a method for the selective analysis of MMA that exploits the significant difference in fragmentation patterns of di-butyl derivatives of the isomers MMA and SA in a tandem mass spectrometer when analyzed in positive ion mode. Tandem mass spectra of di-butyl derivatives of MMA and SA are very distinct; this allows selective analysis of MMA in the presence of SA. The instrumental analysis is performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in positive ion mode, which is, in combination with selective extraction of acidic compounds, is highly selective for organic acids with multiple carboxyl groups (dicarboxylic, tricarboxylic, etc.). In this method organic acids with a single carboxyl group are virtually undetectable in the mass spectrometer; the only organic acid, other than MMA, that is detected by this method is its isomer, SA. Quantitative measurement of MMA in this method is performed using a deconvolution algorithm, which mathematically resolves the signal corresponding to MMA and does not require chromatographic resolution of the MMA and SA peaks. Because of its high selectivity, the method utilizes isocratic chromatographic separation; reconditioning and re-equilibration of the chromatographic column between injections is unnecessary. The above features of the method allow high-throughput analysis of MMA with analysis cycle time of 1 min.

**Key words** Methylmalonic acid, Succinic acid, Isomers, Derivatization, Liquid chromatography, Tandem mass spectrometry, Data analysis, Deconvolution

## 1 Introduction

Methylmalonyl-CoA (MMA-CoA) is an intermediate in the metabolic pathway converting propionyl-CoA to succinyl-CoA (SA-CoA) [1]. Vitamin $B_{12}$ is an essential cofactor for the enzymatic carbon rearrangement of MMA-CoA to SA-CoA, and the

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_18, © Springer Science+Business Media New York 2016

Lack of vitamin $B_{12}$ leads to elevated concentrations of MMA. Therefore, measurement of free methylmalonic acid (MMA) plays an important role in diagnosing vitamin $B_{12}$ deficiency, which can lead to serious and often irreversible neurological and cognitive disorders [1–3] as well as megaloblastic anemia [1]. A moderately increased MMA concentration (greater than 0.4 μmol/L in serum or plasma and greater than 3.6 mmol/mol creatinine in urine) is an early indicator of acquired vitamin $B_{12}$ deficiency; a massive elevation of MMA in serum, plasma, or urine (100 to 1000 fold above the concentrations characteristic for vitamin $B_{12}$ deficiency) is indicative of methylmalonic acidemia, an inborn metabolic disorder [2]. A true prevalence of vitamin $B_{12}$ deficiency is difficult to estimate because published reports are based on diverse inclusion criteria and methods. However, the prevalence of vitamin $B_{12}$ deficiency reported for elderly individuals in the Framingham Heart Study was 12 % [4].

Both, serum MMA and serum cyanocobalamin measurements can be used to detect $B_{12}$ deficiency; serum MMA has been used to assess status of vitamin $B_{12}$ in tissue, and was shown to be a better biomarker of vitamin $B_{12}$ deficiency than serum cyanocobalamin. Advantages of measuring MMA instead of cobalamin include (1) the concentration of vitamin $B_{12}$ in serum or plasma may not adequately reflect tissue cobalamin status; (2) the concentration of methylmalonic acid in serum is up to 1000-fold greater than serum cyanocobalamin concentration; (3) increased rather than decreased concentration is found in vitamin $B_{12}$ deficiency; and (4) methylmalonic acid is more stable than cyanocobalamin.

The major obstacle for MMA analysis in biological fluids is the potential interference from other low molecular weight organic acids, especially from the naturally occurring structurally related isomer, SA, which is typically present in samples at a higher concentration than MMA. SA interference is difficult to overcome because the chromatographic characteristics and mass spectra of SA are almost identical to those of MMA.

Traditionally, the primary method of choice for measuring MMA was gas chromatography-mass spectrometry (GC/MS) [5–7]. The main disadvantage of GC-MS methods is relatively low throughput of the analysis. Using LC-MS/MS organic acids are typically analyzed in negative ion mode so that all organic acids are ionized in the ion source and can be detected [8–10]. The majority of endogenous organic acids are chromatographically retained much longer than MMA, because of this LC-MS/MS methods for MMA utilizing negative ion mode detection require extensive column conditioning and re-equilibration between every injection. Compared to other published methods, this LC-MS/MS method [11, 12] allows increasing throughput of analysis by five to tenfold. The major advantage of this LC-MS/MS method is in its specificity for short chain dicarboxylic

acids (*see* **Note 1**), while all other acids are transparent to the MS/MS detection [11, 12]. The method is based on unique fragmentation of the di-butyl MMA derivative (Fig. 1); the specific fragmentation, in conjunction with use of mathematical deconvolution, allows high-throughput quantitative analysis of MMA without the need of chromatographic separation of the peaks of MMA and SA [13, 14].

We describe here a rapid method for the selective analysis of MMA in serum, plasma, and urine that is based on unique fragmentation of di-butyl MMA combined with a mathematical deconvolution approach, which allows quantitation of MMA in the presence of SA without chromatographic separation. The instrumental analysis time of this method is 1 min per sample.

## 2 Materials

### 2.1 Samples

1. Serum (serum or serum separator tube) and plasma (sodium heparin or EDTA). Samples are stable for 4 days if refrigerated or 6 months frozen at or below −20 °C.
2. Timed (24-h) or random urine. Samples are stable for 4 days refrigerated or 6 months frozen at or below −20 °C, *see* **Note 2**.

### 2.2 Reagents and Buffers

1. MMA, SA, ammonium formate, formic acid, phosphoric acid, methanol, 2-propanol, methyl-tert-butyl ether (MTBE), $K_2HPO_4$, $NaH_2PO_4$, sodium chloride, urea, creatinine (Sigma-Aldrich, St Louis, MO).
2. Methyl-$d_3$-malonic acid (MMA $d_3$) (Cambridge Isotope, Tewksbury, MA).
3. 3 M Hydrochloric acid in 1-butanol (Regis Technologies, Morton Grove, IL), *see* **Note 3**.
4. Extraction solvent (3 % phosphoric acid in MTBE): To a 4 L bottle of MTBE, add 84 mL concentrated phosphoric acid (85 %, specific gravity 1.685 g/mL). Cap and mix thoroughly. Stable for 2 weeks at ambient temperature.
5. Dialyzed plasma (MMA and SA free). (Basematrix 53, SeraCare Life Sciences, Milford, MA). Stable for 1 year at or below −20 °C.
6. Synthetic urine. In a 1 L glass beaker containing 1 L water, dissolve 2.8 g $K_2HPO_4$, 0.55 g $NaH_2PO_4$, 7 g of NaCl, 20 g of urea, and 1 g of creatinine. Mix solution until completely dissolved. Stable for 2 years at or below −20 °C.
7. Mobile phase A (5 mM ammonium formate): To a 1 L beaker containing 300 mL water, add 0.315 g ammonium formate. Fill to 1 L with water, mix for 15 min, and filter through a 0.5 μm filter. Stable for 2 weeks refrigerated or 2 days at ambient temperature.

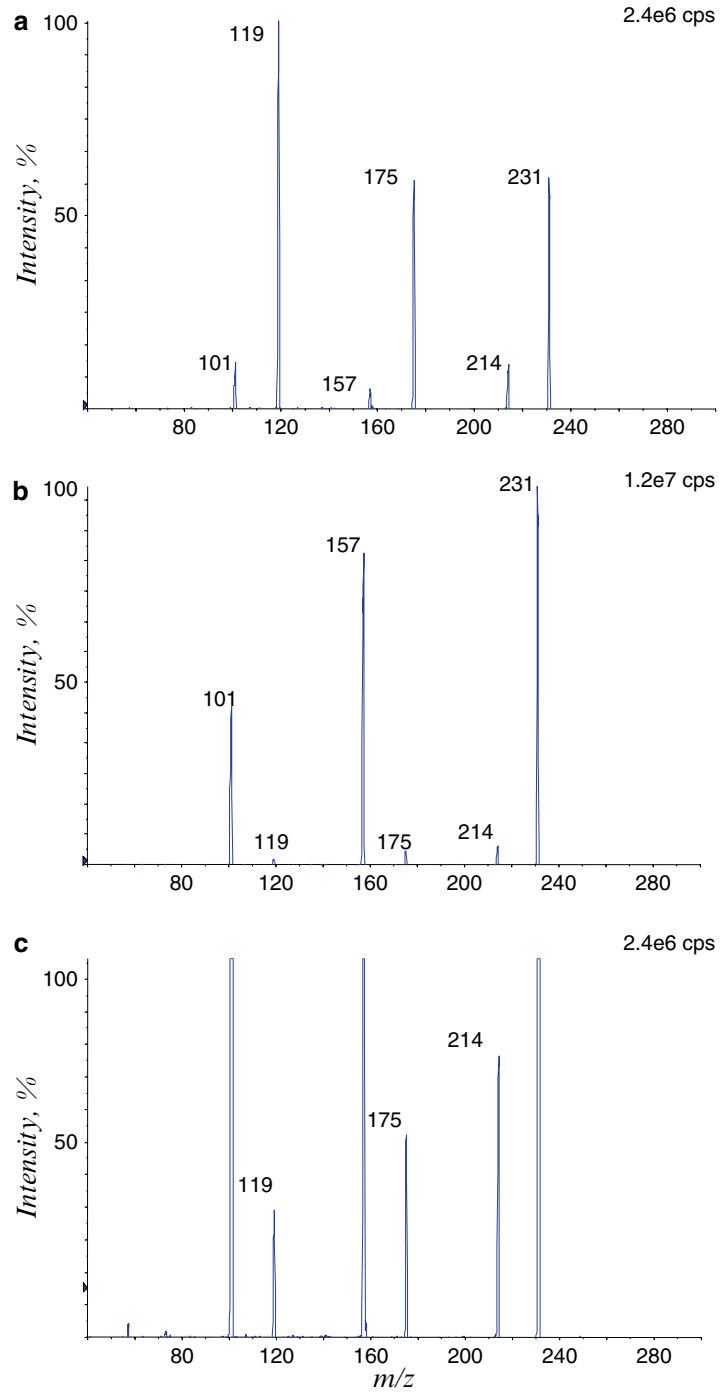



**Fig. 1** Product ion mass spectra of molecular ion $[M+H]^+$ of the n-dibutyl esters of (**a**) MMA, (**b**) SA, and (**c**) mixture of MMA and SA, ratio of the concentrations 1:50, which is comparable to physiologically observed concentrations

8. Mobile phase B (5 mM ammonium formate in methanol): To a 1 L beaker containing 300 mL methanol, add 0.315 g ammonium formate. Fill to 1 L with methanol, mix for 15 min, and filter through a 0.5 μm filter. Stable for 5 days at ambient temperature.
9. Needle wash solution (methanol:2-propanol:water, 60:20:20 (v/v)): Using separate graduated cylinders measure 600 mL methanol, 200 mL 2-propanol, and 200 mL water; transfer into a 1 L glass bottle, mix, and cap. Stable for 10 days at ambient temperature.

### 2.3 Standards and Calibrators

1. MMA stock calibration standard, 10 mmol/L: Weigh 0.0118 g methylmalonic acid, transfer to a 10 mL volumetric flask, and fill to volume with methanol. Mix by inversion until the solid is dissolved. Stable for 1 year at or below –20 °C.
2. MMA working calibration standard, 10 μmol/L: Add 100 μL of 10 mmol/L MMA stock calibration standard into a 100 mL volumetric flask containing approximately 50 mL water. Fill to volume with nanopure water. Aliquot 400 μL in microcentrifuge tubes. Stable for 1 year at or below –20 °C.
3. MMA $d_3$ stock internal standard, 10 mmol/L: Weigh 0.0121 g (12.1 mg) of methylmalonic acid $d_3$ (MMA $d_3$) to a 10 mL volumetric flask. Fill to volume with methanol. Stable for 2 years at or below –20 °C.
4. MMA $d_3$ working internal standard, 15 μmol/L: To a 500 mL volumetric flask, add 400 mL nanopure water and 750 μL methylmalonic acid $d_3$ stock internal standard. Fill to volume with nanopure water and mix. Aliquot 5 mL in polypropylene tubes. Stable for 1 year at or below –20 °C.
5. SA stock standard, 10 mmol/L: Weigh 0.0118 g succinic acid in a 10 mL volumetric flask. Fill to volume with methanol. Stable for 2 years at or below –20 °C.
6. SA injection standard: 100 μmol/L: Transfer 100 μL of SA stock standard into a 10 mL glass tube and evaporate the solvent at room temperature. Add 40 μL of the derivatizing reagent and incubate at 70 °C for 10 min. Evaporate the derivatizing reagent; reconstitute the residue with 5.0 mL water: methanol 15:85. Stable for 3 months refrigerated.

### 2.4 Quality Controls (QC)

1. Dialyzed plasma spiked with succinic acid, 6 μmol/L (used as a matrix for preparation of quality controls): Add 120 μL succinic acid stock standard to 200 mL dialyzed plasma. Mix for 30 min. Stable for 6 months at or below –20 °C.
2. MMA stock standard for preparation of QC, 10 mmol/L: Weigh 0.0118 g methylmalonic acid, add to a 10 mL volumetric flask, and fill to volume with methanol. Mix by inversion until completely dissolved. Stable for 1 year at or below –20 °C.

3. MMA working standard for preparation of QC, 1 mmol/L: In a 10 mL volumetric flask add 1000 µL MMA stock standard, add nanopure water to volume, and mix. Stable for 1 week refrigerated.
4. Control I, Serum/Plasma, 0.4 µmol/L MMA, and 6 µmol/L SA: Add 500 mL dialyzed plasma into a 1 L beaker with a stir bar and begin mixing. Add 200 µL of MMA working standard for preparing QC (1 mmol/L) and stir for 15 min. Add 300 µL succinic acid stock standard (10 mmol/L), cover, and mix for additional 30 min Aliquot in 1.5 mL microcentrifuge tubes. Stable for 1 year at or below –20 °C.
5. Control II, Serum/Plasma, 1 µmol/L MMA, and 6 µmol/L SA: Add 500 mL dialyzed plasma in a beaker with a stir bar, add 50 µL MMA stock for preparation of QC, and mix for 15 min. Add 300 µL succinic acid stock standard, cover, and mix for additional 30 min. Aliquot 1.5 mL into microcentrifuge tubes. Stable for 1 year at or below –20 °C.
6. Negative Control. Dialyzed plasma (free of MMA, determined using this method).
7. Control III, urine, 10 µmol/L MMA, and 6 µmol/L SA: In a 500 mL beaker pour 250 mL of synthetic urine, add 250 µL of MMA stock standard for preparation of QC, and mix for 15 min. Add 150 µL succinic acid stock standard and fill to volume with synthetic urine, cover, and mix for additional 30 min. Aliquot 1.5 mL into microcentrifuge tubes. Stable for 1 year at or below –20 °C.
8. Control IV, urine, 20 µmol/L MMA, and 6 µmol/L SA: In a 500 mL beaker pour 250 mL of synthetic urine, add 500 µL of MMA stock standard for preparation of QC, and mix for 15 min. Add 150 µL succinic acid stock standard and fill to volume with synthetic urine, cover, and mix for additional 30 min. Aliquot 1.5 mL into microcentrifuge tubes. Stable for 1 year at or below –20 °C.

### 2.5 Equipment

1. Triple quadrupole mass spectrometer AB3200 with TurboV ion source (AB Sciex, Foster City, CA) with built-in switching valve.
2. Binary HPLC pump series 1260 (Agilent Technologies, Santa Clara, CA), vacuum degasser, autosampler CTC PAL (Carrboro, NC) equipped with fast wash station.
3. Vortex with adaptor for microcentrifuge tubes.
4. Evaporator for 96-well plates.
5. Centrifuge for microcentrifuge tubes.
6. Centrifuge with buckets for 96-well plates.
7. Shaker for 96-Well Plates.

### 2.6 Supplies

1. Microcentrifuge tubes, 2 mL (Eppendorf, Westbury, NY).
2. Deep 96-well plates (2 mL well volume) and sealing mats for the plates (Phenomenex, Torrance, CA).
3. Transfer pipettes.
4. HPLC Column Luna C18 30 mm × 3 mm, 5 μm particles; SecurityGuard cartridge holder and C18 cartridges (Phenomenex, CA).

## 3 Methods

### 3.1 Procedure

1. Label a set of 2 mL microcentrifuge tubes.
2. Prepare calibrators and negative control by adding working calibration standard and dialyzed plasma to the corresponding tubes:
   (a) Aliquot in the tubes 500 μL of dialyzed plasma.
   (b) Add in the tubes working calibration standard in amounts according to Table 1.
3. Aliquot patient samples and controls:
   (a) Add 500 μL of serum or plasma patient sample of control to labeled tubes.
   (b) Add 50 μL of urine sample and control to the corresponding tubes.
   (c) Add 450 μL of nanopure water to the urine sample and the urine controls.
4. Add to each tube 50 μL of working internal standard.
5. Add to each tube 1 mL extraction solvent (MTBE/3 % phosphoric acid) and close lids.
6. Set tubes in adaptor of vortex and shake for 5 min.

**Table 1**
**Preparation of calibration standards**

| Concentration of standard, μmol/L | Working calibration standard, μL | Dialyzed plasma, μL |
|---|---|---|
| 0.20 | 10 | 500 |
| 0.40 | 20 | 500 |
| 0.80 | 40 | 500 |
| 1.00 | 50 | 500 |
| 1.50 | 75 | 500 |
| 2.00 | 100 | 500 |

7. Centrifuge the tubes at 14,000 rpm for 3 min.
8. Transfer the top organic layer from each tube to the corresponding well in the 96-well plate, *see* **Note 4**.
9. Set 96-well plate on evaporator and evaporate organic phase (50 °C) until completely dry.
10. Add in each well of the plate 40 μL of derivatizing reagent (3 M HCl in 1-butanol) and cover the plate with sealing mat.
11. Incubate the plate at 70 °C for 10 min.
12. Remove the plate from the incubator and take off the mat.
13. Set 96-well plate on evaporator and evaporate organic (50 °C), *see* **Note 5**.
14. Add in each well 200 μL of reconstitution solvent and cover plate with the sealing mat.
15. Set plate on shaker for 96-well plates and vortex on medium setting for 3 min.
16. Centrifuge plate for 1 min at 4000 × *g*.
17. Inject the samples.

### 3.2 LC-MS/MS

1. Mobile phase bottle A—water with 5 mM ammonium formate.
2. Mobile phase bottle B—methanol with 5 mM ammonium formate.
3. Mobile phase program is summarized in Table 2, *see* **Note 6**.
4. Injection volume 20 μL.
5. Autosampler syringe wash solution: methanol, 60 % /isopropanol, 20 %/water, 20 %.
6. Mass transitions are listed in Table 3.
7. Voltages and gases flow rates for the mass spectrometer were optimized for maximum sensitivity and were as follows:
   (a) Ionspray voltage: 5000 V.
   (b) Ion source temperature: 450 °C.
   (c) Nebulizer gas: 60, heating gas: 60.

**Table 2**
**Mobile phase program (flow diverted to waste before 0.3 min and after 1.0 min)**

| Step | Time, min | Flow rate, μL/min | A, % | B, % |
|---|---|---|---|---|
| 0 | 0 | 750 | 15 | 85 |
| 1 | 1.0 | 750 | 15 | 85 |

**Table 3**
**Mass transitions**

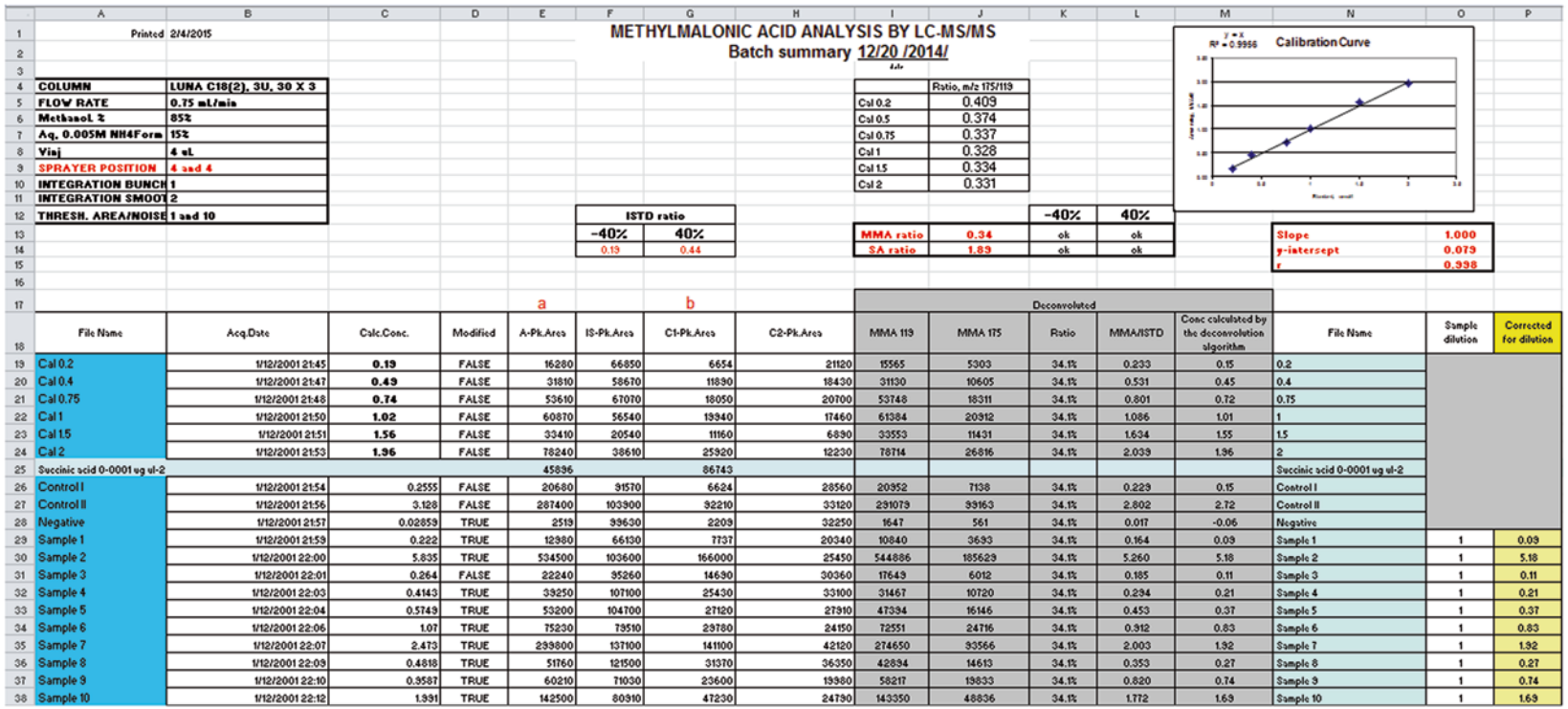
| Compound | Primary mass transition, *m/z* | Secondary mass transition, *m/z* |
|---|---|---|
| MMA | 231.2 to 119.1 | 231.2 to 175.1 |
| MMA $d_3$ | 234.2 to 122.1 | 234.2 to 178.1 |

Dwell time 150 ms

(d) Collision gas: 6.
(e) Declustering potential: 50 V.
(f) Entrance potential: 10 V.
(g) Collision energy: 15 V.
(h) Collision cell exit potential: 6 V.
(i) Mass analyzers $Q_1$ and $Q_3$ are set for unit resolution (0.7 Da width at 50 % height).

### 3.3 Data Analysis

1. Data analysis is performed using software Analyst 1.5 (AB Sciex, Foster City, CA) and spreadsheet for deconvolution of signal corresponding to the peak area of MMA from the total peak area of MMA + SA. Calculations are performed using peak areas of the two mass transitions of MMA and MMA d3. The algorithm and equations used for deconvolution of the signal corresponding to MMA are described in **Note 7**.
2. Export from Analyst™ to Excel worksheet (Fig. 2) summary table with peak areas of mass the transitions *m/z* 231.2 to 119.1 and *m/z* 231.2 to 175.1 (MMA/SA); and *m/z* 234.2 to 122.1 and *m/z* 234.2 to 178.1 (MMA d3).
3. Coefficients of regression equation and correlation coefficient are displayed in cells $O_{13}$–$O_{15}$ (Fig. 2).
4. The calculated deconvoluted peak area corresponding to the signal of MMA is shown in columns "I" (*m/z* 231.2 to 119.1) and "J" (*m/z* 231.2 to 175.1).
5. Calculated MMA concentration is shown in column "M".
6. The test results are considered acceptable if the correlation coefficient (*r*) for the calibration curve is greater than 0.995. The calculated ratio of the peak areas of two mass transitions MMA d3 should be within 30 % of the mean value of the ratio observed in the calibration standards of the batch [13, 14]. Concentration of MMA in the negative control must be below the limit of quantitation of the method; concentration of MMA in the controls should be within the limits of the QC rules established by the laboratory.



Printed 2/4/2015

**METHYLMALONIC ACID ANALYSIS BY LC-MS/MS**
**Batch summary 12/20 /2014/**

| | |
|---|---|
| COLUMN | LUNA C18(2), 3U, 30 X 3 |
| FLOW RATE | 0.75 mL/min |
| Methanol % | 85% |
| Aq. 0.005M NH4Form | 15% |
| Vinj | 4 uL |
| SPRAYER POSITION | 4 and 4 |
| INTEGRATION BUNCH | 1 |
| INTEGRATION SMOOT | 2 |
| THRESH. AREA/NOISE | 1 and 10 |

| | Ratio, m/z 175/119 |
|---|---|
| Cal 0.2 | 0.409 |
| Cal 0.5 | 0.374 |
| Cal 0.75 | 0.337 |
| Cal 1 | 0.328 |
| Cal 1.5 | 0.334 |
| Cal 2 | 0.331 |

Calibration Curve

| ISTD ratio | |
|---|---|
| -40% | 40% |
| 0.19 | 0.44 |

| | | -40% | 40% |
|---|---|---|---|
| MMA ratio | 0.34 | ok | ok |
| SA ratio | 1.89 | ok | ok |

| | |
|---|---|
| Slope | 1.000 |
| y-intersept | 0.079 |
| r | 0.998 |

| | | | | | a | | b | | Deconvoluted | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | File Name | Acq.Date | Calc.Conc. | Modified | A-Pk.Area | IS-Pk.Area | C1-Pk.Area | C2-Pk.Area | MMA 113 | MMA 175 | Ratio | MMA/ISTD | Conc calculated by the deconvolution algorithm | File Name | Sample dilution | Corrected for dilution |
| 19 | Cal 0.2 | 1/12/2001 21:45 | 0.19 | FALSE | 16280 | 66850 | 6654 | 21120 | 15565 | 5303 | 34.1% | 0.233 | 0.15 | 0.2 | | |
| 20 | Cal 0.4 | 1/12/2001 21:47 | 0.49 | FALSE | 31810 | 58670 | 11890 | 18430 | 31130 | 10605 | 34.1% | 0.531 | 0.45 | 0.4 | | |
| 21 | Cal 0.75 | 1/12/2001 21:48 | 0.74 | FALSE | 53610 | 67070 | 18050 | 20700 | 53748 | 18311 | 34.1% | 0.801 | 0.72 | 0.75 | | |
| 22 | Cal 1 | 1/12/2001 21:50 | 1.02 | FALSE | 60870 | 56540 | 19340 | 17460 | 61384 | 20912 | 34.1% | 1.086 | 1.01 | 1 | | |
| 23 | Cal 1.5 | 1/12/2001 21:51 | 1.56 | FALSE | 33410 | 20540 | 11160 | 6830 | 33553 | 11431 | 34.1% | 1.634 | 1.55 | 1.5 | | |
| 24 | Cal 2 | 1/12/2001 21:53 | 1.96 | FALSE | 78240 | 38610 | 25920 | 12230 | 78714 | 26816 | 34.1% | 2.039 | 1.96 | 2 | | |
| 25 | Succinic acid 0-0001 ug ul-2 | | | | 45836 | | 86743 | | | | | | | Succinic acid 0-0001 ug ul-2 | | |
| 26 | Control I | 1/12/2001 21:54 | 0.2555 | FALSE | 20680 | 91570 | 6624 | 28560 | 20952 | 7138 | 34.1% | 0.229 | 0.15 | Control I | | |
| 27 | Control II | 1/12/2001 21:56 | 3.128 | FALSE | 287400 | 103900 | 92210 | 33120 | 291079 | 99163 | 34.1% | 2.802 | 2.72 | Control II | | |
| 28 | Negative | 1/12/2001 21:57 | 0.02859 | TRUE | 2519 | 99630 | 2209 | 32250 | 1647 | 561 | 34.1% | 0.017 | -0.06 | Negative | | |
| 29 | Sample 1 | 1/12/2001 21:59 | 0.222 | TRUE | 12380 | 66130 | 7737 | 20340 | 10840 | 3693 | 34.1% | 0.164 | 0.09 | Sample 1 | 1 | 0.09 |
| 30 | Sample 2 | 1/12/2001 22:00 | 5.835 | TRUE | 534500 | 103600 | 166000 | 25450 | 544886 | 185623 | 34.1% | 5.260 | 5.18 | Sample 2 | 1 | 5.18 |
| 31 | Sample 3 | 1/12/2001 22:01 | 0.264 | FALSE | 22240 | 95260 | 14690 | 30360 | 17643 | 6012 | 34.1% | 0.185 | 0.11 | Sample 3 | 1 | 0.11 |
| 32 | Sample 4 | 1/12/2001 22:03 | 0.4143 | TRUE | 39250 | 107100 | 25430 | 33100 | 31467 | 10720 | 34.1% | 0.234 | 0.21 | Sample 4 | 1 | 0.21 |
| 33 | Sample 5 | 1/12/2001 22:04 | 0.5749 | TRUE | 53200 | 104700 | 27120 | 27910 | 47334 | 16146 | 34.1% | 0.453 | 0.37 | Sample 5 | 1 | 0.37 |
| 34 | Sample 6 | 1/12/2001 22:06 | 1.07 | TRUE | 75230 | 79510 | 29780 | 24150 | 72551 | 24716 | 34.1% | 0.912 | 0.83 | Sample 6 | 1 | 0.83 |
| 35 | Sample 7 | 1/12/2001 22:07 | 2.473 | TRUE | 239800 | 137100 | 141100 | 42120 | 274650 | 93566 | 34.1% | 2.003 | 1.92 | Sample 7 | 1 | 1.92 |
| 36 | Sample 8 | 1/12/2001 22:09 | 0.4818 | TRUE | 51760 | 121500 | 31370 | 36350 | 42834 | 14613 | 34.1% | 0.353 | 0.27 | Sample 8 | 1 | 0.27 |
| 37 | Sample 9 | 1/12/2001 22:10 | 0.9587 | TRUE | 60210 | 71030 | 23600 | 19980 | 58217 | 19833 | 34.1% | 0.820 | 0.74 | Sample 9 | 1 | 0.74 |
| 38 | Sample 10 | 1/12/2001 22:12 | 1.991 | TRUE | 142500 | 80910 | 47230 | 24790 | 143350 | 48836 | 34.1% | 1.772 | 1.69 | Sample 10 | 1 | 1.69 |

**Fig. 2** Example of an Excel worksheet for calculating concentrations of isomers from unresolved chromatographic peaks

7. Method performance characteristics: total CV of the method is below 10 %; limit of quantitation 0.1 μmol/L; upper limit of linearity 150 μmol/L.
8. Reference intervals: serum/plasma < 0.4 μmol/L; urine <3.6 mmol MMA/mol creatinine.

## 4 Notes

1. Detection in this method is specific for organic acids with multiple carboxyl groups (dicarboxylic, tricarboxylic); organic acids with a single carboxyl group are not detectable by the method.
2. Urine samples analyzed for MMA should also be tested for creatinine; concentrations of MMA in urine samples are reported in the units *mmol/mol of creatinine*.
3. 3 M Hydrochloric acid in 1-butanol is anhydrous.
4. During separation of the organic phase after the extraction, it is important to not transfer the aqueous phase.
5. During the evaporation it is acceptable if solution in the wells is not completely evaporated.
6. MMA extraction utilized in this method is specific for acidic compounds and detection is specific for polycarboxylic acids; because of this, the method performs adequately with isocratic chromatographic separation. Reconditioning and re-equilibration of the chromatographic column between injections in this method is not needed.
7. MMA concentration can be determined using an Excel spreadsheet (Fig. 2); alternatively, software can be developed to calculate the isomers' concentration. The algorithm for determining MMA concentration from the total peak intensity of MMA and SA [13, 14] is based on the following assumptions: (1) signal in the mass transitions originates only from the MMA and SA (*see* **Note 1**); (2) the total acquired signal is a linear combination of the signal from MMA and SA; and (3) magnitude of the ratios of the two mass transitions of MMA and SA is significantly different. The following information is required for determining the concentration of MMA:
   (a) Ratio of the peak areas of the mass transitions *m/z* 175/119 of a pure standard of MMA.
   (b) Ratio of the peak areas of the mass transitions *m/z* 175/119 of a pure standard of SA.
   (c) Peak area of mass transitions *m/z* 231.2 to 119.1 and *m/z* 231.2 to 175.1, corresponding to the signal from MMA/SA.

(d) Peak area of mass transitions *m/z* 234.2 to 122.1 and *m/z* 234.2 to 178.1 corresponding to the signal from MMA $d_3$.

(e) The slope, y-intercept, and $R^2$ for the calibration curve.

The algorithm determines

- Deconvoluted peak areas corresponding to MMA.
- The concentration of MMA.
- QC metrics for assessment of the performance of the method.

A description of the algorithm and derivation of the equations for calculation of the concentrations of the isomers from the total peak intensities of unresolved chromatographic peaks and an example of the calculations is shown below.

A model describing the relationship between signal intensities of coeluting chromatographic peaks can be presented as a system of linear equations:

$$M_{175} = R_M \times M_{119} \tag{1}$$

$$S_{175} = R_S \times S_{119} \tag{2}$$

$$I_{119} = M_{119} + S_{119} \tag{3}$$

$$I_{175} = M_{175} + S_{175} \tag{4}$$

where $M_{119}$ and $M_{175}$ are peak areas of the mass transitions *m/z* 231/119 and *m/z* 231/175 of MMA; $S_{119}$ and $S_{175}$ are peak areas of the mass transitions *m/z* 231/119 and *m/z* 231/175 of SA, respectively; $R_M$ and $R_S$ are ratios of the peak areas of mass transition *m/z* 231/175 and *m/z* 231/119 of the standards of MMA and SA, respectively.

$I_{119}$ and $I_{175}$ are total peak area of mass transitions *m/z* 231/175 and *m/z* 231/119, respectively in analyzed samples.

Equations (1)–(4) have four unknowns; and because the number of the unknowns is equal to the number of the equations, this system of equations has a single solution.

Rearranging Eqs. 1 and 2 gives:

$$M_{119} = M_{175} / R_M \quad \text{and} \quad S_{119} = S_{175} / R_S \tag{5}$$

After substitution of the two values in Eq. 3

$$I_{119} = M_{175} / R_{\mathrm{MMA}} + S_{175} / R_{\mathrm{SA}} \tag{6}$$

Solution of the system of equations (Eqs. 1–4) for the deconvoluted peak area of MMA:

$$I_{119\,M} = \left(-R_S \times I_{119} + I_{175}\right) / \left(R_M \quad R_S\right) \tag{7}$$

$$I_{175\,M} = R_M \times \left(-R_S \times I_{119} + I_{175}\right) / \left(R_M \quad R_S\right) \tag{8}$$

Solution of the system of equations (Eqs. 1–4) for the deconvoluted peak area of SA is:

$$I_{119S} = \left(R_M \times I_{119} - I_{175}\right) / \left(R_M \quad R_S\right) \tag{9}$$

$$I_{175S} = R_S \times \left(R_M \times I_{119} - I_{175}\right) / \left(R_M \quad R_S\right) \tag{10}$$

At the MS/MS acquisition conditions utilized in the method, typical ratios of the mass transition *m/z* 175/119 for MMA and SA are $RM = 0.35 \pm 0.05$ and $RS = 2.0 \pm 0.1$, respectively. The actual values of the ratios are determined for each batch of samples and used for the calculations in the spreadsheet (Fig. 2).

Figure 3 shows example of chromatograms representing performance of the algorithm for MMA analysis in presence of SA in human plasma sample. The solid line represents the total intensity

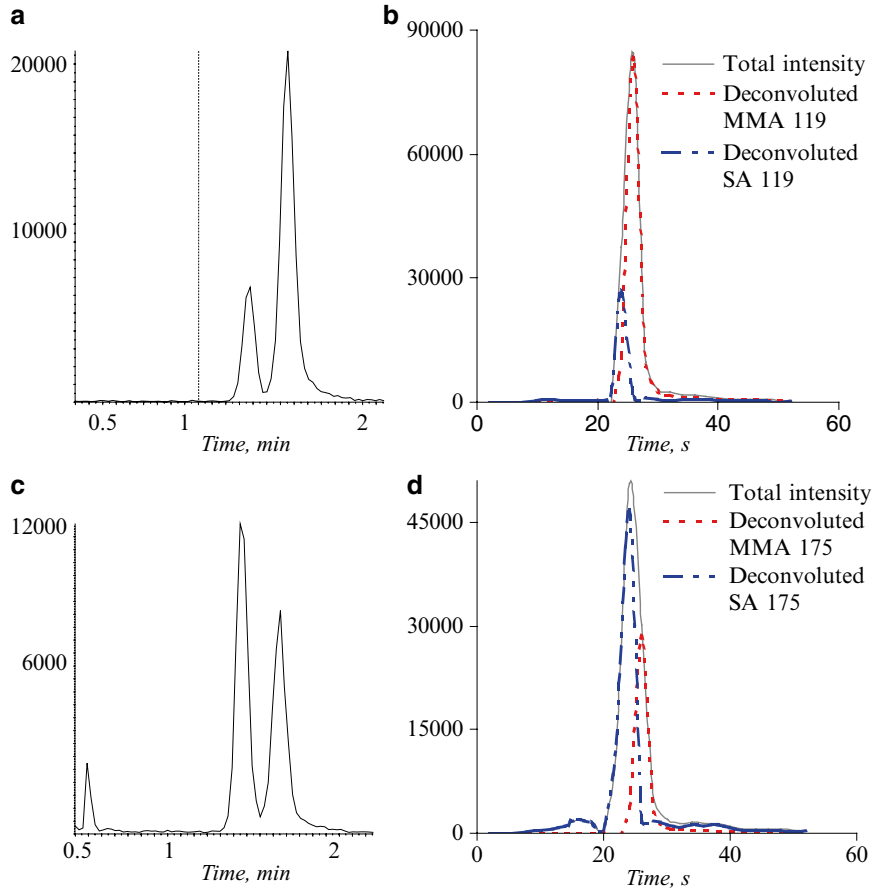



**Fig. 3** MRM chromatograms of a patient sample containing 0.21 μmol/L of MMA and 4.0 μmol/L of SA (**a** and **b** are transitions *m/z* 231 to 119, **c** and **d** are transitions *m/z* 231 to 175). Chromatograms **a** and **c** are for the method utilizing chromatographic separation of MMA and SA; chromatograms **b** and **d** are for the method utilizing the deconvolution and no chromatographic separation. *Solid line* (**b** and **d**) corresponds to acquired data; *dotted lines* are deconvoluted peaks corresponding to MMA and SA (calculated from individual scans utilizing Eqs. 7 and 8)

of the mass transitions of the unresolved peaks that is acquired by the instrument. The intensity of each individual transition (dotted lines) was calculated utilizing the *RM* and *RS* ratios obtained from injections of pure standards of MMA and SA, total area of the peaks $I_{119}$, $I_{175}$, and the derived formulas (Eqs. 7 and 8). As Fig. 3 shows there is a good agreement between the peak intensities determined the utilized algorithm versus intensities observed in the method utilizing chromatographic separation (Fig. 3a, c).

Below is an example of calculating peak areas of the mass transitions of MMA *m/z* 231/119 and 231/175. The following data are used in this example:

$RM = 0.3$; $RS = 2.0$—ratios of the peak areas of the mass transitions of MMA and SA, respectively.

$I_{119} = 125{,}000$; $I_{175} = 60{,}000$; $\mathrm{IS}_{122} = 115{,}000$; $\mathrm{IS}_{178} = 34{,}000$—peak areas of the mass transitions monitored in the method.

MMA peak area (mass transition *m/z* 231/119) calculated

$$I_{119\,M} = \left(-R_S \times I_{119} + I_{175}\right) / \left(R_M \quad R_S\right)$$
$$= \left(-2.0 \times 125{,}000 + 60{,}000\right) / \left(0.3 \quad 2.0\right)$$

using Eq. 7: $= 111{,}760$

MMA peak area (mass transition *m/z* 231/175) calculated

$$I_{175\,M} = R_M * \left(-R_S * I_{119} + I_{175}\right) / \left(R_M \quad R_S\right)$$
$$= 0.3 * \left(-2.0 * 125{,}000 + 60{,}000\right) / \left(0.30 \quad 2.0\right)$$

using Eq. 8: $= 33{,}529.$

The above two values, $I_{119}\,M$ and $I_{175}\,M$, are the deconvoluted peak areas of MMA from the total signal from the peaks of MMA and SA.

In order to determine the MMA concentration in the sample, peak area must be normalized to the peak area of the internal standard, and the MMA/ISTD ratio must be calculated.

$$M_{119} / \mathrm{IS}_{122} = 111{,}760 / 115{,}000 = 0.972$$
$$M_{175} / \mathrm{IS}_{175} = 33{,}529 / 34{,}000 = 0.786$$

These ratios are used to calculate concentration of MMA using linear regression equations determined from the calibration standards analyzed in the batch of samples. Ratio of the peak areas of two mass transitions of MMA d3 is calculated for evaluation of the specificity [15].

Data demonstrating comparison of the quantitation with chromatographically resolved peaks of isomers, and the method utilizing the described mathematical approach have been published [13, 14].

The deconvolution algorithm can be applied for quantitation of unresolved chromatographic peaks and is not unique to the analysis of MMA and SA. Deconvolution can be applied for quantitative analysis of any molecules with identical mass fragments, which produce a distinct ratio of the product ions.

## References


1. Cox EV, White AM (1962) Methylmalonic acid excretion: an index of vitamin $B_{12}$ deficiency. Lancet 2:853–856
2. Fenton WA, Rosenberg LE (2001) Disorders of propionate and methylmalonate metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, Inc, New York, pp 2177–2193
3. Barness LA, Young D, Mellman WJ, Kahn SB, Williams WJ (1963) Methylmalonate excretion in a patient with pernicious anemia. N Engl J Med 268:144–146
4. Lindenbaum J, Rosenberg IH, Wilson PW, Stabler SP, Allen RH (1994) Prevalence of cobalamin deficiency in the Framingham elderly population. Am J Clin Nutr 60:2–11
5. Stabler SP, Marcell PD, Podell ER, Allen RH, Lindenbaum J (1986) Assay of methylmalonic acid in the serum of patients with cobalamine deficiency using capillary gas chromatography-mass spectrometry. J Clin Invest 77:1606–1612
6. McCann MT, Thompson MM, Gueron IC, Lemieux B, Giguere R, Tuchman M (1996) Methylmalonic acid quantification by stable isotope dilution gas chromatography-mass spectrometry from filter paper urine samples. Clin Chem 42:910–914
7. Kushnir MM, Komaromy-Hiller G (2000) Optimization and performance of a rapid GC-MS analysis for methylmalonic acid determination in serum and plasma. J Chromatogr B 741:231–241
8. Yuan C, Gabler J, El-Khoury JM, Spatholt R, Wang S (2012) Highly sensitive and selective measurement of underivatized methylmalonic acid in serum and plasma by liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 404:133–140
9. Pedersen TL, Keyes WR, Shahab-Ferdows S, Allen LH, Newman JW (2011) Methylmalonic acid quantification in low serum volumes by UPLC-MS/MS. J Chromatogr B 879: 1502–1506
10. Magera MJ, Helgeson JK, Matern D, Rinaldo P (2000) Methylmalonic acid measured in plasma and urine by stable-isotope dilution and electrospray tandem mass spectrometry. Clin Chem 46:1804–1810
11. Kushnir MM, Komaromy-Hiller G, Shushan B, Urry FM, Roberts WL (2001) Analysis of dicarboxylic acids by tandem mass spectrometry. High throughput quantitative measurement of methylmalonic acid in serum, plasma and urine. Clin Chem 47:1993–2002
12. Shushan B, Kushnir MM, Komaromy-Hiller G (2000) Method of analyzing dicarboxylic acids. US patent 6,692,971
13. Kushnir MM, Rockwood AL, Nelson GJ (2004) Simultaneous quantitative analysis of isobars by tandem mass spectrometry from unresolved chromatographic peaks. J Mass Spectrom 39:532–540
14. Kushnir MM, Rockwood AL, Nelson GJ (2002) Methods for data analysis in tandem mass spectrometry. US patent 7,158,903
15. Kushnir MM, Rockwood AL, Nelson GJ, Yue B, Urry FM (2005) Assessing analytical specificity in quantitative analysis using tandem mass spectrometry. Clin Biochem 38:319–327

# Chapter 19

# Quantitation of 5-Methyltetrahydrofolate in Cerebrospinal Fluid Using Liquid Chromatography-Electrospray Tandem Mass Spectrometry

**Erland Arning and Teodoro Bottiglieri**

## Abstract

We describe a simple stable isotope dilution method for accurate and precise measurement of cerebrospinal fluid (CSF) 5-methyltetrahydrofolate (5-MTHF) as a clinical diagnostic test. 5-MTHF is the main biologically active form of folic acid and is involved in regulation of homocysteine and DNA synthesis. Measurement of 5-MTHF in CSF provides diagnostic information regarding diseases affecting folate metabolism within the central nervous system, in particular inborn errors of folate metabolism. Determination of 5-MTHF in CSF (50 μL) was performed utilizing high performance liquid chromatography coupled with electrospray positive ionization tandem mass spectrometry (HPLC-ESI-MS/MS). 5-MTHF in CSF is determined by a 1:2 dilution with internal standard (5-MTHF-$^{13}C_5$) and injected directly onto the HPLC-ESI-MS/MS system. Each assay is quantified using a five-point standard curve (25–400 nM) and has an analytical measurement range of 3–1000 nM.

**Key words** 5-Methyltetrahydrofolate, Cerebral folate deficiency, Methylation, Mass spectrometry

## 1 Introduction

5-Methyltetrahydrofolate (5-MTHF) is the predominant form of folate in cerebrospinal fluid (CSF). Testing for 5-MTHF in CSF is useful to determine a deficiency of folate in the central nervous system. Low 5-MTHF levels are associated with inborn errors of metabolism affecting folate metabolism and in dietary deficiency of folate. Disorders associated with low folate include anemia, developmental delay, seizures, depression, dementia, cerebral folate deficiency, and Kearns-Sayre syndrome [1]. More recent reports indicate that there is reduced uptake of 5-MTHF across the blood–brain barrier due to the presence of auto-antibodies to the folate receptor at the choroid plexus. Patients with cerebral folate deficiency (CFD) are characterized by normal plasma folate in the

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_19, 

presence of decreased concentration of 5-MTHF in CSF [2, 3]; patients with CFD have neurological complications. The following is a simple high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for determination of cerebrospinal fluid 5-MTHF as a clinical diagnostic test.

## 2 Materials

### 2.1 Samples

Human lumbar CSF—Specimen drawn any time during the day will be acceptable. No patient preparation is required. Optimal volume of 1.0 ml (minimum 0.25 mL) CSF should be collected in tube 2 (or 1, 4 or 5) of the collection kit provided by the testing laboratory or in a regular CSF collection tube. If the CSF is clear, the sample should be immediately frozen at the bedside on dry ice. If blood contaminated, the sample should be placed on wet ice, centrifuged within 5 min, and the clear CSF transferred to another vial and frozen on dry ice as soon as possible. CSF is stored at –80 °C until time of testing.

### 2.2 Solvents and Reagents

1. Magnesium chloride × $6H_2O$, ACS grade.
2. Sodium phosphate dibasic anhydrous, ACS grade.
3. Mobile Phase A (0.1 % formic acid in HPLC-grade water): In a hood add 1 mL of formic acid to a 1 L volumetric flask, bring to volume with water, and mix. Stable at room temperature, 18–24 °C, up to 3 months.
4. Mobile Phase B (0.1 % formic acid in methanol): In a hood, add 1 mL of formic acid to a 1 L volumetric flask, bring to volume with methanol, and mix. Stable at room temperature, 18–24 °C, up to 3 months.
5. 10× Artificial CSF (aCSF): 1450 mM NaCl, 27 mM KCl, 10 mM $MgCl_2$, 12 mM $CaCl_2$, 20 mM $Na_2HPO_4$.
   (a) Weigh the following and combine in 100 mL volumetric flask containing 50 mL water: 8.474 g NaCl, 0.201 g KCl, 0.203 g $MgCl_2$, 0.176 g $CaCl_2$, 0.284 g $Na_2HPO_4$.
   (b) Bring to volume with water.
   (c) Add small magnetic stir bar and mix on magnetic stirrer until dissolved.
   (d) Adjust pH to 7.4 with 85 % phosphoric acid.
   (e) Store at 2–8 °C for up to 1 year.
6. 1× aCSF: Add 1 mL 10× aCSF to a 10 mL volumetric flask and bring to volume with water. 1× aCSF is stable for up to 8 h at 2–8 °C and must be made fresh daily.

### 2.3 Internal Standards and Standards

1. Primary standard: 5-MTHF ((6S)-5-Methyl-5,6,7, 8-tetrahydrofolic acid, calcium salt) (Schircks Laboratories).
2. Primary internal standard (I.S.): 5-MTHF-$^{13}C_5$ (Calcium-L-Mefolinate-$^{13}$C5) (Merck Eprova).
3. 5-MTHF Standard Stock Solution (1 mM): Add 49.8 mg 5-MTHF to 100 mL volumetric flask, bring to volume with water containing 1 mg/mL ascorbic acid. Wrap flask with foil and sonicate for 5 min. Store in 125 μL aliquots at −80 °C for up to 4 years (*see* **Note 1**).
4. 5-MTHF-$^{13}C_5$ I.S. Stock Solution (1 mM): Add 5 mg 5-MTHF-$^{13}$C5 to 10 mL volumetric flask, bring to volume with water containing 1 mg/mL ascorbic acid. Wrap flask with foil and sonicate for 5 min. Store in 125 μL aliquots at −80 °C for up to 4 years (*see* **Note 1**).
5. I.S. Working Solution (5-MTHF-$^{13}C_5$ prepared in water containing ascorbic acid and dithiothreitol): Add 2 μL 1 mM 5-MTHF-$^{13}C_5$ to 2 mL of water containing 40 mg ascorbic acid and 18 mg dithiothreitol in a 2 mL screw-top tube and mix by vortex. Working internal standard may be stored in the refrigerator at 0–10 °C for up to 8 h. Volume of internal standard may be increased to process the number of specimens within the assay.

### 2.4 Calibrators and Controls

1. Calibrators: 5-MTHF Working Standard Curve, dilute stock solution 1 mM 5-MTHF as follows:
   (a) Dilution A (100 μM): Add 100 μL of 1 mM 5-MTHF stock solution to 900 μL of 1× aCSF and mix well by vortex.
   (b) Dilution B (10 μM): Add 100 μL Dilution A to 900 μL of 1× aCSF and mix well by vortex.
   (c) Dilution C (1 μM): Add 100 μL Dilution B to 900 μL of 1× aCSF and mix well by vortex.
   (d) Working Standard Curve (25–400 nM): Add 400 μL of Dilution C to 600 μL of 1× aCSF and mix well by vortex. Perform four additional serial dilutions by adding 500 μL of previous standard to 500 μL of 1× aCSF. This will provide a calibration curve of (400, 200, 100, 50, 25 nM). Working standard curve may be stored in the refrigerator at 4 °C for up to 8 h (*see* **Note 2**).
2. Normal Control: (5-MTHF = 80–240 nM target value):
   (a) Prepare 10 mL pooled CSF.
   (b) Assay pooled CSF to quantitate the native concentration of 5-MTHF.

(c) Spike or dilute pooled CSF with diluted stock standard or water to obtain a final concentration of 80–240 nM 5-MTHF. Store in 80 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).

3. Abnormal Control: (5-MTHF = 20–40 nM target value):
   (a) Prepare 10 mL pooled CSF.
   (b) Assay pooled CSF to quantitate the native concentration of 5-MTHF.
   (c) Spike or dilute pooled CSF with diluted stock standard or water to obtain a final concentration of 20–40 nM 5-MTHF. Store in 80 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).

### 2.5 Analytical Equipment and Supplies

1. Shimadzu Prominence liquid chromatograph system with ABSciex 4000QTRAP® with Analyst software version 1.6.2.
2. Analytical Column: Phenomenex Synergi-Hydro, 4 µm, 150 × 3 mm.
3. Guard Column: Phenomenex Security Guard, 5 µm, 4 × 3 mm.
4. 1.5 mL microcentrifuge tubes.

## 3 Methods

### 3.1 Sample Preparation

1. To labeled 1.5 mL microcentrifugal units, pipette 50 µL sample (calibrators, controls, patient CSF).
2. Add 50 µL of 5-MTHF-$^{13}C_5$ I.S. Working Solution.
3. Cap and vortex mix tubes at maximum speed for 3 s.
4. Centrifuge for 10 min at 14,000 × *g*.
5. Transfer 90 µL of prepared sample into corresponding work list position in 96-well microtiter plate and cover with silicone cover.
6. Place completed 96-well microtiter plate onto refrigerated autosampler (4 °C).
7. Inject 10 µL of sample onto HPLC-ESI-MS/MS. Representative HPLC-ESI-MS/MS ion chromatograms for 5-MTHF and I.S. are shown in Figs. 1 and 2 (*see* **Note 3**).

### 3.2 Data Analysis

1. Instrumental operating parameters are given in Table 1.
2. Data are analyzed using Analyst software version 1.6.2 (AB Sciex).
3. Standard curves are generated based on linear regression of the analyte/I.S. peak-area ratio ($y$) versus analyte concentration ($x$) using the primary ions indicated in Table 2.

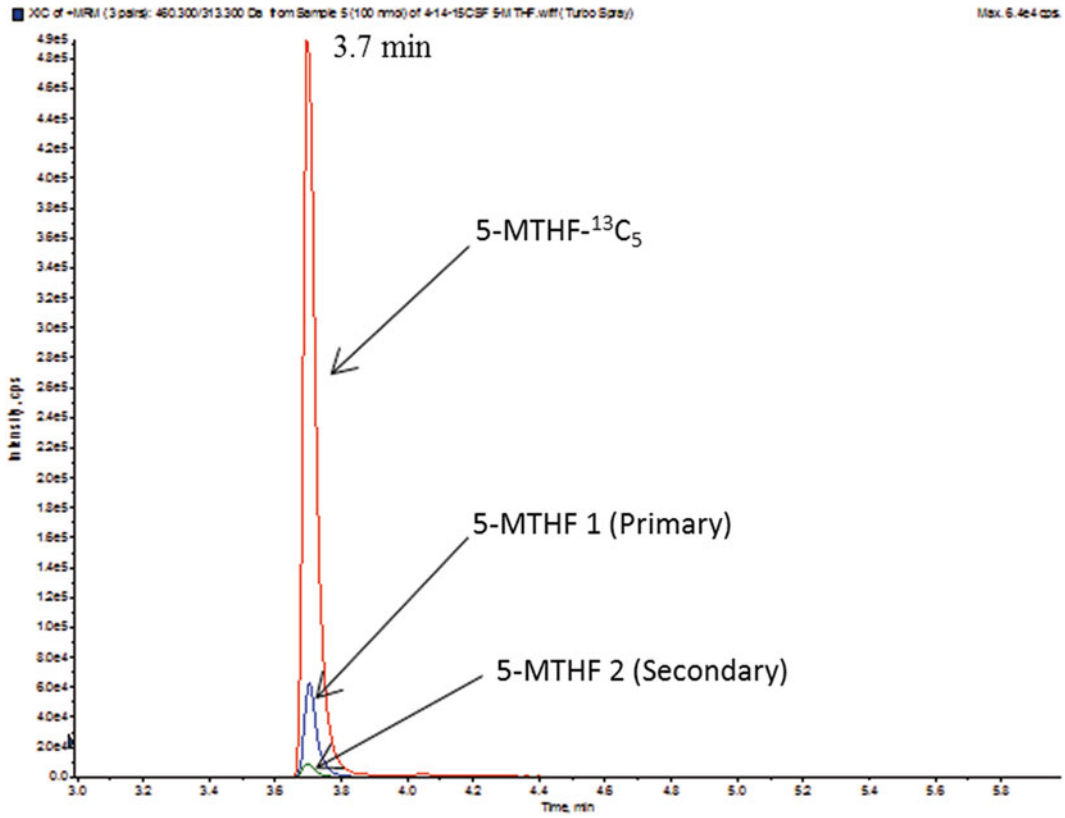



**Fig. 1** HPLC-ESI-MS/MS ion chromatogram of 5-MTHF 100 nM standard. [5-MTHF 1 (*m/z* 460.3 > 313.3), 5-MTHF 2 (*m/z* 460.3 > 194.5), and 5-MTHF-13C5 (*m/z* 465.3 > 313.3)]

4. Acceptability of each run is confirmed if the calculated control concentrations fall within two standard deviations of the target values. Inter-day precision was evaluated by repeated analysis of bi-level QC material analyzed in duplicate over a period of 20 different days.
5. Liquid chromatography retention time window limits for 5-MTHF and 5-MTHF-$^{13}C_5$ are set at 3.7 (±0.2) min.
6. The assay has a lower limit of quantitation of 3 nM for 5-MTHF, with imprecision of <6 % over the entire range. *See* **Note 4** for information regarding ion suppression studies. *See* Table 3 for age-specific reference range [4].

## 4 Notes

1. Individual sets of 5-MTHF Standard and Internal Standard Stock Solutions and controls can be pre-aliquoted and frozen until use in each analytical run. For each set pipette specified

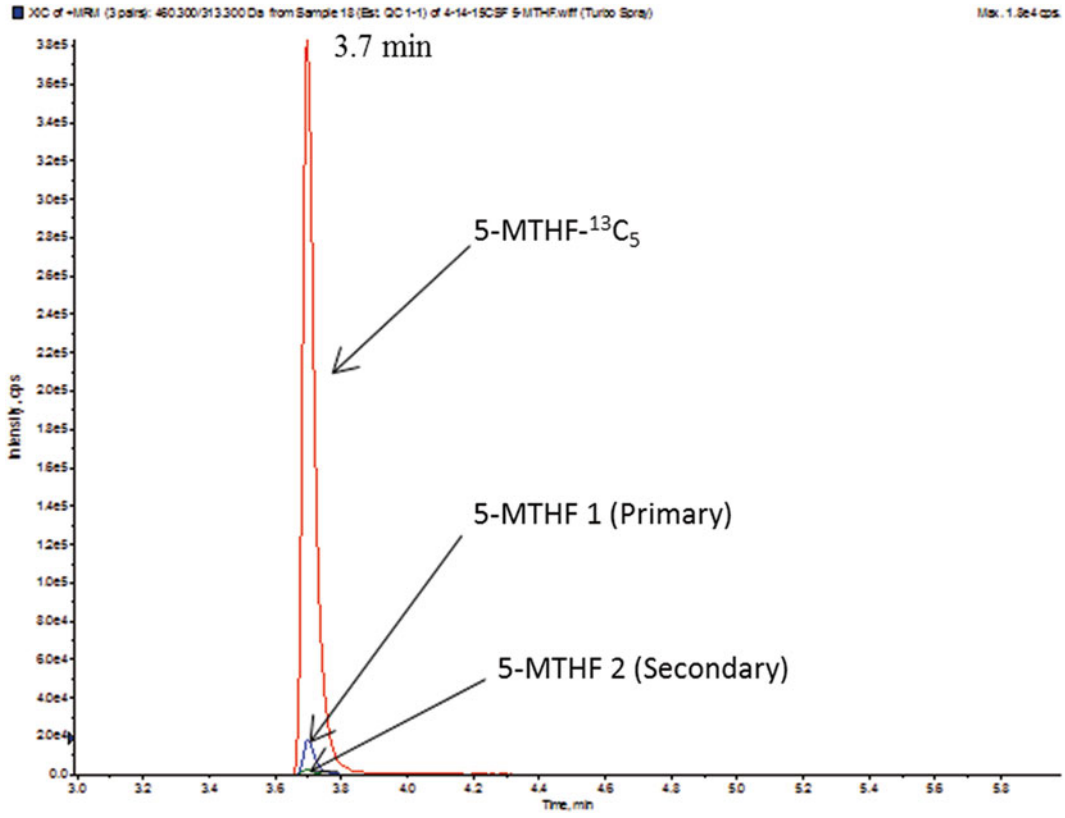



**Fig. 2** HPLC-ESI-MS/MS ion chromatogram of 5-MTHF abnormal QC (30 nM). [5-MTHF 1 (*m/z* 460.3 > 313.3), 5-MTHF 2 (*m/z* 460.3 > 194.5), and 5-MTHF-13C5 (*m/z* 465.3 > 313.3)]

volume of stock standards/control solution into 1.5 mL microfuge tubes and freeze at −80 °C until use. Thaw completely before use. Stable for 4 years at −80 °C.

2. A new standard curve should be prepared with each analytical run to optimize method performance.
3. The controls are analyzed at the beginning of analysis, every five unknowns and at the end of the assay as analysis verification.
4. Ion suppression effects were evaluated by sample infusion method. No significant interferences or ion suppression was identified.

**Table 1**
**HPLC-ESI-MS/MS operating conditions**

| | | |
|---|---|---|
| (a) HPLC (5-MTHF)[a] | | |
| Column temp | 40 °C | |
| Flow rate | 0.375 mL/min | |
| Gradient | Time (min) | Mobile phase A (%) |
| | 0 | 100 |
| | 1.5 | 0 |
| | 2 | 0 |
| | 2.1 | 100 |
| (b) MS/MS tune settings[b] | | |
| Entrance potential (V) | 10 | |
| Curtain gas (psi) | 20 | |
| CAD gas | Medium | |
| Ion spray (V) | 5500 | |
| Temp (°C) | 500 | |
| GS 1 (psi) | 50 | |
| GS 2 (psi) | 50 | |
| Resolution Q1 and Q3 | Unit | |

[a]Optimized for Shimadzu prominence liquid chromatography system equipped with Phenomenex Synergi-Hydro, 4 μm, 150×3 mm analytical column; Mobile phase A: 0.1 % formic acid in water; Mobile phase B: 0.1 % formic acid in methanol
[b]Optimized for ABSciex 4000QTRAP®. Tune settings may vary slightly between instruments

**Table 2**
**HPLC-ESI-MS/MS operating conditions**

| | MRM transition | | | | | |
|---|---|---|---|---|---|---|
| **Compound** | **Q1 (*m/z*)** | **Q3 (*m/z*)** | **Dwell time (ms)** | **DP (V)** | **CE (V)** | **CXP (V)** |
| 5-MTHF 1 | 460.3[a] | 313.3[a,b] | 150 | 96 | 29 | 18 |
| 5-MTHF 2 | 460.3[a] | 194.5[a,c] | 150 | 96 | 48 | 8 |
| 5-MTHF-$^{13}C_5$ | 465.3[a] | 313.1[a,b] | 150 | 96 | 29 | 18 |

[a]Optimized *m/z* may change based on tuning parameters and instrument used
[b]Primary ion for 5-MTHF quantitation
[c]Secondary ion used for MRM ratio confirmation

**Table 3**
**Age-specific reference range for CSF 5-MTHF**

| Age | 5-MTHF (nmol/L) |
|---|---|
| 0–1 year ($n = 12$) | 63–129 |
| 2–3 years ($n = 32$) | 44–122 |
| 4–18 years ($n = 19$) | 42–81 |

Table modified from reference [3]

## References

1. Serrano M, Perez-Duenas B, Montoya J, Ormazabal A, Artuch R (2012) Genetic causes of cerebral folate deficiency: clinical, biochemical and therapeutic aspects. Drug Discov Today 17(23-24):1299–1306
2. Ramaekers VT, Blau N (2004) Cerebral folate deficiency. Dev Med Child Neurol 46(12): 843–851
3. Ramaekers VT, Hausler M, Opladen T, Heimann G, Blau N (2002) Psychomotor retardation, spastic paraplegia, cerebellar ataxia and dyskinesia associated with low 5-methyltetrahydrofolate in cerebrospinal fluid: a novel neurometabolic condition responding to folinic acid substitution. Neuropediatrics 33(6):301–308
4. Ormazabal A, Garcia-Cazorla A, Perez-Duenas B, Gonzalez V, Fernandez-Alvarez E, Pineda M, Campistol J, Artuch R (2006) Determination of 5-methyltetrahydrofolate in cerebrospinal fluid of paediatric patients: reference values for a paediatric population. Clin Chim Acta 371: 159–162

# Chapter 20

# Quantitative Organic Acids in Urine by Two Dimensional Gas Chromatography-Time of Flight Mass Spectrometry (GCxGC-TOFMS)

**Lawrence Sweetman, Paula Ashcraft, and Jeanna Bennett-Firmin**

## Abstract

Seventy-six organic acids in urine specimens are determined with quantitative two dimensional Gas Chromatography-Time of Flight Mass Spectrometry (GCxGC-TOFMS). The specimen is treated with urease to remove urea then derivatized to form pentafluorobenzyl oximes (PFBO) of oxoacids. The sample is then treated with ethyl alcohol to precipitate proteins and centrifuged. After drying the supernatant, the organic acids are derivatized to form volatile trimethylsilyl (TMS) derivatives for separation by capillary two dimensional Gas Chromatography (GCxGC) with temperature programming and modulation. Detection is by Time of Flight Mass Spectrometry (TOFMS) with identification of the organic acids by their mass spectra. Organic acids are quantitated by peak areas of reconstructed ion chromatograms with internal standards and calibration curves. Organic acids are quantified to determine abnormal patterns for the diagnosis of more than 100 inherited disorders of organic acid metabolism. Characteristic abnormal metabolites are quantified to monitor dietary and other modes of treatment for patients who are diagnosed with specific organic acid disorders.

**Key words** Urine organic acids, Trimethylsilyl derivatives (TMS), Mass spectrometry

## 1 Introduction

Qualitative analysis of organic acids in urine by gas chromatography–mass spectrometry (GCMS) is an important laboratory procedure for the diagnosis of inherited disorders of organic acid, amino acid, and fatty acid oxidative metabolism. Quantitative analysis of urinary organic acids is helpful for diagnosis and is essential for monitoring the clinical management of diagnosed patients. Critical steps for quantitative analysis are the extraction of the organic acids from urine and derivatization for GCMS analysis. Organic solvent extraction is commonly used but has the disadvantages of large differences in extraction efficiency of different organic acids and interferences from extracted inorganic acids and other compounds. Liquid partition chromatography provides more uniform extraction

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_20, © Springer Science+Business Media New York 2016

efficiency and removes inorganic acids but is a laborious and time-consuming procedure [1]. An alternative simplified preparation method is to treat the urine with urease to remove the large amount of urea that interferes with the GCMS analysis followed by derivatization and GCMS. The chromatogram includes amino acids and sugars in addition to the organic acids and can increase the range of inherited disorders that can be detected. However, it has the disadvantage of a much more complicated GCMS profile with more compounds co-eluting making quantitation of the organic acids more difficult [2–4]. The analysis of organic acids with solvent extraction and derivatization has been improved by replacing GCMS with two dimensional GC-time of flight mass spectrometry (GCxGC-TOF-MS) using two columns of different polarity to obtain two dimensions of separation, decreasing the number of co-eluting peaks [5]. We have developed a quantitative urine organic acid analysis combining the simplicity of urease treatment, pentafluorobenzyloximation, trimethylsilyl derivatization (TMS), and enhanced peak separation by GCxGC-TOFMS. Two dimensional separation improves the separation and identification of acids from their deconvoluted spectra. Organic acids are quantitated by peak areas of reconstructed ion chromatograms with internal standards and calibration curves. This urease treatment of urine, TMS derivatization followed by GCxGC-TOFMS method can be extended to include amino acids, sugars, purines, pyrimidines etc. as an improvement of the urease GCMS procedure [2].

## 2 Materials

### 2.1 Samples

Random clean-catch urine collected into a sterile container is acceptable. No preservatives or stabilizing agents should be added to the urine. Optimal sample volume is 3 mL; the minimum sample volume is 1 mL. The sample should be stored at 2–8 °C immediately and frozen at −20 °C within 4 h of collection. The urine sample may be stored at −20 °C for up to 1 year without compromise of the sample integrity except for the loss of oxo (keto) acids.

### 2.2 Reagents

1. Urease 12KU type C-3: Use equal milliliter amounts of deionized water to the printed weight on the urease vial (e.g., Urease contents weight is 7.53 mg, add 7.53 mL of water). Aliquot and store in the −20 °C freezer.
2. Acetic Acid 1:100 dilution with water—add 9.9 mL of $H_2O$ to 100 μL of Acetic Acid.
3. *o*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamineHCl (PFBO) (Sigma, 250 mg F.W. 249.6): Dissolve 10 mg in 1 mL of 1:100 Acetic Acid: $H_2O$, depending on the number of samples being processed (40 μL needed per sample). Prepare fresh daily no more than 1 h prior to use.

4. TriSil (Pierce). One mL ampules, store at 2–8 ° C, stable 1 year.
5. BSTFA (Supelco). One mL ampules, store at 2–8 °C, stable 1 year.
6. BSTFA:TriSil Derivatization mixture: Equal volumes of BSTFA and TRISIL are transferred from the sealed glass ampules into a Screw-Cap vial and gently mixed. Store at 2–8 °C. Stable for 2 weeks.
7. Sodium Bicarbonate, $NaHCO_3$, 20 mM: Dissolve 840.1 mg in 500 mL deionized water. Store in a Nalgene bottle with screw cap at 2–8 °C, stable for 1 year.
8. Hydrochloric Acid, HCl, 20 mM. Dilute 0.16 mL of concentrated reagent grade hydrochloric acid up to 100 mL with deionized water. Store at room temperature, stable for 1 year.

### 2.3 Standards and Calibrators

Seventy-six standard acids (Table 1) are divided into five different calibration mixtures so that no organic acids in a mixture coelute (i.e., different retention indices, RI). *See* Table 2 for example. Where possible, authentic high purity standards are purchased from biochemical manufacturers (*see* **Note 1**). In a few cases when not commercially available, standards have been synthesized. The purity of these compounds is always checked by GCxGC-TOFMS prior to using them in the calibration procedure.

The stock standards are typically prepared as 10 mM solutions in aqueous or methanol solvents that are most appropriate for good solubility and stability. Solvent A is 20 mM sodium bicarbonate which is used for as many acids as possible to give stable, nonvolatile sodium salts of the acids. Solvent B is 20 mM HCl, used primarily for 2-oxoacids which are unstable in sodium bicarbonate. Solvent C is methanol for fatty acids and longer chain dicarboxylic acids which are not soluble in water. The stock standards are stable indefinitely at −20 °C, with a few exceptions (oxoacids).

To calibrate the instrument (performed every 6 months), stock standards are combined into three different solvent mixtures for each of the five calibration mixtures. Each solvent mix will contain 46 times the volume of each acid that is needed for a 1× calibration. *See* Table 2 for example. This is sufficient to use these mixes for the complete calibrations of levels 0.02×, 0.1×, 0.2×, 1×, 3×, 5×, and 10× (*see* Table 3 for setup). The different concentration levels are set up, extracted, and analyzed using the same methodology as patient samples. The Calibration module in Leco's ChromaTof 4.1 software is utilized to create the calibration curves that will quantitate the 76 compounds in patient and control samples (*see* **Note 2**).

### 2.4 Quality Controls

1. Artificial urine is prepared to use as a blank and for calibration. Creatinine is excluded due to possible interference with calibration of some compounds. In a clean 1000 mL beaker, add 500 mL of distilled water. Add 9.1 g of urea to the water and mix until all crystals are dissolved. Add 3.75 g of sodium chloride,

**Table 1**
**Standards**

| Pre- | Name | Synonym | Supp. | Mol. wt. |
|---|---|---|---|---|
| | Acetoacetic Li salt | Lithium acetoacetate | Sigma | 108.00 |
| *N*- | Acetylaspartic | | Aldrich | 175.10 |
| *N*- | Acetyltyrosine | | Sigma | 223.20 |
| | Aconitic | *cis*-Aconitic | Sigma | 174.10 |
| | Adipic | | Sigma | 146.14 |
| | Benzoic-Na salt | | Aldrich | 144.10 |
| | Butyrylglycine | | ten Brink | |
| | Citric, anhydrous | | Sigma | 192.10 |
| | Decanoic | Capric | Sigma | 172.27 |
| | Ethylmalonic | | Sigma | 132.10 |
| | Fumaric (free acid) | | Aldrich | 116.07 |
| | Glutaconic | | ICN | 130.10 |
| | Glutaric | | Sigma | 132.10 |
| DL | Glyceric hemi Ca salt | | Sigma | 125.10 |
| | Glycolic | | Sigma | 76.05 |
| | Glyoxylic Na salt | | Sigma | 114.00 |
| | Hexanoylglycine | | ten Brink | 173.21 |
| | Hippuric | Benzoylglycine | Sigma | 179.18 |
| 3- | Hydroxy-3-methylglutaric | HMG | Sigma | 162.10 |
| 2- | Hydroxy-3-methylvaleric Na salt | | Sigma | 154.10 |
| 2- | Hydroxyadipic | | UAM | 162.16 |
| 3- | Hydroxybutyric Na salt | beta-Hydroxybutyric-Na salt | Sigma | 126.10 |
| 4- | Hydroxybutyric Na salt | gamma-Hydroxybutyric-Na | Sigma | 126.10 |
| 4- | Hydroxycyclohexylacetic | | UAM | 158.19 |
| 2- | Hydroxyglutaric di-Na salt | L-a-hydroxyglutaric | Sigma | 192.10 |
| 3- | Hydroxyglutaric | | ten Brink | 192.10 |
| 3- | Hydroxyisobutyric | beta-Hydroxyisobutyric | UAM | 104.12 |
| 2- | Hydroxyisocaproic | alpha-hydroxyisocaproic | Sigma | 132.20 |
| 2- | Hydroxyisovaleric | alpha-Hydroxyisovaleric | Fluka | 118.13 |
| 3- | Hydroxyisovaleric | beta-Hydroxyisovaleric | UAM | 118.13 |

(continued)

**Table 1**
**(continued)**

| Pre- | Name | Synonym | Supp. | Mol. wt. |
|---|---|---|---|---|
| 5- | Hydroxymethyluracil | | Sigma | 142.10 |
| 2- | Hydroxyphenylacetic | *o*-Hydroxyphenylacetic | Sigma | 152.10 |
| 4- | Hydroxyphenylacetic | *p*-Hydroxyphenylacetic | Sigma | 152.10 |
| 4- | Hydroxyphenyllactic | *p*-Hydroxyphenyllactic | Sigma | 182.20 |
| 4- | Hydroxyphenylpyruvic | *p*-Hydroxyphenylpyruvic | Sigma | 180.16 |
| 3- | Hydroxypropionic | Ethylhydracrylic | Fluka | 90.08 |
| 3- | Hydroxyvaleric K salt (94 %) | | UAM | 156.00 |
| | Isobutyrylglycine | | ten Brink | |
| | Isocitric-3 Na salt | | Sigma | 258.10 |
| | Isovalerylglycine | | UAM | 159.21 |
| | Lactic | L-(+)-Lactic | Sigma | 90.08 |
| | Malic | | Sigma | 134.10 |
| | Malonic | | Sigma | 104.10 |
| 2- | Methyl-3-hydroxybutyric (80 %) | | UAM | 118.13 |
| 2- | Methyl-3-hydroxyvaleric | 3-Hydroxy-2-methylvaleric | UAM | 132.16 |
| 2- | Methylacetoacetic | | Med. Isotopes | 116.11 |
| 2- | Methylbutyrylglycine | | ten Brink | 159.00 |
| 2- | Methylcitric | | UAM | 206.15 |
| 3- | Methylcrotonylglycine | | ten Brink | |
| 3- | Methylglutaconic (82 %) | | UAM | 156.00 |
| 3- | Methylglutaric | | Sigma | 146.14 |
| | Methylmalonic | | Aldrich | 118.09 |
| | Methylsuccinic | | Sigma | 132.10 |
| | Mevalonic Lactone | Mevalonolactone | Aldrich | 130.15 |
| | Octanoic | Caprylic | Sigma | 144.20 |
| | Orotic | | Sigma | 178.10 |
| 2- | Oxo-3-methylvaleric Na salt | 3-Methyl-2-oxopentanoic | Aldrich | 152.10 |
| 2- | Oxoadipic | alpha-Ketoadipic | Sigma | 160.10 |
| 2- | Oxoglutaric | alpha-Ketoglutaric | Sigma | 168.10 |
| 2- | Oxoisocaproic Na salt | Na 4-methy-2-oxopentanoic | Aldrich | 152.10 |

(continued)

**Table 1**
**(continued)**

| Pre- | Name | Synonym | Supp. | Mol. wt. |
|---|---|---|---|---|
| 2- | Oxoisovaleric Na salt | 3-Methyl-2-oxobutanoic | Aldrich | 138.10 |
| 5- | Oxoproline | Pyroglutamic | Sigma | 129.10 |
| | Phenylacetic | Benzeneacetic | Aldrich | 136.20 |
| 3- | Phenyllactic | DL-beta-Phenyllactic | Sigma | 166.20 |
| | Phenylpyruvic Na salt | beta-Phenylpyruvic Na | Sigma | 186.10 |
| | Pimelic | Heptanedioic | Sigma | 160.20 |
| | Propionylglycine | | UAM | 131.15 |
| | Pyruvic Na salt | | Sigma | 110.00 |
| | Sebacic | Decanedioic | Sigma | 202.25 |
| | Suberic | Octanedioic | Sigma | 174.20 |
| | Suberylglycine | | ten Brink | 231.00 |
| | Succinic | | Sigma | 118.10 |
| | Succinylacetone | 4,6-Dioxoheptanoic | Sigma | 158.16 |
| | Thymine | | Sigma | 126.10 |
| | Tiglylglycine | | UAM | 157.19 |
| | Uracil | | Sigma | 112.10 |

2.25 g of potassium chloride, and 2.4 g of sodium phosphate. Mix until all crystals are dissolved. Add 50 mg of albumin powder to the solution. Mix until clear. Add 267 mg of alanine (3 mmol/g creatinine) and 676 mg of glycine (9 mmol/g creatinine). Mix until the amino acids are dissolved. Check pH with indicator paper. The pH within normal urine is 5–7. If the pH is outside of range, the pH may be lowered with 1 N hydrochloric acid or raised with 1 N sodium hydroxide. Measure out 10 mL of artificial urine into a 40 mL trace clean vial, add 10 mL of deionized water, mix. Label as Calibration 1:2 Artificial Urine (AU). This will be used in Calibration setup. Store at −20 °C. Aliquot stock into 1.5 mL microtubes. Label and store at −20 °C. This will be used as the Blank at setup. 25 μL will be used at setup + 25 μL of deionized water. Aliquot remaining stock into 50 mL tubes. Label and store at −20 °C.

2. Normal and Abnormal controls are prepared by collecting approximately 1 L of urine from previously analyzed normal patient samples or from healthy pediatric volunteers. Determine creatinine concentration of pooled urine. Take appropriate volume aliquot (25, 50, or 100 μL) based on creatinine result and

**Table 2**
**Example of a calibration mixture**

| Pre- | Name (purity %) | RI DB5MS | RI DB5 | M+ | T *m/z* | Q *m/z* | Solv. | Mix | 1× nmol | mM | 1× nmol | 1× µL Bicarb | × µL MeOH | 1× µL HCl | 46× µL Bicarb | 46× µL MeOH | 46× µL HCl |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glycolic 2TMS | 1068 | 1077 | 220 | 177 | 205 | Bicarb | 1 | 50 | 10 | 50 | 5.00 | | | 230 | | |
| 3- | Hydroxybutyric 2TMS | 1153 | 1163 | 248 | 191 | 117 | Bicarb | 1 | 100 | 10 | 100 | 10.00 | | | 460 | | |
| | Benzoic 1TMS | 1249 | 1253 | 194 | 179 | 105 | Bicarb | 1 | 25 | 10 | 25 | 2.50 | | | 115 | | |
| 3- | Methylglutaric 2TMS | 1418 | 1428 | 290 | 204 | 69 | Bicarb | 1 | 25 | 10 | 25 | 2.50 | | | 115 | | |
| | Butyrylglycine 1TMS 95 % pure | 1441 | 1445 | 217 | 158 | 145 | Bicarb | 1 | 25 | 9.5 | 25 | 2.63 | | | 121 | | |
| | Butyrylglycine 2TMS | 1476 | 1493 | 289 | 172 | 102 | Ok | 1 | Ok | Ok | Ok | | | | | | |
| | Malic | 1480 | | 350 | 245 | 233 | Bicarb | 1 | 25 | 10 | 25 | 2.50 | | | 115 | | |
| | Tiglylglycine 2TMS 96 % pure | 1547 | 1564 | 301 | 286 | 184 | Bicarb | 1 | 25 | 9.6 | 25 | 2.60 | | | 120 | | |
| | Tiglylglycine 1TMS | 1569 | 1571 | 229 | 229 | 214 | Ok | 1 | Ok | Ok | Ok | | | | | | |
| 2- | Hydroxyglutaric 3TMS | 1565 | 1583 | 364 | 203 | 247 | Bicarb | 1 | 25 | 10 | 25 | 2.50 | | | 115 | | |
| 2- | Oxo-3-methylvaleric PFBO-S 1TMS Quan S | 1600 | 1620 | 397 | 369 | 200 | HCl-FR | 1 | 25 | 10 | 25 | | | 2.50 | | | 115 |
| 2- | Oxo-3-methylvaleric PFBO-A 1TMS No Quan | 1612 | 1632 | 397 | 369 | 200 | Ok | 1 | OK | OK | OK | | | | | | |
| 5- | Hydroxymethyluracil 3TMS | 1662 | 1682 | 358 | 358 | 343 | Bicarb | 1 | 25 | 10 | 25 | 2.50 | | | 115 | | |
| 4- | Hydroxyphenyllactic 3TMS | 1893 | 1920 | 398 | 308 | 179 | Bicarb | 1 | 25 | 10 | 25 | 2.50 | | | 115 | | |

(continued)

**Table 2**
**(continued)**

| Pre- | Name (purity %) | RI DB5MS | RI DB5 | M+ | T *m/z* | Q *m/z* | Solv. | Mix | 1× nmol | mM | 1× nmol | 1× µL Bicarb | × µL MeOH | 1× µL HCl | 46× µL Bicarb | 46× µL MeOH | 46× µL HCl |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Suberylglycine(-$H_2O$) 2TMS | | 2030 | 357 | 184 | 342 | Ok | 1 | Ok | Ok | Ok | | | | | | |
| *N*- | Acetyltyrosine 3TMS | 2087 | 2120 | 439 | 260 | 218 | Bicarb | 1 | 50 | 10 | 50 | 5.00 | | | 230 | | |
| *N*- | Acetyltyrosine 2TMS | 2125 | 2143 | 367 | 308 | 352 | Ok | 1 | Ok | Ok | Ok | | | | | | |
| | Suberylglycine 3TMS | 2217 | 2243 | 447 | 330 | 432 | Ok | 1 | Ok | Ok | Ok | | | | | | |
| | Suberylglycine 2TMS 90 % pure SD STD | 2254 | 2271 | 375 | 360 | 189 | $H_2O$ | 1 | 25 | 9 | 25 | 2.78 | | | 128 | | |
| 4- | Hydroxyphenylpyruvic PFBO-S 2 TMS No Q | 2259 | 2288 | 519 | 277 | 190 | Ok | 1 | OK | OK | OK | | | | | | |
| 4- | Hydroxyphenylpyruvic PFBO-A 2 TMS Q A | 2281 | 2304 | 519 | 277 | 190 | HCl-FR | 1 | 50 | 2 | 50 | | | 25.00 | | | 1150 |
| | Count | 20.00 | 20.00 | | 21.00 | 21.00 | | | 14.00 | 14.00 | 14.00 | 12.00 | 0.00 | 2.00 | 12 | 0 | 2 |
| | | | | | | Total µL of stds | | | | | | 43.01 | 0.00 | 27.50 | 1979 | 0 | 1265 |
| | | | | | | µL 1:2 AU, MeOH or HCl for dilution | | | | | | 56.99 | 50.00 | 22.50 | 2621[a] | 2300 | 1035 |
| | | | | | | Total volume, µL stds plus solvent | | | | | | 100.00 | 50.00 | 50.00 | 4600 | 2300 | 2300 |
| | | | | | | | | | | | | Date Mix 1 stock expires | | | | | |
| | | Date Mix 1 stock prepared | | | | | | | | | | | Mix 1 | Stock | Bicarb | MeOH | HCl |

[a]Use the 1:2 artificial urine for dilution

**Table 3**
**Mixture setup**

| Component | 0× | 0.02× | 0.1× | 0.2× | 1× | 3× | 5× | 10× |
|---|---|---|---|---|---|---|---|---|
| | µL | µL | µL | µL | µL | µL | µL | µL |
| 1:2 Artificial urine | 150 | 130 | 50 | 130 | 50 | 0 | 0 | 0 |
| OA Mix 1 MeOH | 0 | 5 | 25 | 5 | 25 | 75[a] | 125[a] | 250[a] |
| OA Mix 1 AU | 0 | 10 | 50 | 10 | 50 | 150 | 250 | 500 |
| OA Mix 1 HCl | 0 | 5 | 25 | 5 | 25 | 75 | 125 | 250 |
| Total microliters $H_2O$, bicarb and HCl | 150 | 150 | 150 | 150 | 150 | 225 | 375 | 750 |
| | | Use a 1:10 dilution of Mix | Use full strength Mix | | | | | |

[a]Dry down under a gentle stream of nitrogen before adding MIX1 AU and MIX1 HCl volumes

run a full organic acid analysis. Review the GCxGC-TOFMS results and determine that the following commonly found organic acids are present at a level of at least 5 mmol/mol creatinine. Monitor these analytes over a period of time (at least 20 determinations over 1–2 months): Lactic, Malic, Glycolic, *N*-Acetylaspartic, 3-HPA, MMA, 3-HIBA, 4-Hydroxyphenyllactic, 3-HIVA, 3HBA ***** *See* **Note**, Succinic, Glyceric, Adipic, 2-Hydroxyglutaric, 4-Hydroxyphenylacetic, 3-Hydroxyadipic, Aconitic, Isocitric. Those analytes >5 mmol/mol creatinine will be used for the normal control. Take 400 mL aliquot of the pooled urine to spike as the abnormal control. The following organic acids and their desired concentrations are used to spike Normal Urine (the organic acids should be of the highest grade, purity, and quality available): Lactic 200 nmoles/µmole creatinine, Methylmalonic 200 nmoles/µmole creatinine, 2-Hydroxyisocaproic 100 nmoles/µmole creatinine, Ethylmalonic 90 nmoles/µmole creatinine, Glutaric 100 nmoles/µmole creatinine, 2-Hydroxyglutaric 90 nmoles/µmole creatinine, 4-Hydroxyphenylacetic 160 nmoles/µmole creatinine, Orotic 50 nmoles/µmole creatinine, 4-Hydroxyphenyllactic 100 nmoles/µmole creatinine, *N-Acetylaspartic* 80 nmoles/µmole creatinine, 3-HBA ***** *see* **Note** 200–300 nmoles/µmole creatinine. ***** *Note*: 3-HBA is spiked when the normal pooled urine contains less than 100 nmol/mol creatinine.

### 2.5 Internal Standards

1. Intermediate: 10 mM 2-Methylsuccinic-d6 (Cambridge, F.W.138.16) in 20 mM HCl (used for all other analytes not covered by the other two Internal Standards).

2. Intermediate: 10 mM 2-Oxocaproic (2-ketohexanoic) (Sigma, F.W.152.16) in 20 mM HCl (used for oxoacids).
3. Working Internal Standard: Combine equal volumes of the 10 mM 2-methylsuccinic-d6 and 10 mM 2-oxocaproic to make a final concentration of 5 mM each in a 20 mM HCl solution. Aliquot and store at −20 °C, stable for 6 months.
4. Working Internal Standard: 5 mM Heptanoic-d5 (CDN Isotopes, F.W. 135.18) in Methanol. Aliquot and store at −20 °C, stable for 6 months (used for octanoic and decanoic).

### 2.6 Supplies

1. 1.5 mL microtubes (extraction).
2. 1.5 mL tubes with screw caps (urease).
3. Wheaton Scintillation Vials with screw caps (PFBO and calibration mixes).
4. Glass transfer pipets.
5. VWR Precleaned Trace Clean Clear Borosilicate 20 mL vials with Teflon-lined closure (Stock Standards).
6. Agilent certified vials (used for internal standards and reaction vials).
7. Xpertek 0.1 mL autosampler vials, plastic with glass inner cone.
8. Xpertek 11 mm Teflon faced silicon/rubber seals (for autosampler and reaction vials).
9. SGE Syringe 10F-CTC-0.63 10 μL.
10. Columns-Primary column, Restek 30 m × 0.25 mm ID—BPX50 0.25 μm, secondary column, Restek 25 m × 0.32 mm ID—BPX5 0.025 μm (2 m used in the secondary oven).
11. Liquid nitrogen, very dry nitrogen gas and air, Ultra high purity Helium gas.

### 2.7 Equipment

Pegasus 4D GCxGC-TOFMS System (Leco, St. Joe, MI) with autosampler (Gerstel).

Concentrator.

Adjustable and fixed volume pipettes.

Crimper and Decapper.

## 3 Methods

### 3.1 Stepwise Procedure

1. Analyze urine samples for creatinine (LC-MS-MS method is used).
2. Pull an Artificial Urine Control, a Normal Urine Control, an Abnormal Urine Control, urease, urine samples, and internal standards out and allow them to come to room temperature.

3. Obtain 1.5 mL microtubes for sample setup; verify that the tubes are clean and without any particulate matter in it before using. Label tubes.
4. Prepare PFBO soln (10 mg/mL). Each sample requires 40 μL of PFBO. PFBO must be made fresh daily and should not be made up more than 1 h prior to setting up samples. Vortex the PFBO solution and verify that all of the PFBO is dissolved prior to use.
5. Add 10 μL of combined working internal standard 5 mM Methylsuccinic-d6 and 5 mM 2-oxocaproic acid to each tube. Add 10 μL of 5 mM Heptanoic-d5 internal standard to each tube.
6. Vortex the urine sample and pipette the urine into appropriately labeled 1.5 mL microtube. Pipette an aliquot of urine (plus deionized water if indicated) based on Creatinine result.
   (a) 0.2–0.99 mmoles take 0.200 mL of urine
   (b) 1.0–4.0 mmoles take 0.100 mL of urine
   (c) 4.0–13.0 mmoles take 0.050 mL of urine
   (d) >13.0 mmoles take 0.025 mL of urine + 0.025 mL deionized $H_2O$

   Any samples with creatinine less than 0.2 will need to be tested for proper sample type; if deemed urine, the sample will need to be concentrated before it can be tested. Concentration procedure: take a large aliquot of urine (at least 1500 μL), place in a scintillation vial, and dry down overnight in a vacuum concentrator. Reconstitute with deionized water at a concentration 10× less (150 μL of water).
7. Add 40 μL of working urease to each tube, cap, vortex, and place in a 37 °C incubator for 10 min.
8. Add 40 μL of PFBO to each sample, cap, vortex, and incubate at room temperature for 30 min.
9. Add 900 μL of 200 proof ethyl alcohol to each tube, cap, and vortex. Centrifuge tubes at 2,400 × *g* for 10 min.
10. Transfer solution to a prelabeled Agilent certified reaction vial, transfer as much of the liquid as possible without disturbing the pellet at the bottom of the vial, place on a concentrator, and evaporate under nitrogen at 50 °C until completely dry (~30 min).
11. Pipette 300 μL of BSTFA:TriSil Derivatization mixture into each vial. Cap and vortex. Place in an oven at 60–65 °C for 30 min.
12. Remove the samples from the oven and allow the samples to cool to room temperature prior to transferring to the GC vials.

13. Pipette 100 μL of the derivatized sample to a labeled autosampler vial. Cap with a GC autosampler cap immediately. Place on instrument to run.
14. One microliter of sample is injected using a 1:2 ratio in split mode. The inlet temperature is at 280 °C with Helium as the carrier gas. A flow rate of 1 mL/min for the entire run is used using the corrected constant flow via pressure ramps. The initial oven temperature is 75 °C; 5 min after the injection the oven temperature is raised to 280 °C at a rate of 4 °C/min and held at 280 °C for 5 min. The Secondary oven temperature offset is 25 °C with the modulator offset at 30 °C. Modulation timing is set for 6 s periods with 0.6 s hot pulse and 2.40 s cool time. Transfer line and source temperatures are set at 250 °C and 230 °C respectively. Mass Range is 45–750 with an acquisition rate of 200 spectra/s. The acquisition voltage setting is 1500 with the electron energy of –70.

### 3.2 Data Analysis

1. The identification criteria for organic acids (and internal standards), which are quantified by areas of extracted ion chromatograms, include a combination of: a quantitation ion and a confirming ion which are unique to that organic acid peak retention times ratios of areas of extracted ion chromatograms and total spectra. Additionally for accurate identification, the retention time of the quantitation ion extracted ion chromatogram must be near the center (expected retention time of the acid) of the 7 s time range of the primary chromatogram and 0.05 s of the secondary. The quantitation ion and confirming ion of each organic acid were chosen to be as uniquely characteristic as possible within this narrow time window. Therefore, the retention times for the extracted ion chromatograms must be very close and the ratios of areas for the confirming ion as percent of the quantitation ion should be within 45 % (relative) of the expected values in the calibration table; most compounds will be within 20 %. In the identification of each organic acid, the full ion spectrum is compared to the authentic spectra in the mass spectral library and calibration reference and a match of greater than 700 is considered to be acceptable for identification.
2. However, because there are many co-eluting compounds in urine (*see* **Notes 3** and **4**) which can lower the library match, the definitive criteria for identification from ion ratios and the total spectra are at the discretion of carefully trained highly experienced technicians who can recognize characteristic masses and % intensities to identify the different compounds in mixed spectra. A representative GCxGC-TOFMS Chromatogram and a 2D-chromatogram (contour plot) of the TMS derivatives of all compounds seen are shown in Fig. 1. GCxGC-TOFMS selected ion chromatogram is shown in Fig. 2 (*see* **Note 3**).

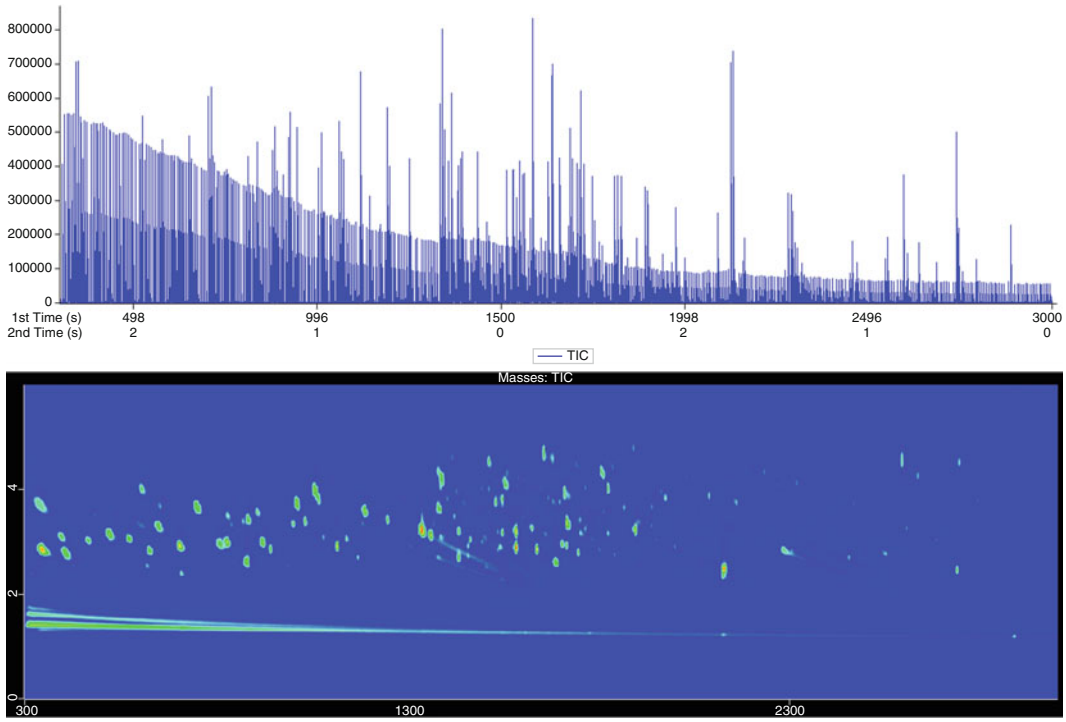



**Fig. 1** GCxGC-TOFMS Chromatogram and 2D-chromatogram (contour plot) of the TMS derivatives of all compounds seen

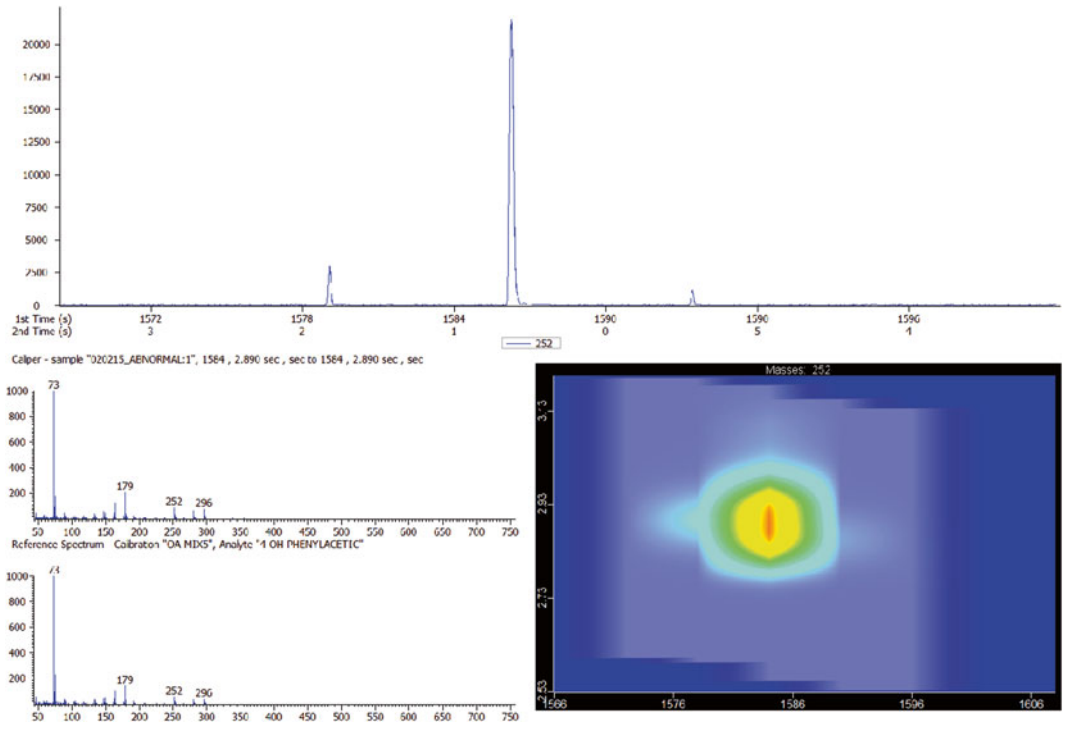



**Fig. 2** Selected ion chromatogram of a TMS derivative of 4-OH phenylacetic

3. Analyze data using ChromTof Software (Leco, St. Joseph, MI) with a data processing method which includes the calibration curves for the 76 analytes previously performed. The quantifying ions previously established are used to construct standard curves of the peak area ratios (calibrator/internal standard pair) vs. concentration. These curves are then used to determine the concentrations of the controls and unknown samples. All results are reported in mmol/mole of creatinine; a calculation factor is created using the creatinine value and volume of sample taken to calculate the final result. This factor is multiplied by the result from the calibration curve to give the final result in mmol/mole of creatinine.
4. The linearity/limit of quantitation of the method is 0.25–500 mmol. Samples in which the analyte concentrations exceed the upper limit of quantitation should have a smaller aliquot taken.
5. A typical calibration curve has correlation coefficient ($R^2$) of >0.99.
6. Typical intra- and inter-assay imprecision is <10 %.
7. Quality control: The analytical run is considered acceptable if the calculated concentrations of analytes monitored in the controls are within ±20 % of target values. The quantifying ion in the sample is considered acceptable if the ratios of qualifier ions to quantifying ion are within ±30 % of the ion ratios for the calibrators.

## 4 Notes

1. Calibration preparation of ketoacids can be difficult; many of the ketoacids are very unstable. We buy fresh standards for these compounds each time a calibration is performed.
2. Many of the compounds and most of the ketoacids make multiple TMS derivatives; be aware that these compounds will have a curve for each derivative and the concentration of the compound will have to be divided between the derivatives. Some of the compounds will have a constant percentage of multiple derivatives across a calibration curve; in these cases the largest derivative may be used for the calibration curve instead of having multiple curves for one compound.
3. During data analysis review of the 2D contour plot is very helpful in determining if there are large interfering peaks that may obstruct the ability to see the compounds being reviewed or shift the peaks outside the retention time window. Look for large smears and wrap around peaks.

4. The majority of the 76 compounds are separated with the GCxGC method but there are a few that do not, such as 3-HBA and 3-HIBA, Hexanoylglycine and *N*-acetylaspartic, 2-hydroxyisocaproic and 2-hydroxy-3-methylvaleric to name a few.

## References


1. Sweetman L (1991) Organic acid analysis. In: Hommes FA (ed) Techniques in diagnostic human biochemical genetics: a laboratory manual. Wiley-Liss Inc, New York, pp 143–176, Chapter 11
2. Shoemaker JD, Elliott WH (1991) Automated screening of urine samples for carbohydrates, organic acids and amino acids after treatment with urease. J Chromatogr 562: 125–138
3. Matsumoto I, Kuhara T (1996) A new chemical diagnostic method for inborn errors of metabolism by mass spectrometry—rapid, practical, and simultaneous urinary metabolites analysis. Mass Spectrom Rev 15:43–57
4. Lo SF, Young V, Rhead WJ (2010) Identification of urine organic acids for the detection of inborn errors of metabolism using urease and gas chromatography-mass spectrometry (GC-MS). Methods Mol Biol 603:433–443
5. Kouremenos KA, Pitt J, Marriott PJ (2010) Metabolic profiling of infant urine using comprehensive two-dimensional gas chromatography: application to the diagnosis of organic acidurias and biomarker discovery. J Chromatogr A 1217:104–111

# Chapter 21

# High Sensitivity Measurement of Pancreatic Polypeptide and Its Variant in Serum and Plasma by LC-MS/MS

**Hernando Escobar, Mark M. Kushnir, Alan L. Rockwood, and A. Wayne Meikle**

## Abstract

Aliquots of serum or plasma samples are combined with stable isotope labeled internal standard. Pancreatic polypeptide (PP) and its truncated variant PP3-36 are enriched by incubation with anti-PP antibody conjugated to magnetic beads. Peptides are eluted from beads in acidic buffer and the samples analyzed using liquid chromatography coupled with tandem mass spectrometry. Instrumental analysis of PP and PP3-36 is performed using electrospray ionization ESI in positive ion mode and multiple reaction monitoring (MRM) acquisition.

**Key words** Pancreatic polypeptide, Peptide hormone, Mass spectrometry, Tandem mass spectrometry, Clinic biomarker

## 1 Introduction

Pancreatic polypeptide (PP) is secreted by pancreatic islets after food ingestion [1]. Meal composition is associated with different concentration of PP released in circulation [2]. PP can be elevated in blood of patients with acute pancreatitis [3], endocrine pancreatic tumors [4, 5], gastrinomas [6], and Alzheimer disease patients [7, 8], supporting its valuable use as a predictive and prognostic biomarker. An endogenous truncated form of PP, PP3-36, has been found as the only variant circulating in patient samples [2]. Limitations caused by poor specificity of commercial radioimmunoassays can be overcome using immune-capture and liquid chromatography-tandem mass spectrometry as a sensitive and robust analysis for identification and quantification of intact PP and its endogenous variant in serum and plasma.

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_21, 

## 2 Materials

### 2.1 Samples

Blood is collected in serum separation tubes (red cap), plasma with EDTA (purple cap) or sodium heparin (green cap). Samples are kept on ice. Serum or plasma should be separated from cells within 1 h and frozen at −20 °C. Samples are stable refrigerated at 4 °C no more than 8 h; on ice no more than 24 h; or frozen at −20 °C or below up to 1 year.

### 2.2 Reagents and Buffers

1. Deionized water. Water purified with a Barnstead® NANOpure® system with up to 18.2 MΩ-cm.
2. Human PP, purity ≥95 % (ANASPEC, Fremont, CA). Peptide must contain certificate of analysis, amino acid analysis, and net weight per vial. Keep at −70 °C until use.
3. Isotopically labeled PP Internal standard (PPIS): APL*EPVYPGDNATPEQMAQYAADL*RRYINML*TRPR Y-NH2 (L* = [6C13, N15]), purity ≥95 % (United Biosystems, Herndon, VA). Peptide must contain certificate of analysis, amino acid analysis, and net weight. Keep at −70 °C until use.
4. N-truncated PP peptide, PP3-36: LEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY -NH2, purity ≥95 % provider for synthesis: United Biosystems. Peptide must contain certificate of analysis, amino acid analysis, and net weight per vial. Keep at −70 °C until use.
5. Bovine serum albumin, BSA, purity ≥96 %. Sigma-Aldrich.
6. PP (human) Purified IgG Antibody from rabbit, 400 μg (Phoenix Pharmaceuticals, Burlingame, CA).
7. Alternative antibody: PP purified IgG antibody from rabbit, 400 μg (Bachem, Torrance, CA).
8. Dynabeads® M-280 Tosyl activated magnetic beads. 10 mL (Life Technologies, Carlsbad, CA).
9. L-Leucine-N-FMOC (U-13C6, 97–99 %; 15 N, 97–99 %). Cambridge Isotope Laboratories, Andover, MA. Used to synthesize labeled IS.
10. Sodium phosphate monobasic ≥99.0 %, $NaH_2PO_4 \times H_2O$. Sigma-Aldrich (St Louis, MO).
11. Sodium phosphate dibasic, ≥99.0 % ($Na_2HPO_4 \times 2H_2O$). Sigma-Aldrich.
12. Ammonium sulfate, ≥99.0 %. Sigma-Aldrich.
13. Trizma buffer 1×: 50 mM Trizma, 100 mM NaCl, $NaN_3$ 0.09 %, pH 7.6, HCl adjusted, prepared with deionized water.
14. Trizma buffer 3×: 150 mM Trizma, 300 mM NaCl, $NaN_3$ 0.09 %, pH 7.6, HCl adjusted, prepared with deionized water.

15. Deionized water degassed (used to dissolve pure lyophilized peptides): In a glass Erlenmeyer flask from a funnel system, heat 50 mL deionized water to boil. Connect vacuum until reach room temperature and transfer to a 50 mL tube. Allow nitrogen flux until use. Prepare fresh.
16. Pooled human serum (either serum or plasma can be used) depleted from PP and its immunoreactive variants using PP antibody: filter 300 mL of pooled serum with a disposable filter, 0.45 μm nylon membrane, capacity 500 mL. Add magnetic beads with conjugated anti-PP antibody (volume corresponding to the amount of the conjugated antibody 5 μg). Incubate with rotation for 1 h at 4 °C. Set tube with the pool on magnetic stand and allow the beads to get attracted to the magnet; remove the depleted serum into a clean container. To preserve add to the serum 0.27 g sodium azide (0.09 % w/v, *see* **Note 1**).
17. Acetic acid 5 %. Add 5 mL of Acetic acid (99.9 %) to 100 mL deionized water. Stable refrigerated for 6 months.
18. Trizma buffer 3×, pH 7.6: 150 mM Trizma, 300 mM NaCl, $NaN_3$ 0.09 %, adjust pH 7.6 with HCl, use deionized water to dissolve. Dissolve in 900 mL of deionized water 18.15 g. Trizma, 26.3 g NaCl and 0.9 g $NaN_3$, adjust pH to 7.6 with concentrated HCl, add water to 1 L. Stable refrigerated for 6 months.
19. Trizma buffer 1×, pH 37.6: 50 mM Trizma, 100 mM NaCl, $NaN_3$ 0.09 %, adjust pH 7.6 with HCl, use deionized water to dissolve. Add 250 mL of Trizma buffer 3× to 750 mL of deionized water, mix solution. Stable refrigerated for 6 months.
20. Trizma buffer 0.2×: Add 10 mL of Trizma buffer 1× into a 50 mL plastic tube. Fill with deionized water to 50 mL. Prepare fresh daily.
21. BSA 1 % stock: Weight 0.2 g BSA, dilute in deionized water to 20 mL. Aliquot 300 μL in LoBind™ tubes. Stable for 1 year at −70 °C.
22. BSA 0.005 % (used as matrix for preparation of the working calibration standard): Add 150 μL of 1 % BSA to 30 mL deionized water in a 50 mL plastic tube. Prepare fresh daily.
23. Acetic acid 5 % with BSA 0.0025 % (to elute peptides from antibody): Add 50 μL 1 % BSA to 20 mL 5 % acetic acid in a 50 mL plastic tube. Prepare fresh daily.
24. Mobile phase A ($H_2O$, 5 % ACN, 10 mM acetic acid): Add 570 μL acetic acid (99.9 %) to 950 mL with deionized water, add 50 mL acetonitrile. Filter through 0.45 μm membrane.
25. Mobile phase B: (ACN, 10 mM acetic acid): Add 570 μL acetic acid (99.9 %) to 1000 mL high purity acetonitrile. Filter through 0.45 μm membrane using a glass funnel system.

26. Coupling antibody to magnetic beads performed according to the manufacturer recommended procedure supplied with Dynabeads® M-280 (Life Technologies, Grand Island, NY). Final concentration of antibody into the beads should be 1 μg/5 μL. In the method each sample analyzed receives 5 μL of beads coupled to antibody for a total of 5 μg of antibody.
27. CTC-PAL wash solution 1: Deionized water with 5 % Acetonitrile, 0.1 % acetic acid.
28. CTC-PAL wash solution 2: Acetonitrile with 0.1 % acetic acid.

### 2.3 Standard, Calibrators, and Quality Control Solutions

1. PP, PP3-36, and PPIS stock solutions: Remove vials with lyophilized peptides from freezer and equilibrate at room temperature. Add 1 mL of 0.005 % BSA (prepared in degassed water). Preserve stock solutions during preparation with nitrogen in order to prevent oxidation of PP and PP3-36 purging with nitrogen with final tight capping of each tubes (0.5 mL protein extra low binding screw cap tubes from Bioplastics, Durham, NC). Keep vials tightly capped at −70 °C until use. For stability information (*see* **Note 2**).
2. Working solutions of PP (2, 20, 200 pg/μL):
   Prepare PP solutions at 2500 pg/μL with 0.005 % BSA using a dilution based on the stock solution concentration (*see* **Notes 3** and **4**). Prepare fresh, daily in LoBind™ tubes (Eppendorf, Hauppauge, NY). Make the dilutions below:
   (a) Dilution 1 (200 pg/μL): Add 80 μL of 2500 pg/μL working solution to 920 μL of 0.005 % BSA. Mix.
   (b) Dilution 2 (20 pg/μL): Add 100 μL of 200 pg/μL peptide (Dilution 1) to 900 μL 0.005 % BSA. Mix.
   (c) Dilution 3 (2 pg/μL): Add 100 μL of 20 pg/μL peptide (Dilution 2) to 900 μL 0.005 % BSA. Mix.
3. Working Solutions of PP3-36 (2, 20, 200 pg/μL):
   Prepare PP3-36 solutions at 2500 pg/μL with 0.005% BSA using a dilution based on the stock solution concentration (*see* **Note 3** and **4**). Prepare fresh, daily in LoBind™ tubes (Eppendorf). Make the dilutions below:
   (a) Dilution 1 (200 pg/μL): Add 80 μL of 2500 pg/μL working solution to 920 μL of 0.005 % BSA. Mix.
   (b) Dilution 2 (20 pg/μL): Add 100 μL of 200 pg/μL peptide (Dilution 1) to 900 μL 0.005 % BSA. Mix.
   (c) Dilution 3 (2 pg/μL): Add 100 μL of 20 pg/μL peptide (Dilution 2) to 900 μL 0.005 % BSA. Mix.
4. Mixture of combined calibration standard (PP and PP3-36) is prepared from the working solutions of PP and PP3-36: (final concentrations: 1, 10, 100, and 1250 pg/μL)

(a) Mixture 1 (100 pg/μL, each peptide): Add 400 μL of Dilution 1 PP, and 400 μL Dilution 1 PP3-36 in a 1.5 mL microcentrifuge tube. Mix.

(b) Mixture 2 (10 pg/μL, each peptide): Add 400 μL of Dilution 2 PP, and 400 μL Dilution 2 PP3-36 in a 1.5 mL microcentrifuge tube. Mix.

(c) Mixture 3 (1 pg/μL, each peptide): Add 400 μL of Dilution 3 of PP, and 400 μL Dilution 3 of PP3-36 in a 1.5 mL microcentrifuge tube. Mix.

(d) Mixture 4 (1250 pg/μL). Used to prepare Quality Control 2. Add 400 μL of working solution, 2500 pg/μL of PP, and 400 μL working solution, 2500 pg/μL of PP3-36, in a 1.5 mL microcentrifuge tube. Mix.

5. PP Internal Standard Solution (PPIS), 1000 pg/μL: Prepare with 0.005 % BSA using a dilution based on the stock solution concentration (*see* **Note 5**). Prepare fresh, daily in LoBind™ tubes (Eppendorf).
6. Working PP Internal Standard Solution (PPIS), 10 pg/μL: in LoBind™ microcentrifuge tubes add 10 μL of 1000 pg/μL PPIS solution to 990 μL 0.005 % BSA. Mix. Prepare fresh daily.
7. Calibration standards: Calibration standards for PP and PP3-36 are prepared and analyzed with every batch of samples. Calibration standards are prepared in depleted serum at concentrations of 30, 50, 100, 200, 500, and 1000 pg/mL for PP and PP3-36 (*see* Table 1).
8. Quality control (QC) samples: This assay uses three controls along the run to confirm assay accuracy. One negative control and two controls with low and high peptides levels are analyzed with every batch of samples. The QC samples are prepared by spiking PP and PP3-36 into patient serum pools.

**Table 1**
**Calibration standards preparation**

| Calibration std conc. (pg/mL) | Add working calibration std Mix 1, 1 pg/μL (μL) | Add working calibration std Mix 2, 10 pg/μL (μL) | Add working calibration std Mix 3, 100 pg/μL (μL) | Add serum PP depleted (μL) |
|---|---|---|---|---|
| 30 | 30 | | | 450 |
| 50 | | 5 | | 450 |
| 100 | | 10 | | 450 |
| 200 | | 20 | | 450 |
| 500 | | | 5 | 450 |
| 1000 | | | 10 | 450 |

(a) Negative control: pooled PP and PP3-36 depleted serum/plasma.

(b) Control level 1 (low): 30 pg/mL PP and PP3-36. 50 mL of pooled, depleted serum is spiked with 15 µL of 100 pg/µL of PP and PP3-36 (Mixture 3). Aliquot 500 µL in 1.5 mL microcentrifuge LoBind vials and store at −70 °C. Stable for 6 months.

(c) Control level 2 (high): 500 pg/mL PP and PP3-36. 50 mL of pooled, depleted serum is spiked with 20 µL of 1250 pg/µL of target peptides (Mixture 4). Aliquot 500 µL in 1.5 mL microcentrifuge LoBind™ tubes and store at −70 °C. Stable for 6 months.

### 2.4 Equipment

1. Triple quadrupole mass spectrometer API 5500 (ABSciex) with TurboV ion source. Software Analyst 1.6.1 or newer version.
2. Binary HPLC pumps series 1200 SL (Agilent Technologies), vacuum degasser, autosampler CTC-PAL (LEAP Technologies) equipped with fast wash station.
3. Centrifuge.
4. Sample rocker mixer for 1.5 mL microcentrifuge tubes.
5. Microcentrifuge for 0.5 and 1.5 mL tubes.
6. Analytical balance.

### 2.5 Supplies

1. 1.5 mL microcentrifuge LoBind™ tubes (Eppendorf).
2. Polypropylene 0.3 mL vials (Wheaton, Millville, NJ).
3. PTFE/rubber cap (National Scientific, Rockwood, TN).
4. Filter Units 500 mL, 0.45 µm nylon membrane (Nalgene, Rochester, NY).
5. Glass funnel filter with vacuum adapter. Sigma-Aldrich.
6. Guard cartridge Poroshell 300SB-C18 with Guard Column cartridge holder (Agilent Technologies, Santa Clara, CA).
7. HPLC column for analytical separation Poroshell 300SBC18 column 2.1 × 75 mm, 5 µm (Agilent Technologies).

## 3 Methods

### 3.1 Procedure for Sample Preparation (See Note 7)

1. Prepare calibrators according to Table 1.
2. 450 µL of patient samples and controls are aliquoted in 1.5 mL microcentrifuge protein LoBind™ vials.
3. Add to each tube 225 µL of Trizma buffer 3×.
4. Add 10 µL of working internal standard.

5. Resuspend magnetic beads and add 5 µL to each tube.
6. Incubate for 1 h at 4 °C. Ensure beads are suspended by gentle vortexing.
7. Remove supernatant. Set tubes on magnet stand and remove supernatants with a pipette, changing tips for each sample.
8. Wash the beads twice with 500 µL Trizma 1×. Set tubes on magnet stand and remove supernatants with a pipette, changing tips for each sample. Mix samples between the washes.
9. Wash the beads once with 500 µL Trizma 0.2× with deionized water (*see* **Note 6**). Set tubes on magnet stand and remove supernatants with a pipette, changing tips for each sample.
10. Add 40 µL of 5 % acetic acid with 0.0025 % BSA to elute peptides. Incubate at room temperature for 10 min (without vortexing).
11. Set tubes on magnet stand and transfer the supernatants to polypropylene 0.3 mL vials (do not transfer the beads). Seal the vials with PTFE/rubber caps. Centrifuge vials at 2000×*g* for 30 s.
12. Set the vials to the CTC-PAL autosampler for LC-MS/MS analysis.

### 3.2 HPLC and Autosampler CTC-PAL Conditions

1. HPLC conditions are provided in Table 2. Column temperature is 70 °C.
2. CTC-PAL Autosampler conditions are provided in Table 3. Injection volume is 10 µL.

### 3.3 Mass Spectrometer Conditions

Voltages and gases flow rates for the mass spectrometer were optimized for maximum sensitivity; mass transitions used for PP and PP3-36 and the internal standards are listed in Table 4.

Other mass spectrometer parameters

(a) Source Temperature (at set point): 600.0 °C
(b) Resolution Q1: Unit
(c) Resolution Q3: Unit
(d) CUR: 25
(e) Ion source voltage: 5500
(f) Ion source temperature: 600
(g) Gas heater 1: 50
(h) Gas heater 2: 55

Mass transitions used for qualification and quantitation are shown in Fig. 1. Product ion mass spectrum of PP is shown in Fig. 2.

**Table 2**
**Mobile phase program for chromatographic separation**

| Step | Time | Flow rate (µL/min) | %A ($H_2O$ + 10 mM acetic acid, 5 % ACN) | %B (Acetonitrile + 10 mM acetic acid) |
|---|---|---|---|---|
| 1 | 0.00 | 700 | 95.0 | 5.0 |
| 2 | 0.50 | 700 | 79.0 | 21.0 |
| 3 | 2.00 | 700 | 42.0 | 58.0 |
| 4 | 2.01 | 700 | 5.0 | 95.0 |
| 5 | 4.00 | 700 | 5.0 | 95.0 |
| 6 | 4.01 | 700 | 95.0 | 5.0 |
| 7 | 4.50 | 700 | 95.0 | 5.0 |
| 8 | 4.51 | 700 | 5.0 | 95.0 |
| 9 | 6.00 | 700 | 5.0 | 95.0 |
| 10 | 6.01 | 700 | 95.0 | 5.0 |
| 11 | 8.00 | 700 | 95.0 | 5.0 |

**Table 3**
**Autosampler settings CTC-PAL autosampler. Mode LC-Inj DLW Standard_Rev05**

| | |
|---|---|
| Volume (µL) | 1 |
| Front volume (µL) | 1 |
| Rear volume (µL) | 1 |
| Filling speed (µL/s) | 5 |
| Pullup delay (ms) | 3000 |
| Inject to | LCVlv1 |
| Injection speed (µL/s) | 5 |
| Pre-inject delay (ms) | 500 |
| Post-inject delay (ms) | 500 |
| Needle gap valve clean (mm) | 3 |
| Valve clean time solvent 2 (s) | 2 |
| Post clean time solvent 2 (s) | 2 |
| Valve clean time solvent 1 (s) | 2 |
| Post clean time solvent 1 (s) | 2 |
| Stator wash | 0 |
| Delay stator wash (s) | 120 |
| Stator wash time solvent 2 (s) | 2 |
| Stator wash time solvent 1 (s) | 2 |

**Table 4**
**Parameters optimized for MRM transitions selected for PP, PP3-36, and PPIS (*see* Note 8)**

| Q1 Mass (Da) | Q3 Mass (Da) | Dwell time (ms) | DP | EP | CE | CXP | ID |
|---|---|---|---|---|---|---|---|
| 837.3 | 953.0 | 70 | 45 | 8 | 37 | 22 | PP 953 |
| 837.3 | 411.4 | 70 | 45 | 8 | 35 | 22 | PP 411 |
| 803.5 | 853.7 | 70 | 168 | 8 | 35 | 11 | PP3-36 853 |
| 803.5 | 197.2 | 70 | 168 | 8 | 41 | 10 | PP3-36 197 |
| 841.4 | 957.7 | 70 | 47 | 6 | 35 | 23 | PPIS 957 |
| 841.4 | 418.2 | 70 | 47 | 6 | 37 | 19 | PPIS 418 |

*DP* declustering potential, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential

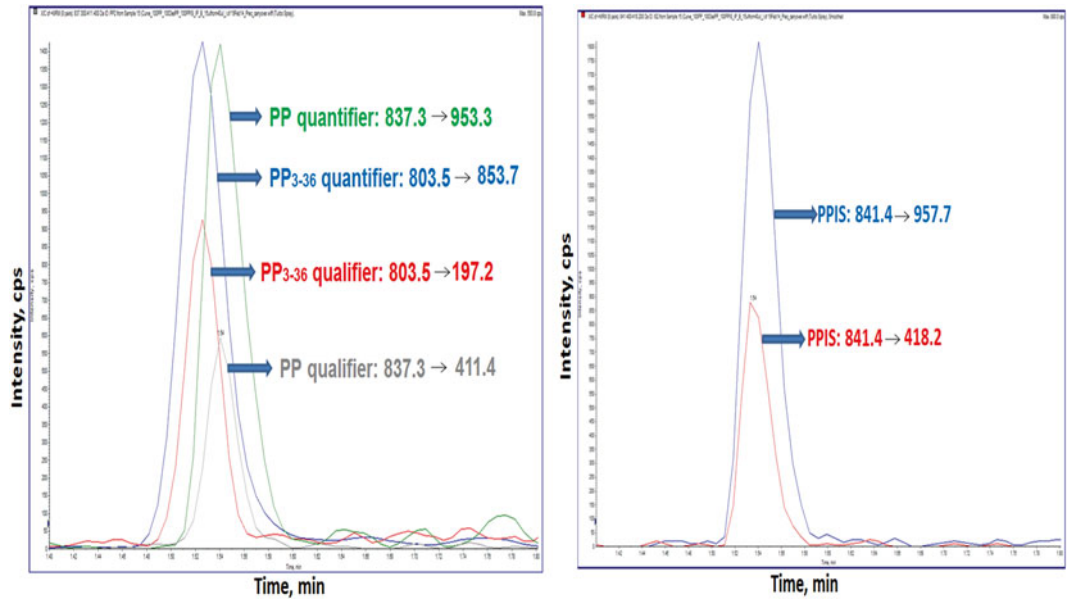



**Fig. 1** Chromatograms of PP, PP3-36, and the internal standard in serum sample, spiked with 100 pg/mL of PP, PP3-36, and PPIS. The MRM transitions are: PP primary (*m/z*: 837.3 → 953.3) and PP secondary (*m/z* 837.3 → 411.4); PP3-36 primary (*m/z*: 803.5 → 853.7) and PP3-36 secondary (*m/z*: 803.5 → 197.2). For PPIS the primary and secondary mass transitions are *m/z*: 841.4 → 957.7 and 841.4 → 418.2 respectively

### *3.4 Data Analysis*

1. Evaluate each chromatogram for acceptable peak shape.
2. Internal standard peak area in every injection should be greater than 4000 cps.
3. Method performance characteristics: analytical measurement range 10–1000 pg/μL for PP and PP3-36; total imprecision of the method is less than 20 %.

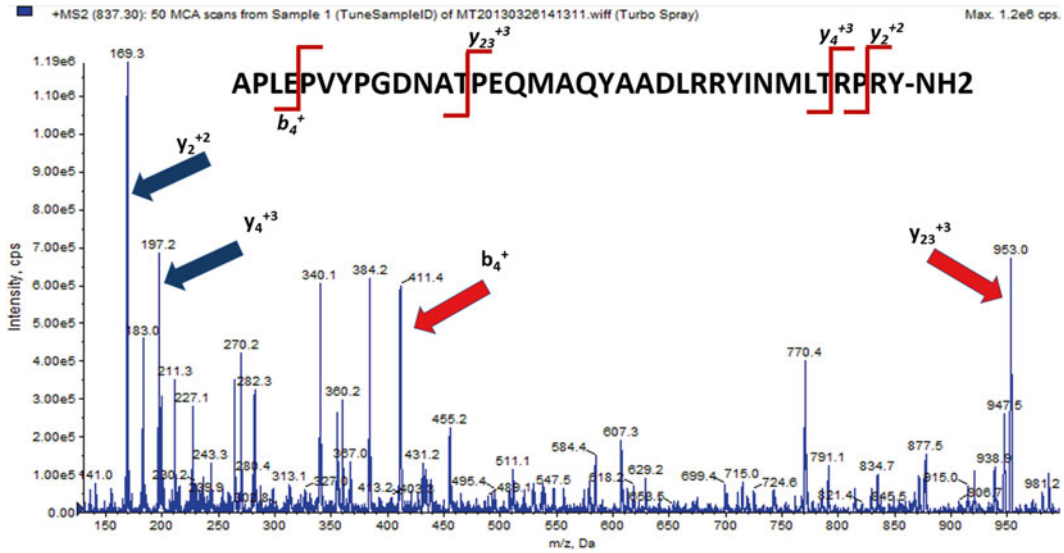



**Fig. 2** Product ion scan mass spectrum of PP obtained using low-energy CID. *Arrows* point on the major product ions used for the MRM transitions of PP

4. Correlation coefficient ($r$) for the calibration curve is greater than 0.995.
5. Concentration of PP and PP3-36 in the negative control should be below the limit of quantitation for the method.
6. Ratio of the concentrations determined from the two mass transitions used in the method should be within 0.7–1.3.
7. Patient samples should not be injected if calibrators and controls are not acceptable.
8. Patient sample following injection of a sample with concentration of PP and PP3-36 greater than 100,000 pg/mL requires evaluation for carryover.
9. Calculations are performed with mass spectrometer software using peak area ratios. Calibration curve; linear regression line forced through origin; no weighting applied. Calibration is performed with every batch of samples.
10. Concentrations are determined from the quantitative transitions of PP and PP3-36. Specificity of the analysis is evaluated using ratios of concentrations determined from the primary and the secondary mass transitions.

## 4 Notes

1. Sodium azide is highly acutely toxic by all routes of exposure. Dilute solutions of sodium azide (less than 0.1 %) are used in research laboratories as a biocide presenting no extraordinary dangers to the user, but at those concentrations they are eye and skin irritants. Any waste containing sodium azide may react with lead and copper plumbing to form potentially explosive metal oxides, dispose flushing with at least 100-fold excess of water to prevent azide build-up in the sewer. Federal, State, and Local laws related with disposal of Hazardous Materials should be reviewed.
2. Peptide stock aliquots are stable up to 6 months at −70 °C, 24 h at RT and 48 h at 4 °C; they can bear only one freeze-thaw cycle.
3. Concentration of peptide in the stock solution will be determined by purity and free peptide content reported by provider (e.g., if 0.5 mg of peptide were provided in a vial with reported purity of 95 % and 60 % free peptide content, the stock solution prepared by this protocol (ng/μL) will be: (500 μg × 0.95 × 0.6)/1000 μL = 0.285 μg/μL = 285 ng/μL).
4. Once PP and PP3-36 stock solution are prepared, convert units from ng/μL to pg/μL by multiplying the concentration by 1000. If *X* is the stock concentration in pg/μL, prepare a working solution with 2500 pg/μL following this protocol for 5 μL of the stock standard:
   (a) Calculate volume of 0.005 % BSA (*D*) solution needed: $D = X/2500$.
   (b) Volume of 0.005 % BSA = $D \times 5 - 5$ (μL). Add 5 μL of peptide stock solution to the 0.005 % BSA calculated (*D*).
   (c) Example: Peptide stock solution with 285 ng/μL equal to 285,000 pg/μL:
      - $D = 285{,}000/2500 = 114$
      - Volume of 0.005 % BSA needed = $114 \times 5 - 5 = 565$ μL
      - Add 5 μL of 285 ng/μL peptide stock to 565 μL of 0.005 % BSA to obtain a 2500 pg/μL working solution.
5. With Internal Standard IS also convert units for stock solution from ng/μL to pg/μL by multiplying the concentration by 1000. If *X* is the IS stock concentration in pg/μL, prepare a 1000 pg/μL working solution following this protocol using 10 μL of the stock standard:
   (a) Calculate volume of 0.005 % BSA (*D*) solution needed: $D = X/1000$.

(b) Volume of 0.005 % BSA = $D \times 10 - 10$ (μL). Add 10 μL of peptide PPIS stock solution to the 0.005 % BSA calculated ($D$).

(c) Example: Peptide PPIS stock solution with 150 ng/μL equal to 150,000 pg/μL. $D = 150{,}000/1000 = 150$.

- Volume of 0.005 % BSA needed = $150 \times 10 - 10 = 1490$ μL
- Add 10 μL of 150 ng/μL PPIS stock to 1490 μL of 0.005 % BSA to obtain 1000 pg/μL PPIS solution.

6. The mass spectrometry signal is affected by tris base compound, so a dilution reduces its presence before eluting the peptides with acid.
7. Preparation of peptide standards, calibrators, controls, and samples involves some grade of complexity. An automation is desirable to improve the work and performance into the laboratory.
8. Use internal standard MRM transition ISPP 957 (IS1) to calculate PP 953 (PP1, quantifier ion). Use internal standard MRM transition ISPP2 418 (IS2) to calculate PP 411 (PP2, qualifier ion). Use internal standard MRM transition IS1 to calculate both PP3-36 (quantifier and qualifier ion).

## References


1. Tseng PH, Wu MS, Kao JH (2010) Recent advances in clinical application of gut hormones. J Formos Med Assoc 109:859–861
2. Escobar H, Kushnir MM, Ray JA, Merrell MA, Gomez G, Fietkau R, Alan L, Rockwood AL, Meikle WA (2014) Measurement of pancreatic polypeptide and its peptide variant in human serum and plasma by immunocapture-liquid-chromatography-tandem mass spectrometry. Reference intervals and practical assay considerations. Biochem Physiol 3:140
3. Pappas TN, Yovos JG, Ellison EC, O'Dorisio TM, Cataland WG, Pace WG, Carey LC (1981) Pancreatic polypeptide in acute pancreatitis and small-bowel infarction in dogs. Dig Dis Sci 26:1013–1018
4. Adrian TE, Uttenthal LO, Williams SJ, Bloom SR (1986) Secretion of pancreatic polypeptide in patients with pancreatic endocrine tumors. N Engl J Med 315:287–291
5. Lamberts SW, Hofland LJ, Nobels FR (2001) Neuroendocrine tumor markers. Front Neuroendocrinol 22:309–339
6. Ardill JES, O'Dorisio TM (2010) Circulating biomarkers in neuroendocrine tumors of the enteropancreatic tract: application to diagnosis, monitoring disease, and as prognostic indicators. Endocrinol Metab Clin North Am 40:777–790
7. Soares HD, Potter WZ, Pickering E, Kuhn M, Immermann FW, Shera DM, Ferm M, Dean RA, Simon AJ, Swenson F, Siuciak JA, Kaplow J, Thambisetty M, Zagouras P, Koroshetz WJ, Wan HI, Trojanowski JQ, Shaw LM (2012) Plasma biomarkers associated with the apolipoprotein E genotype and Alzheimer disease. Arch Neurol 69: 1310–1317
8. Henriksen K, O'Bryant SE, Hampel H, Trojanowski JQ, Montine TJ, Jeromin A, Blennow K, Lönneborg A, Wyss-Coray T, Soares H, Bazenet C, Sjögren M, Hu W, Lovestone S, Karsdal MA, Weiner MW (2013) Blood-based biomarker: the future of blood-based biomarkers for Alzheimer's disease. Alzheimers Dement 10:115–131

# Chapter 22

# Quantitation of Parathyroid Hormone in Serum or Plasma by Liquid Chromatography-Tandem Mass Spectrometry

**Hemamalini Ketha and Ravinder J. Singh**

## Abstract

Parathyroid hormone (PTH), an 84 amino acid peptide hormone, is an important regulator of calcium homeostasis. Quantitation of PTH in serum is useful for the diagnosis of primary hyperparathyroidism, hypoparathyroidism, and for monitoring osteodystrophy in patients with renal failure. The biological activity of PTH arises from binding of PTH (N terminus) to its target receptor (D'Amour et al., Kidney Int 68: 998–1007, 2005). Several C-terminal and N-terminal fragments circulate in normal subjects. Recent studies have demonstrated that accurate quantitation of PTH fragments may be of clinical value. In this chapter a mass spectrometry based method for quantitation of PTH(1–84) is described. This method involves immunoaffinity capture of PTH followed by trypsinization and quantitation of PTH-specific tryptic peptides by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The N-terminal tryptic peptide, PTH(1–13) as surrogate of 1–84 PTH, is used for quantitation.

**Key words** LC-MS/MS, Parathyroid hormone quantitation, Immunocapture LC-MS/MS

## 1 Introduction

Parathyroid hormone (PTH) is an 84 amino acid peptide hormone secreted by the parathyroid gland. The major physiological role of PTH is to regulate calcium homeostasis in the body. The binding of PTH to its target receptor is responsible for its biological activity [1, 2]. The clinical utility of serum PTH quantitation lies in its ability to diagnose a wide spectrum of diseases including primary hyperparathyroidism, hypoparathyroidism and for monitoring osteodystrophy in patients with renal failure. In a normal subject, in addition to intact PTH, several PTH fragments are found in circulation. These include C-terminal fragments (70–95 %) and N-terminal fragments (~5 %). PTH fragments including PTH(34–84) have been shown to play important role in calcium homeostasis and bone physiology [3]. It has been shown that the PTH (fragments) to PTH(1–84) ratio is useful to differentiate between hyperparathyroid-associated bone loss and non-dynamic bone disease [4–6].

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_22, 

PTH was first quantitated using a radioimmunoassay (RIA) [7, 8]. The epitopes towards which the antibodies were directed were present on the mid- or carboxyl-terminal portion of the PTH molecule. The RIAs for PTH have been termed as the first-generation PTH assays. Then second-generation immunoassays involving two antibodies measuring PTH(1–84) and other C-terminal fragments were introduced for PTH analysis [9]. The oldest assays were less specific and were incapable of differentiating between various PTH fragments. The sensitivity and specificity of the later generation PTH immunoassays (second and third generation) to distinguish between various PTH fragments has improved [10].

In this chapter we describe an immunoaffinity purification coupled liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for serum PTH(1–84) quantitation [11]. The antibodies are immobilized on polystyrene beads and treated with serum and $^{15}$N labeled recombinant PTH ($^{15}$NPTH) internal standard (IS). The immobilized antibodies bind PTH containing the 44–84 amino acid portion of the molecule. Then, the beads are washed to remove nonspecific material, PTH bound to the antibody digested by trypsin followed by LC-MS/MS analysis on a triple quadrupole mass spectrometer. The N-terminal tryptic peptide PTH(1–13) and the $^{15}$N labeled recombinant tryptic peptide $^{15}$NPTH(1–13) from the PTH IS are monitored using a multiple reaction monitoring (MRM) method. Quantification is performed using calibrators made from certified recombinant PTH(1–84) spiked into stripped serum along with the $^{15}$N labeled recombinant PTH. A linear calibration standard curve constructed from the peak area ratio (sample/IS) is used to calculate the concentration of the sample.

This method can be useful for quantification of PTH in samples where a PTH value cannot be determined with an immunoassay due to an interference with various antibodies. The assay can be of clinical value where knowing the accurate concentration of N-terminal PTH will aid in deciding treatment options.

## 2 Materials

### 2.1 Samples

Serum or plasma samples are acceptable sample types for this method. Samples have to be maintained frozen prior to analysis and up to three freeze-thaw cycles are acceptable. Samples that are refrigerated or those maintained at room temperature are unacceptable.

### 2.2 Reagents and Buffers

1. 50 mM Ammonium Bicarbonate, pH 8.0 (stable for 6 months at room temperature).
2. 1 mg/mL Trypsin stock (Sigma Aldrich). Store at −80 °C for 2 years.

3. Reaction Stopping Reagent: Add 100 μL of formic acid to 10 mL 50 mM Ammonium Bicarbonate and mix thoroughly (stable for 1 month at room temperature).
4. Phosphate Buffered Saline (PBS) (Roche). Store at room temperature. Expiration: until date printed on box. Dilute appropriately with water (stable at room temperature for 3 months).
5. Mobile Phase A: 100 % water, 0.1 % formic acid. Stable at room temperature for 1 month.
6. Mobile Phase B: 100 % methanol, 0.1 % formic acid. Stable at room temperature for 1 month.
7. Stripped Serum Seracon II stripped (SeraCare Life Sciences). For use in blanks and working standard preparation. Store frozen at −20 °C.
8. Standard Diluent (1 % formic acid in water). Store at room temperature for 2 months.
9. Internal standard diluent: 95 % water, 5 % acetonitrile, 0.1 % formic acid.
10. Standards and Calibrators: PTH stock standard (PTH(1–84)) (NIBSC # 95/646);$^{15}$N labeled PTH ($^{15}$NPTH) as internal standard (IS).

### 2.3 Calibrators, Internal Standard, and Quality Control Samples

1. Calibrators ranging in concentration from 0 to 2000 pg/mL are prepared by diluting the NIBSC PTH(1–84) standard material into charcoal-stripped human serum. Prepare a 1 mg/mL stock standard and a 50 ng/mL intermediate stock to use for preparing working calibrators (Table 1). Keep frozen at −20 °C until needed.
2. Dissolve 500 μg $^{15}$NPTH in 1 mL internal standard diluent to make a high stock concentration of 500 μg/mL. (Aliquot and store unused high stock at −80 °C.) Dilute the 500 μg/mL

**Table 1**
**Dilution scheme for preparing PTH(1–84) calibrator samples**

| Calibrator concentration pg/mL | Intermediate 50 ng/mL std (mL) | Stripped serum (mL) |
|---|---|---|
| 0 | 0 | 250 |
| 50 | 0.25 | 249.75 |
| 100 | 0.50 | 249.5 |
| 300 | 1.50 | 248.5 |
| 600 | 3.0 | 247.0 |
| 1000 | 5.0 | 245.0 |
| 2000 | 10.0 | 240.0 |

appropriately to achieve a final concentration of 10 ng/mL in 500 mL charcoal-stripped serum. Store at −80 °C for 2 years.

3. Three levels of quality control samples (Low: 50–100 pg/mL; Medium: 150–200 pg/mL; High: 450–550 pg/mL) are prepared by pooling patient samples at appropriate concentrations. Samples can be aliquoted and stored at −80 °C for 2 years.

### 2.4 Supplies and Equipment

1. Transfer pipettes, vortex and microtiter plate shaker.
2. Incubator shaker that can heat to 37 °C and can shake 250 rpm.
3. Positive pressure manifold capable of handling a 96 well plate.
4. Analytical column: Higgins Analytica, TARGA C18, size 50 × 3.0 mm, 5 μm particle size (Chromtech).
5. IMMULITE™ ¼ inch diameter polystyrene beads, IMMULITE™ 2000 PTH assay (Siemens L2KPP6).
6. Isolute PPT+ filter plate, 96-well (Biotage).
7. Thermo-Cohesive HPLC system (Thermo Scientific).
8. Applied Biosystems API 5000 triple quadrupole mass spectrometer.

## 3 Method

### 3.1 Sample Preparation

1. Thaw the patient samples, calibrators, controls, blanks, and IS ($^{15}$NPTH) on ice and allow PTH antibody beads to reach room temperature.
2. Transfer 1 PTH antibody bead per sample into each well of the 96 well filter plate (Fig. 1).

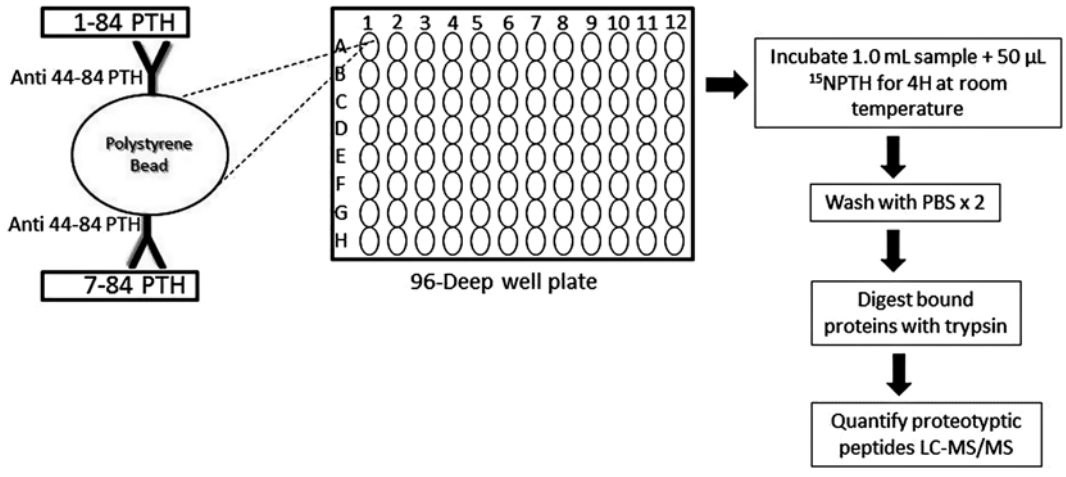



**Fig. 1** Work flow for quantifying PTH(1–84) in serum and plasma

3. Pipette 1.0 mL of each of patient samples, calibrators, controls or blanks into individual well of the filter plate.
4. Add 50 µL IS ($^{15}$NPTH) into each well. Cover the plate with an adhesive plate seal and incubate at ambient temperature on the plate shaker for 4 h with constant shaking.
5. Push serum waste into reservoir by placing the plate on a positive pressure manifold and applying pressure. Blot bottom of plate on paper towels to remove residual liquid.
6. Wash the contents of the 96 well plate twice with 1–2 mL PBS. Allow all the liquid to pass through the filter plate using positive pressure manifold between washes.
7. Add 300 µL trypsin buffer (1 µg in 300 µL 50 mM ammonium bicarbonate) to each well.
8. Cover and seal the plate and incubate at 37 °C on a shaker at 270 rpm for 30 min.
9. Add 30 µL of reaction stopping reagent to each well and incubate the filter plate with shaking at ambient temperature for 5 min on a plate shaker.
10. Place the filter plate over a 96 deep-well collection plate and push the liquid into the 96 deep-well plate using the positive pressure manifold.
11. Seal the top of the 96 deep-well plate and inject supernatant onto the LC system. The prepared samples can be stored in a refrigerator until analyzed.

### 3.2 HPLC Conditions

1. Mobile phase A: 100 % water, 0.1 % formic acid; mobile phase B: 100 % methanol, 0.1 % formic acid (*see* **Note 1**).
2. Flow rate: 250 µL/min.
3. The injected extract is separated on a C18 column, size 50 × 3.0 mm, 5 µm analytical column. Start the gradient at 20 % mobile phase B held for 2 min before ramping to 35 % B (4 min), 42 % B (6 min), 95 % B (2 min), 20 % B (1 min). Then the gradient is held at 20 % mobile phase B for 5 min before the next injection.

### 3.3 Mass Spectrometer Conditions (See Notes 2 and 3) (Table 2)

1. Data is quantitated using the Analyst™ Software (version 1.4.1 or higher) (Applied Biosystems).

### 3.4 Data Analysis and Quantitation

2. Calibration curves are established for every day of analytical run based on linear fit regression of IS peak area/IS concentration (*x*-axis) vs. analyte concentration/IS peak area (*y* axis). Peak area ratios are then used to calculate the analyte concentration by the software.
3. Area of Q1/Q3 pair 486/635 is used for quantitation and 492/693 is used as the IS.

**Table 2**
**MRMs for PTH and internal standard**

| Ion | Q1 Mass (amu) | Q3 Mass (amu) | Time (ms) |
|---|---|---|---|
| 1–13 PTH analyte | 486.200 | 635.400 | 100 |
| 1–13 PTH IS | 492.200 | 643.700 | 100 |

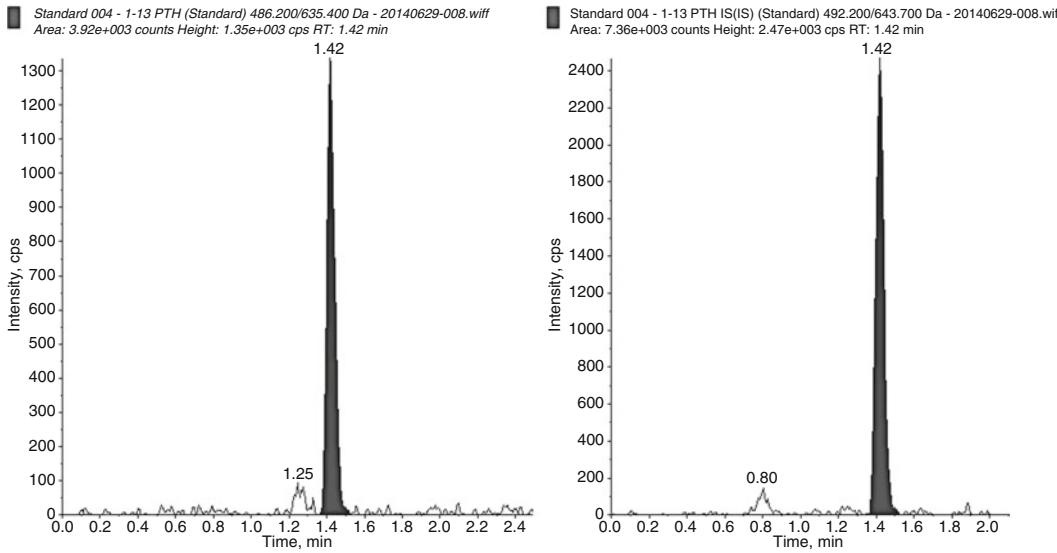



**Fig. 2** Representative chromatograph for PTH(1–13) standard (486.2/635) and $^{15}$NPTH(1–13) IS (492.2/643.7)

4. Calibration curve is acceptable if correlation coefficient is ≥0.99. If one of the calibrators is out of range by greater than ±20 % of expected value then the curve may be plotted after eliminating the calibrator. The lowest and the highest calibrator may not be removed as it will affect the LOQ and reportable range.
5. QC values are acceptable if found to be with ±2SD of the established mean for the lot.
6. The chromatographic retention times are monitored closely (*see* **Notes 4** and **5**). A ±0.03 min shift in retention time on each LC system is considered acceptable.
7. A typical ion extract chromatogram is shown in Fig. 2.

## 4 Notes

1. Acetonitrile will also work and is always good practice to optimize the signal to noise ratio after trying with various gradient ratios.
2. Mass spectrometer source and lens parameters need to be optimized on every instrument as they differ between instruments

of even the same manufacturer. MS/MS parameters can be optimized using the synthetic PTH(1–13) peptide by infusing a peptide solution through a "tee" into a 250 µL/min flow stream consisting of 40 % mobile phase A.

3. Ion suppression on the instrument can be assessed as follows. Connect a syringe pump via a tee to the column effluent and infuse synthetic PTH(1–13) peptide directly into the electrospray source until a constant response is obtained. Then a stripped serum blank and a pooled serum control extracted and prepared after complete sample work-up is injected onto the column to observe the effect of matrix suppression on the response for the continuously infused PTH(1–13).
4. The PTH fragment 1–44 is known to interfere with this assay at levels as low as 500 pg/mL. The significance and presence of this fragment is not fully understood.
5. The PTH fragment 7–84 is known to interfere in immunoassays but the significance and presence of this fragment in circulation is not fully understood [11].

## References


1. D'Amour P, Brossard JH, Rousseau L, Nguyen-Yamamoto L, Nassif E, Lazure C, Gauthier D, Lavigne JR, Zahradnik RJ (2005) Structure of non-(1–84) PTH fragments secreted by parathyroid glands in primary and secondary hyperparathyroidism. Kidney Int 68:998–1007
2. Murray TM, Rao LG, Divieti P, Bringhurst FR (2005) Parathyroid hormone secretion and action: evidence for discrete receptors for the carboxyl-terminal region and related biological actions of carboxyl-terminal ligands. Endocr Rev 26:78–113
3. Zhang CX, Weber BV, Thammavong J, Grover TA, Wells DS (2006) Identification of carboxyl-terminal peptide fragments of parathyroid hormone in human plasma at low-picomolar levels by mass spectrometry. Anal Chem 78:1636–1643
4. Malluche HH, Mawad H, Trueba D, Monier-Faugere MC (2003) Parathyroid hormone assays—evolution and revolutions in the care of dialysis patients. Clin Nephrol 59:313–318
5. Goodman WG, Juppner H, Salusky IB, Sherrard DJ (2003) Parathyroid hormone (PTH), PTH-derived peptides, and new PTH assays in renal osteodystrophy. Kidney Int 63:1–11
6. Goodman WG (2003) New assays for parathyroid hormone (PTH) and the relevance of PTH fragments in renal failure. Kidney Int Suppl S120–S124
7. Berson SA, Yalow RS, Aurbach GD, Potts JT (1963) Immunoassay of bovine and human parathyroid hormone. Proc Natl Acad Sci U S A 49:613–617
8. Berson SA, Yalow RS (2006) General principles of radioimmunoassay. 1968. Clin Chim Acta 369:125–143
9. Silverberg SJ, Gao P, Brown I, LoGerfo P, Cantor TL, Bilezikian JP (2003) Clinical utility of an immunoradiometric assay for parathyroid hormone (1–84) in primary hyperparathyroidism. J Clin Endocrinol Metab 88:4725–4730
10. Terry AH, Orrock J, Meikle AW (2003) Comparison of two third-generation parathyroid hormone assays. Clin Chem 49:336–337
11. Kumar V, Barnidge DR, Chen LS, Twentyman JM, Cradic KW, Grebe SK, Singh RJ (2010) Quantification of serum 1–84 parathyroid hormone in patients with hyperparathyroidism by immunocapture in situ digestion liquid chromatography-tandem mass spectrometry. Clin Chem 56:306–313

# Chapter 23

# Determination of Phenylalanine and Tyrosine by High Performance Liquid Chromatography-Tandem Mass Spectrometry

**Judy Peat and Uttam Garg**

## Abstract

Hyperphenylalaninemia/phenylketonuria (PKU) is one of the most common inborn errors of amino acid metabolism affecting about 1:15,000 infants in the United States. PKU is an autosomal recessive disorder that if untreated results in mental retardation. The most common cause of PKU is deficiency of the enzyme phenylalanine hydroxylase that converts phenylalanine to tyrosine. Tyrosine deficiency results in impaired synthesis of catecholamines and thyroxine. Less commonly, it can result from defects in the synthesis or regeneration of tetrahydrobiopterin (BH4), an essential cofactor for the enzyme phenylalanine hydroxylase. Increased phenylalanine and decreased tyrosine in blood are used in the diagnosis and follow-up of patients with PKU. LC/MS/MS method is described for the quantification of phenylalanine and tyrosine.

**Key words** Hyperphenylalaninemia, Phenylketonuria, PKU, Phenylalanine, Tyrosine, Tandem mass spectrometry, Tetrahydrobiopterin, Phenylalanine hydroxylase

## 1 Introduction

Phenylketonuria (PKU) is one of the most common inborn errors of amino acid metabolism with frequency of 1:15,000 in the United States. The most common cause of PKU is deficiency of the enzyme phenylalanine hydroxylase that converts phenylalanine to tyrosine [1, 2]. This enzyme needs tetrahydrobiopterin (BH4) as an essential cofactor. Defects in the synthesis or regeneration of BH4 may also result in PKU. Infants with PKU generally appear normal at birth and show clinical signs of PKU within few months after birth. The primary phenotype of untreated PKU is mental retardation. It may also be accompanied by seizures, dystonia, irritability, microcephaly, and cardiac malformations [1, 2]. The exact mechanism and pathogenesis of PKU is not known. But it is thought that phenylalanine and its metabolites are toxic to brain. Pathogenesis may also be related to competitive inhibition by phenylalanine of neutral amino acid transport across the blood–brain

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_23, 

barrier. This results in deficiency of tyrosine that is a precursor for synthesis of catecholamines [1–5].

Most of the newborns with PKU are detected through newborn screening by elevated phenylalanine. Confirmation is performed by elevated plasma phenylalanine (>120 μmol/L) and decreased tyrosine, and elevation of the phenylalanine/tyrosine ratio (>3). Increased excretion of phenylalanine metabolites phenylpyruvic, phenyllactic, and phenylacetic acids is also seen in urine organic acids analysis. Although phenylalanine and tyrosine can be measured by spectrophotometric or fluorometric assays, chromatographic methods particularly LC/MS/MS are preferred [6–9]. The levels are measured for the diagnosis and follow-up of patients with PKU. Since phenylalanine is an essential amino acid, its frequent measurement, particularly in growing children, is needed for optimal growth and clinical outcome.

## 2 Materials

### 2.1 Samples

Serum or heparinized plasma.

### 2.2 Reagents and Solvents

1. Bovine Serum Albumin (Sigma).
2. Heptafluorobutyric acid (Sigma).
3. 2.3 M ammonium hydroxide—Pipette 15.8 mL concentrated ammonium hydroxide into a 100 mL volumetric flask containing about 25 mL HPLC water. Mix and qs to 100 mL with HPLC water.
4. Phosphate Buffered Saline System (Sigma PBSS): The system includes the following reagents: 1 M potassium phosphate (monobasic), 1 M potassium phosphate (dibasic), 5 M sodium chloride.
5. Working phosphate buffered saline (PBS): Add 7.6 mL of 1 M potassium phosphate (monobasic), 42.4 mL of 1 M potassium phosphate (dibasic), and 30 mL 5 M NaCl to a 1 L volumetric flask. QS to 1 L with HPLC water. Confirm that the pH is $7.4 \pm 0.1$. Stable for 6 months when stored at room temperature.
6. 7 % bovine serum albumin standard buffer (pH 7.4): In a 100 mL volumetric flask, dissolve 7 g of bovine serum albumin using 80 mL working PBS (phosphate buffered saline). QS to 100 mL with PBS. Store at 2–8 °C. Stable for 1 year.
7. Mobile phase A (0.1 % heptafluorobutyric acid, HBA, in water): To 1 L of HPLC grade water add 1 mL HBA. Mix and degas. Store at room temperature. Stable for 6 months.
8. Mobile phase B (0.1 % heptafluorobutyric acid, HBA, in acetonitrile): To 1 L of HPLC grade acetonitrile add 1 mL HBA. Mix and degas. Store at room temperature. Stable 6 months.

### 2.3 Standards and Internal Standards

1. Primary (1°) Standards (1 mg/mL):
   (a) Phenylalanine: Weigh 10 mg of phenylalanine (Sigma) and add to a 10 mL volumetric flask. Add approximately 8 mL of HPLC water. Sonicate a few minutes until dissolved then qs to 10 mL with HPLC water. Stable for 1 year when stored at –20 °C.
   (b) Tyrosine: Weigh 10 mg of tyrosine (Sigma) and add to a 10 mL volumetric flask. Dissolve with approximately 1 mL 2.3 M ammonium hydroxide. QS to 10 mL with HPLC water for a 1 mg/mL solution. Stable for 1 year when stored at –20 °C.
2. Secondary (2°) Standards (100 μg/mL):
   (a) Dilute the primary phenylalanine standard 1:10 with HPLC water. Stable for 1 year when stored at –20 °C.
   (b) Dilute the primary tyrosine standard 1:10 with HPLC water. Stable for 1 year when stored at –20 °C.
3. Primary Internal Standards (1 mg/mL):
   (a) Phenylalanine $^{13}C_6$: Weigh 10 mg of phenylalanine $^{13}C_6$ (Cambridge Isotope Laboratories) and add to a 10 mL volumetric flask. Add approximately 8 mL HPLC water. Sonicate for few minutes until dissolved, then qs to 10 mL with HPLC water. Stable for 1 year when stored at –20 °C.
   (b) Tyrosine $D_4$: Weigh 10 mg tyrosine $D_4$ (Cambridge Isotope Laboratories) and add to a 10 mL volumetric flask. Dissolve with approximately 1 mL 2.3 M ammonium hydroxide. QS to 10 mL with HPLC water. Stable for 1 year when stored at –20 °C.
4. Working internal standard reagent: In a 100 mL volumetric flask add approximately 80 mL methanol, 1.0 mL of primary phenylalanine $^{13}C_6$, and 0.1 mL primary tyrosine $D_4$. QS to 100 mL with methanol. Stable for 1 year at –20 °C.

### 2.4 Calibrators and Controls

1. Calibrators: Prepare calibrators 1–7 according to Table 1.
2. Quality controls:
   (a) BIORAD Liquichek TDM Levels 1 and 2. Tyrosine is added to Biorad 1 for a target concentration of 0.5 mg/dL and to Biorad 2 for a target concentration of 2.5 mg/dL. Ranges for phenylalanine and tyrosine are established in-house.
   (b) In-house control spiked with phenylalanine (target: 25 mg/dL) and tyrosine (target: 7.5 mg/dL) in 7 % bovine serum albumin. Ranges established in-house.

   Store calibrators and controls at –20 °C to –70 °C. Stable for 6 months.

**Table 1**
**Preparation of calibrators in 7 % bovine serum albumin (BSA)**

| 7 % BSA (mL) | 1° Standard (μL) PHE/TYR | 2° Standard (μL) PHE/TYR | Calibrator (mg/dL) PHE/TYR |
|---|---|---|---|
| 0.940 | –/– | 50/10 | 0.5/0.1 |
| 0.930 | 20/– | –/50 | 2.0/0.5 |
| 0.850 | 50/– | –/100 | 5.0/1.0 |
| 0.880 | 100/20 | –/– | 10.0/2.0 |
| 0.750 | 200/50 | –/– | 20.0/5.0 |
| 0.600 | 300/100 | –/– | 30.0/10.0 |

### *2.5 Analytical Equipment and Supplies*

1. AB Sciex LC/MS/MS 4000Q TRAP with Shimadzu HPLC.
2. Analytical column: Supelcosil, LC-18, 5 cm × 4.6 mm, 5 μm (Supelco).
3. Turbovap LV (Biotage).
4. Microcentrifuge tubes, 1.5 mL graduated tube with flat cap (Fisher Scientific).
5. 13 × 100 mm screw-cap test tubes.
6. Autosampler vials with glass inserts and crimp caps (P.J. Cobert Associates).

## 3 Methods

### *3.1 Stepwise Procedure*

1. Pipette 20 μL sample, calibrator, blank, or control into a labeled microcentrifuge tube.
2. Add 200 μL working internal standard reagent to all tubes.
3. Vortex the tubes and let stand for 5 min.
4. Centrifuge the tubes for 5 min at 10,000 × *g*.
5. Remove 150 μL supernatant from each tube and transfer the extract to a Turbovap tube. Dry the extract for 2 min at 40 °C.
6. Reconstitute the residue with 400 μL mobile phase mixture (80 % HPLC water:20 % ACN:0.1 % HBA).
7. Transfer to labeled autosampler vials.
8. Inject 30 μL on LC/MS/MS for analysis.

### *3.2 Instrument Operating Conditions*

The instrument's operating conditions are given in Tables 2 and 3.

**Table 2**
**HPLC gradient programming and operating conditions**

| Time (min) | Mobile phase A | Mobile phase B |
|---|---|---|
| 0.10 | 80 | 20 |
| 3.00 | 40 | 60 |
| 3.10 | 10 | 90 |
| 5.00 | 10 | 90 |
| 6.00 | 80 | 20 |
| 8.00 | Stop | |

Flow rate: 0.60 mL/min; Column temperature: 40 °C

**Table 3**
**MS/MS parameters and operating conditions**

| Q1 Mass | Q3 Mass | Dwell (ms) | DP | CE | CXP | Compounds |
|---|---|---|---|---|---|---|
| 166.1 | 120.0 | 100 | 86 | 17 | 10 | PHE-1(quant ion) |
| 166.1 | 102.9 | 100 | 86 | 37 | 18 | PHE-2 (qual ion) |
| 172.2 | 126.0 | 100 | 111 | 17 | 10 | PHE-$^{13}C_6$ |
| 182.1 | 165.0 | 200 | 86 | 13 | 12 | TYR-1(quant ion) |
| 182.1 | 136.1 | 200 | 86 | 19 | 10 | TYR-2 (qual ion) |
| 186.1 | 169.1 | 200 | 81 | 16 | 14 | TYR-$D_4$ |

Scan type: MRM; Polarity: Positive; Ion Source: Turbo Spray; Resolution Q1 and Q3: unit; CUR: 30.00; TEM: 400.00; GS1: 45.00; GS2: 40.00; ihe: ON; CAD: Medium; IS: 4000.00; EP: 10.00

### 3.3 Data Analysis

1. Data are analyzed using Analyst software (AB Sciex).
2. Standard curves are generated based on linear regression of the analyte/IS peak area ratio ($y$) versus analyte concentration ($x$) using the quantifying ion listed in Table 3 (*see* **Note 1**).
3. Typical coefficient of correlation is >0.99.
4. Typical ion chromatogram is shown in Fig. 1.
5. Runs are accepted if calculated controls concentrations fall within two standard deviations of the target values.

## 4 Note

1. Two measurement units (mg/dL and μmol/L) are frequently used. Conversion of μmol/L to mg/dL can be done using following formulas

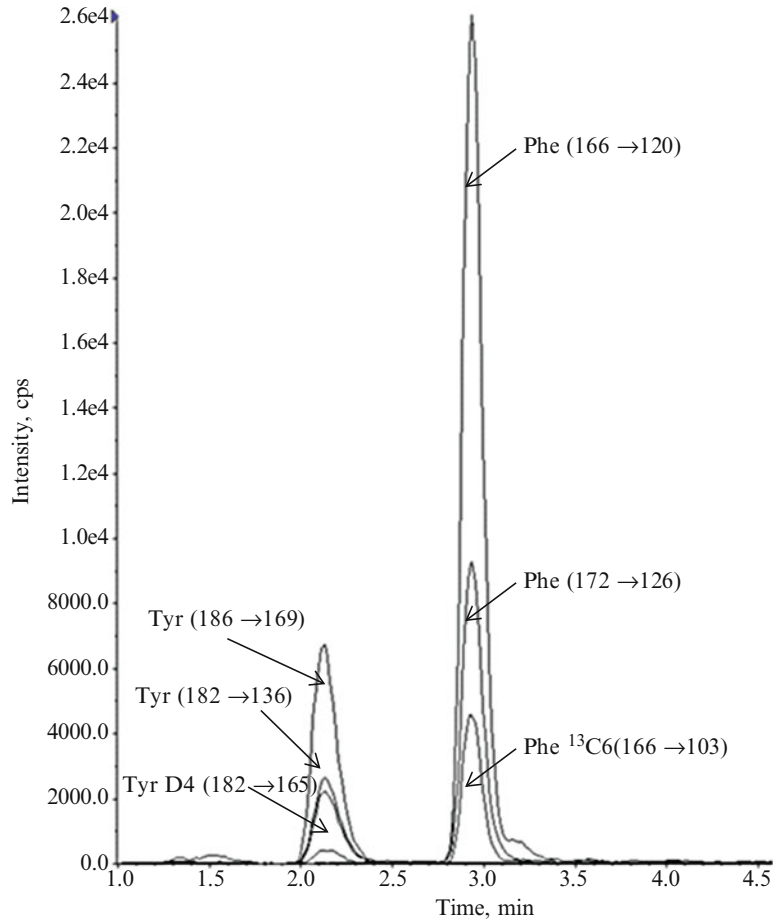



**Fig. 1** Ion chromatogram for tyrosine and phenylalanine

(a) Phenylalanine: μmol/L = mg/dL × 60.5

(b) Tyrosine: μmol/L = mg/dL × 55.2.

## References

1. Blaskovics ME, Schaeffler GE, Hack S (1974) Phenylalaninaemia. Differential diagnosis. Arch Dis Child 49:835–843
2. Blau N, Thony B, Spada M, Ponzone A (1996) Tetrahydrobiopterin and inherited hyperphenylalaninemias. Turk J Pediatr 38:19–35
3. Blau N (2008) Defining tetrahydrobiopterin (BH4)-responsiveness in PKU. J Inherit Metab Dis 31:2–3
4. Koch R, Moats R, Guttler F, Guldberg P, Nelson M Jr (2000) Blood–brain phenylalanine relationships in persons with phenylketonuria. Pediatrics 106:1093–1096
5. Steinfeld R, Kohlschutter A, Ullrich K, Lukacs Z (2003) A hypothesis on the biochemical mechanism of BH(4)-responsiveness in phenylalanine hydroxylase deficiency. Amino Acids 25:63–68
6. Chace DH, Millington DS, Terada N, Kahler SG, Roe CR, Hofman LF (1993) Rapid diagnosis of phenylketonuria by quantitative analysis for phenylalanine and tyrosine in neonatal blood spots by tandem mass spectrometry. Clin Chem 39:66–71

7. De Silva V, Oldham CD, May SW (2010) L-Phenylalanine concentration in blood of phenylketonuria patients: a modified enzyme colorimetric assay compared with amino acid analysis, tandem mass spectrometry, and HPLC methods. Clin Chem Lab Med 48:1271–1279
8. Hardy DT, Hall SK, Preece MA, Green A (2002) Quantitative determination of plasma phenylalanine and tyrosine by electrospray ionization tandem mass spectrometry. Ann Clin Biochem 39:73–75
9. Lee H, Park S, Lee G (2006) Determination of phenylalanine in human serum by isotope dilution liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 20: 1913–1917

# Chapter 24

# Urine Purine Metabolite Determination by UPLC-Tandem Mass Spectrometry

Qin Sun

## Abstract

Inborn errors of purine metabolism, either deficiencies of synthesis or catabolism pathways, lead to a wide spectrum of clinical presentations: urolithiasis (adenine phosphoribosyltransferase), primary immune deficiency (adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency), severe intellectual disability, and other neurological symptoms (Lesch-Nyhan disease, adenylosuccinase deficiency, and molybdenum cofactor deficiency). A rapid quantitative purine assay was developed using UPLC-MS/MS to determine purine nucleoside and base concentrations in urine. Taking advantages of ultra performance liquid chromatography, we achieved satisfactory analyte separation and recovery with a polar T3 column in a short run time with no requirement of time-consuming sample preparation or derivatization. This targeted assay is intended for diagnosis and management of purine diseases, newborn screening follow-up of SCID, and evaluation of autism spectrum disorders.

**Key words** Purine, Adenine, Adenosine, Xanthine, Hypoxanthine, Inosine, Guanine, Succinyladenosine, Mass spectrometry, UPLC

## 1 Introduction

Purine metabolism involves nucleotide biosynthesis (de novo or salvage pathways) and degradations. The de novo multistep pathway uses precursors of glycine, glutamine, and $CO_2$ to synthesize the nucleotide, inosine monophosphate (IMP), which later can be interconverted to adenosine monophosphate (AMP) and guanosine monophosphate (GMP). Energy expensive nucleosides (inosine, adenosine, guanosine) and bases (xanthine, hypoxanthine, adenine, guanine) are salvaged, by two phosphoribosyltransferases, to corresponding nucleotides. Collectively with deoxynucleotides, they provide key building blocks for DNA, RNA, and many coenzymes. They also play crucial roles in cellular signal transduction, energy conservation, and transport. On the other hand, catabolism of purine nucleotides produces uric acid which is excreted as end product in urine. Intracellular concentrations of purine are

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_24, 

balanced as results of catabolic and anabolic processes. Although fluctuation of purine intermediates and uric acid is expected due to diet and exercises, significant abnormalities of urinary excretions are pathognomonic biomarkers in purine metabolism disorders [1, 2].

Inborn errors of purine metabolism present with a broad spectrum of clinical phenotypes. Multiple tissues can be affected with immunological, hematological, neurological, and renal symptoms. Among purine disorders, hypoxanthine guanine phosphoribosyltransferase 1 (HGPRT) deficiency is the most prevalent one. While severe Lesch-Nyhan syndrome (OMIM 300322) is characterized by cerebral palsy-like motor dysfunction and self-injurious behavior, mild variation of the defect in the same HGPRT enzyme (OMIM 300323) results in gout [3, 4]. The similar phenotype variations are common in other purine diseases. Adenylosuccinase deficiency (ADSL, OMIM 103050) shows encephalopathy, seizure, and hypotonia. Mild ADSL cases may be associated with autism spectrum behaviors. Adenosine deaminase (ADA, OMIM 102700) and purine nucleoside phosphorylase (PNP, OMIM 613179) deficiencies are two of congenital disorders causing Severe Combined Immunodeficiency (SCID). Adenine Phosphoribosyltransferase (APRT, OMIM 614723) deficiency and Xanthinuria (OMIM 278300) present predominantly with urolithiasis. The list of purine disorders continuously expanded in the last decade. Several comprehensive reviews were recently published describing in detail the clinical features of known purine deficiencies [5–10]. In 2013, the American College of Medical Genetics and Genomics (ACMG) revised the guideline for clinical genetic evaluation in identifying the etiology of autism spectrum disorders (ASD). Adenylosuccinate lyase deficiency (ADSL, OMIM 103050) was listed as one of the metabolic conditions that are associated with ASD phenotype [11, 12]. All purine diseases mentioned above are inherited as autosomal recessive with the exception of Lesch-Nyhan disease (X-linked inheritance). It was reported fewer than 1000 patients had been diagnosed with purine or pyrimidine disorders in 435 million in 18 European countries [13]. However purine deficiencies may be underdiagnosed because physicians are not familiar with the group of disorders. In addition diagnostic tests are only available in few biochemical genetics laboratories. The phenotypic complexity of purine deficiencies presents a diagnostic dilemma clinically. However if diagnosis is made earlier, effective treatments for purine deficiencies, though limited at the moment, can help patients before the onset of irreversible damages: bone marrow transplant for ADA and PNP deficiencies, enzyme replacement for ADA deficiency, cyclic pyranopterin monophosphate (cPMP) for most common molybdenum cofactor deficiency. Besides diagnostic and monitoring purposes, purine tests are also important for newborn screening follow-up. Currently more than 16 states in the United States add SCID screening to

their newborn screening programs. Purine screening can easily confirm SCID that is related to ADA or PNP deficiencies. Finally screening for both purine and pyrimidine (*see* Chapter 25) is helpful for ASD evaluation.

For laboratory diagnosis of purine disorders, both functional studies and analyte tests are available. Enzyme activities for ADA, PNP, and HGPRT can be performed in red blood cells, white blood cells, and skin fibroblast cultured cells. Purine metabolites are mostly determined in urine [14]. Here we describe a rapid assay using ultra performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) to quantitative three purine bases (adenine, xanthine, and hypoxanthine) and five nucleosides (adenosine, deoxyadenosine, guanosine, inosine, and succinyladenosine).

## 2 Materials

### 2.1 Samples

Collect 1.0–5.0 mL of random urine. No preservatives are required. Store the specimen frozen. Specimens may be stored for not more than 1 month. Specimens with a creatinine value of less than 0.1 mg/mL should be rejected.

### 2.2 Reagents

1. Running Buffer A & Sample Dilution Buffer: 2 mM ammonium acetate, 0.1 % Formic acid in deionized water. Store the buffer at room temperature, stable for 2 years.
2. Running Buffer B: 2 mM ammonium acetate, 0.1 % Formic acid in methanol. Store the buffer at room temperature, stable for 2 years.
3. 40 mM Tris–HCl: Mix 0.48 g Tris-base (Sigma, MO), 38 mg magnesium chloride (Sigma, MO) with 70 mL HPLC grade water. Adjust pH to 8.0 with hydrochloric acid. Adjust final volume to 100 mL with water. Store solution at room temperature, stable for 2 years.

### 2.3 Standards and Calibrators

1. Stock analyte solutions: Stock solutions of each analyte are made in running buffer A according to Table 1. Before mixture, concentrations of each analyte stock are determined by UV/VIS spectrophotometer according to their molar absorption coefficients at appropriate pH ranges and specified wavelengths. Prepare a stock solution of each of the above compounds. Store in −80 °C freezer. Stable for 2 years.
2. Standard curve mix: The single standard curve mix is made by adding each analyte to Buffer A for a final concentration of 100 μM for each analyte in a total volume of 50 mL. Vortex each stock analyte solution well prior to making the mix. Aliquot 500 μL into singly labeled microfuge tubes and store in −80 °C freezer.

**Table 1**
**Preparation of analyte stock solutions**

| Analyte | Stock solution | [Expected] | pH, Max UV wavelength | $\varepsilon_{max} \times 10^{-3}$ |
|---|---|---|---|---|
| Adenine | 30 mg in 100 mL Buffer A | 2.2 mM | pH 6–8, 260 nm | 13.3 |
| Xanthine[a] | 12 mg in 1 mL 10 N NaOH[a] | 0.79 mM | pH 2–6, 260 nm | 8.85 |
| Hypoxanthine | 70 mg in 100 mL Buffer A | 5.1 mM | pH 4–7, 260 nm | 7.90 |
| Guanosine | 24 mg in 100 mL Buffer A | 0.85 mM | pH 7, 260 nm | 11.7 |
| Inosine | 120 mg in 100 mL Buffer A | 4.47 mM | pH 3–6, 260 nm | 7.10 |
| Adenosine | 120 mg in 100 mL Buffer A | 4.49 mM | pH 7–12, 260 nm | 14.90 |
| Deoxyadenosine | 55 mg in 100 mL Buffer A | 2.04 mM | pH 2, 258 nm | 14.1 |

*Abs* absorption on UVspec
[a]Dissolve xanthine in 1–2 mL 10 N sodium hydroxide. Mix in glass test tube by shaking until xanthine is completely in solution. Add this solution to ~90 mL buffer, mix, and fill to 100 mL total volume

**Table 2**
**Standard curve construction for urine purines and succinyladenosine**

| Working sol'n | Int. std. | Sample buffer | Concentration (μM) |
|---|---|---|---|
| 5 | 10 | 95 | 5 |
| 10 | 10 | 90 | 10 |
| 25 | 10 | 75 | 25 |
| 50 | 10 | 50 | 50 |
| 75 | 10 | 25 | 75 |
| 100 | 10 | 0 | 100 |

3. Prepare working calibrators according to Table 2.
4. Prepare the following vials for the standard curve using the standard curve and internal standard mixes from the −80 °C freezer. The mixes are aliquoted into single use microfuge tubes. Thaw the standard curve and internal standard solutions to room temperature and sonicate for 2 min. Leave at room temperature during use and discard any remaining mixes as freezing/thawing degrades the analytes.
5. Succinyladenosine (2.6 mM working solution): Add 12 mg of adenylosuccinic acid (Sigma, MO) to 10 mL of 40 mM Tris–HCl buffer (pH 8.0). Add 100 U of 5′-nucleotidase (Sigma, MO). Incubate at 37 ± 1 °C overnight. Check conversion using

tandem mass. Substrate adenylosuccinate displays a 427 $m/z$ peak and succinyladenosine 382 $m/z$. Add another 100 U enzyme if conversion is not complete.

6. Quantitate the succinyladenosine concentration by diluting 200× and measuring its absorbance with the spectrophotometer. The millimolar extinction coefficient is 19.2 at 268 nm. Make an individual standard curve mix for succinyladenosine by diluting in buffer A to a final concentration of 100 μM. Aliquot 500 μL into singly labeled microfuge tubes and store in −80 °C freezer.

### 2.4 Quality Controls and Internal Standards

1. Internal Standard Mix: $^{15}N_2$-adenine (Sigma, MO), $^{13}C5$-adenosine, $^{13}C_{10}$, $^{15}N_5$-guanosine, 1,3-$^{15}N_2$ Xanthine, and $^{15}N_5$ Deoxyadenosine (Cambridge Isotope Laboratories, MA). Prepare a 200 μM solution of each of the above solutions in running buffer A.
2. Each run must contain two levels of spiked controls. Pool urines for a volume of about 100 mL. Put 50 mL each into two 50 mL conical tubes and centrifuge at 3000 × $g$ for 10 min. Transfer urine supernatants into new 50 mL conical tubes. The level of endogenous adenosine in normal urine is very low and a small concentration spike is used to create the low control range. Aliquot 1 mL to labeled microfuge tubes and store in the −80 °C with the assay reagents. Establish control ranges by running ten control samples of each.

### 2.5 Supplies and Equipment

1. Column: Atlantis T3, 5 μm particle, 2.1 × 100 mm (Waters, MA).
2. Acquity UPLC coupled with TQD Tandem Mass Spectrometer (Waters, MA).

## 3 Methods

### 3.1 Stepwise Procedure

1. Allow urine samples to thaw completely and mix thoroughly. Samples may be maintained at room temperature while processing.
2. Prepare each urine sample as follows:
   (a) Sample volume: 50 μL
   (b) Internal standard mix: 10 μL
   (c) Sample buffer: 50 μL
   Mix samples well and briefly centrifuge before loading to LCMS. Injection volume is 3 μL.
3. UPLC setting:
   (a) Flow rate: 0.75 mL/min

**Table 3**
**MRM transitions and analyte tune conditions**

| Analyte | Transition | Dwell | Cone voltage | Collision energy |
|---|---|---|---|---|
| Adenine | 133.8 > 106.8 | 0.5 s | 25 | 17 |
| Xanthine | 150.5 > 107.9 | 0.5 s | 35 | 15 |
| Hypoxanthine | 134.4 > 91.8 | 0.5 s | 30 | 15 |
| Guanosine | 281.8 > 149.8 | 0.5 s | 26 | 18 |
| Inosine | 266.7 > 134.4 | 0.5 s | 32 | 22 |
| Adenosine | 311.8 > 133.8 | 0.5 s | 18 | 16 |
| Deoxyadenosine | 295.7 > 45 | 0.5 s | 18 | 11 |
| Succinyladenosine | 381.8 > 133.8 | 0.5 s | 35 | 18 |
| $^{15}N_2$-Adenine | 135.9 > 107.9 | 0.5 s | 40 | 17 |
| $^{13}C_5$-Adenosine | 316.9 > 133.8 | 0.5 s | 19 | 14 |
| $^{13}C_{10}{}^{15}N_5$-Guanosine | 296.6 > 159.8 | 0.5 s | 31 | 19 |

   (b) Gradient: 0–0.5 min, 100 % A; 0.5–1.5 min, 100 % to 70 % A; 1.5–1.8 min, 70 % B to 100 % B; 1.8–5 min, 100 %B; 5–8 min, equilibrate with 100 %A.
   (c) Column manager temperature: 30 °C
4. TQD mass spectrometry setting:
   (a) Source: ESI+ and ESI– (Xanthine only)
   (b) Cone (V): see Table 3
   (c) Extractor (V): 2
   (d) Collision energy: *see* Table 3

### 3.2 Data Analysis

1. A sample of MRM chromatograms of purine metabolites is shown in Fig. 1. MRM transitions used to quantify these analytes are listed in Table 3.
2. Quantlynx software (Waters, MA) was used to quantitate data (*see* **Notes 1–3**). Linear standard curves based on calibrator/internal standard responses vs. target concentration are established to quantitate unknown patient samples. Acceptable calibration curves have correlation coefficient ($R^2$) > 0.96 but preferable > 0.99.
3. Use dilution factor of 2 in the sample sequence for calculating the final concentration.
4. Normal reference ranges are listed in Table 4.

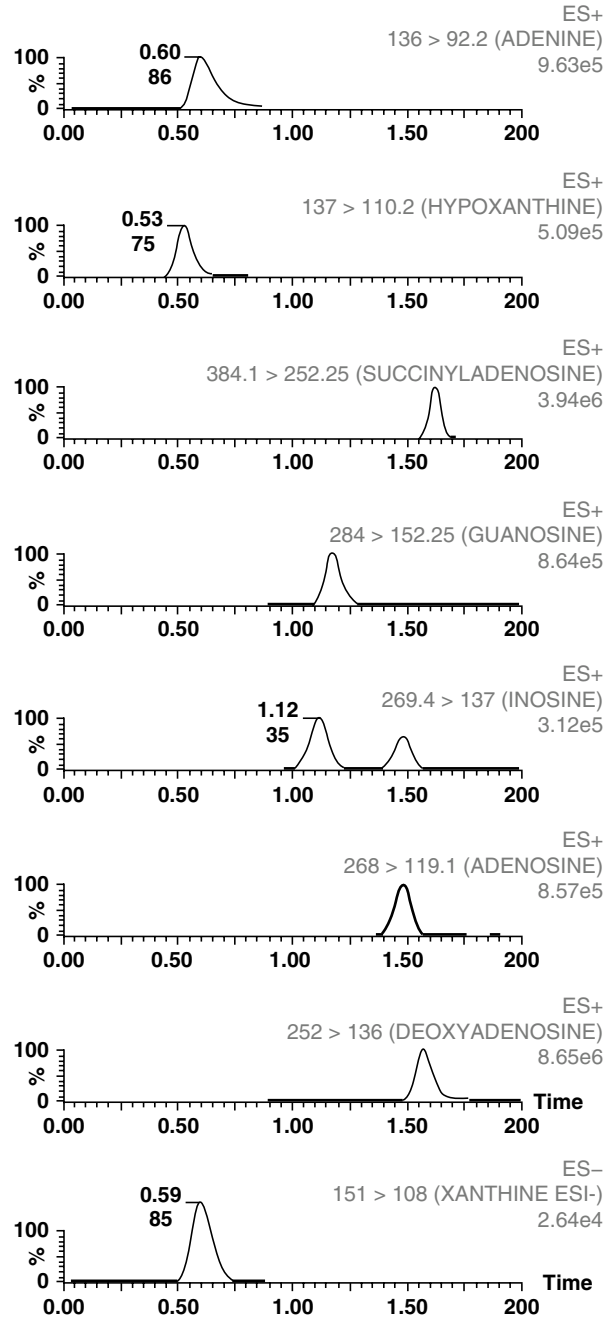



**Fig. 1** UPLC-MS/MS chromatograms of purine analytes. From *top*, hypoxanthine, succinyladenosine, guanosine, inosine, adenosine, deoxyadenosine, and xanthine. Note that actual inosine peak elutes at 1.12 min. The second peak (elutes at around 1.48 min) is an unidentified contaminant found in isotopic adenosine

**Table 4**
**Normal reference ranges[a]**

| Analyte | 0–2 years | 3 to Adult |
|---|---|---|
| Adenine | 0–2.9 | 0–1.3 |
| Adenosine | 0–3.8 | 0–3.4 |
| Deoxyadenosine | 0–4.1 | 0–3.2 |
| Hypoxanthine | 1–30.9 | 0–38.6 |
| Guanosine | 0–7.3 | 0–5.3 |
| Inosine | 0–4.8 | 0–4.8 |
| Succinyladenosine | 0–30.2 | 0–34.6 |
| Xanthine | 0–59 | 0–47.8 |

[a]All values are expressed as mmol/mole creatinine

## 4 Notes

1. Urine purine values are normalized with creatine level of specimen. Diluted urine may cause artificial elevations of analyte concentrations. Extremely low urine samples with creating levels < 0.1 mg/mL are rejected and a replacement sample is required for accurate measurement. Infantile urine samples frequently contain low creatinine and are canceled. To improve chances of sample acceptability, 24 h randomly collected urine is recommended. In such cases, individual urine samples need to be pooled and frozen immediately after collection.
2. Occasionally urine samples may give low responses for some purine analytes while other analytes in the same sample yield reasonable responses. Signals of corresponding internal standards are usually low as well. This signal quenching phenomenon is most likely due to interferences by unknown medications or compounds in the urine sample. One quick solution is to dilute such urine specimen. This can solve the problem in many cases without further modification of LC settings.
3. Succinyladenosine can also be determined in cerebrospinal fluid (CSF) using the same method. A minimum of 100 μL of cerebrospinal fluid (CSF) is needed. Store specimen frozen for no longer than 2 weeks.
4. A list is provided in Table 5 for quick reference of purine disorders and their corresponding diagnostic markers.

**Table 5**
**Diseases associated with elevated analyte concentrations**

| Disease | Diagnostic metabolite(s) |
|---|---|
| Adenosine deaminase deficiency | Deoxyadenosine, adenosine |
| Purine nucleoside phosphorylase deficiency | Inosine, deoxyinosine, guanosine, deoxyguanosine |
| Molybdenum cofactor disease & xanthinuria | Xanthine, hypoxanthine |
| Adenylosuccinase deficiency | Succinyladenosine |
| Lesch-Nyhan disease | Adenine, hypoxanthine |
| APRT deficiency | Adenine, 8-hydroxyadenine, 2,8-dihydroxyadenine |
| PRPP synthetase deficiency | Adenine, hypoxanthine |

## References


1. Micheli V, Camici M, Tozzi MG, Ipata PL, Sestini S, Bertelli M, Pompucci G (2011) Neurological disorders of purine and pyrimidine metabolism. Curr Top Med Chem 11(8):923–947
2. Cameron JS, Moro F, Simmonds HA (1993) Gout, uric acid and purine metabolism in paediatric nephrology. Pediatr Nephrol 7(1):105–118
3. Fu R, Chen CJ, Jinnah HA (2014) Genotypic and phenotypic spectrum in attenuated variants of Lesch-Nyhan disease. Mol Genet Metab 112(4):280–285
4. Fu R, Ceballos-Picot I, Torres RJ, Larovere LE, Yamada Y, Nguyen KV, Hegde M, Visser JE, Schretlen DJ, Nyhan WL, Puig JG, O'Neill PJ, Jinnah HA (2014) Genotype-phenotype correlations in neurogenetics: Lesch-Nyhan disease as a model disorder. Brain 137(Pt 5):1282–1303
5. Balasubramaniam S, Duley JA, Christodoulou J (2014) Inborn errors of purine metabolism: clinical update and therapies. J Inherit Metab Dis 37(5):669–686
6. van den Berghe G, Vencent M-F, Marie S (2012) Disorders of purine and pyrimidine metabolism. In: Blau N, Duran M, Gibson KM, Dionisi-Vici C (eds) Inborn metabolic diseases diagnosis and treatment, 5th edn. Springer-Verlag, Berlin Heidelberg, pp 455–474
7. Jurecka A (2009) Inborn errors of purine and pyrimidine metabolism. J Inherit Metab Dis 32(2):247–263
8. Kelley RE, Andersson HC (2014) Disorders of purines and pyrimidines. Handb Clin Neurol 120:827–838
9. Nyhan WL (2005) Disorders of purine and pyrimidine metabolism. Mol Genet Metab 86(1–2):25–33
10. Van Gennip AH, Bierau H, Nyhan WL (2006) Inborn errors of purine and pyrimidine metabolism. In: Blau N, Duran M, Gibson KM, Dionisi-Vici C (eds) Physician's guide to the treatment and follow-up of metabolic diseases. Springer-Verlag, Berlin, Heidelberg, pp 245–253
11. Schaefer GB, Mendelsohn NJ (2013) Clinical genetics evaluation in identifying the etiology of autism spectrum disorders: 2013 guideline revisions. Genet Med 15(5):399–407
12. Frye RE (2015) Metabolic and mitochondrial disorders associated with epilepsy in children with autism spectrum disorder. Epilepsy Behav 47:147–57
13. Gennip AV (1999) Defects in metabolism of purines and pyrimidines. Ned Tijdschr Klin Chem 24:171–175
14. Ito T, van Kuilenburg AB, Bootsma AH, Haasnoot AJ, van Cruchten A, Wada Y, van Gennip AH (2000) Rapid screening of high-risk patients for disorders of purine and pyrimidine metabolism using HPLC-electrospray tandem mass spectrometry of liquid urine or urine-soaked filter paper strips. Clin Chem 46(4):445–452

# Chapter 25

# Urine Pyrimidine Metabolite Determination by HPLC Tandem Mass Spectrometry

Qin Sun

## Abstract

Pyrimidine diseases result from deficiencies in pyrimidine de novo synthesis, degradation, and salvage pathways. Enzymatic deficiencies in pyrimidine catabolism lead to mitochondrial neurogastrointestinal encephalopathy (MNGIE), pyrimidinuria, dihydropyrimidinuria, ureidopropionic aciduria, and other disorders. While MNGIE presents with gastrointestinal dysmotility, cachexia, and leukoencephalopathy, pyrimidinuria and dihydropyrimidinuria may show symptoms of epilepsy, autism, mental retardation, and dysmorphic features. The application of HPLC-MS/MS facilitates rapid screening of pyrimidine metabolites. Here we describe an LCMS method for determination of uracil, thymine, thymidine, dihydrouracil, and dihydrothymine that are diagnostic biomarkers of MNGIE, pyrimidinuria, and dihydropyrimidinuria.

**Key words** Pyrimidine, Pyrimidinuria, Dihydropyrimidinuria, Ureidopropionic aciduria, Uracil, Thymine, Thymidine, Dihydrouracil and dihydrothymine

## 1 Introduction

Pyrimidines perform fundamental cellular functions in biosynthesis of DNA, RNA, polysaccharides, and phospholipids. Similar to purines, pyrimidine metabolism also involves de novo synthesis, salvage, and degradation pathways. Beginning with carbamoyl phosphate as the precursor, it takes multiple steps to synthesize the central compound, uridine 5′-monophosphate (UMP). UMP can be then converted to other essential nucleotides, cytidine monophosphate (CMP) and thymidine monophosphate (TMP). While pyrimidine nucleosides (cytidine and thymidine) are also converted to related nucleotides by phosphorylation kinases, there is no evidence for salvage of pyrimidine bases so far. Catabolism of CMP, TMP, and UMP yields end products of β-alanine and β-aminoisobutyrate [1].

Totally ten inborn errors of pyrimidine metabolism in pyrimidine synthesis and catabolism pathways have been reported [2–6]. As in purine disorders, pyrimidine deficiencies can present with

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_25, 

widely varied clinical manifestations. Symptoms like hemolytic anemia, development delay, recurrent infections, optic atrophy, scoliosis, hypotonia, and gastrointestinal dysfunction can all be found in pyrimidine diseases. Curiously, neurologic symptoms and epilepsy are more likely presented in pyrimidine degradation deficiencies in addition to other manifestations. Furthermore, dihydropyrimidine dehydrogenase (DPD) deficiency is recognized as one of the metabolic conditions that are associated with autistic spectrum disorders [7].

Because of their broad spectrum of phenotypes, pyrimidine diseases have been misdiagnosed or remained undiagnosed in patients. Only few biochemical genetics laboratories provide targeted diagnostic tests for pyrimidine deficiencies. The correct diagnosis may lead to timely treatment and genetic counseling for the family. It is also critical when considering cancer therapy with pyrimidine analogues in patients with certain pyrimidine degradation diseases as the enzymatic deficiency causes unexpected accumulation of toxic drugs. Few methods were reported for individual disease with thin layer chromatography or gas chromatography–mass spectrometry. With availability of tandem mass spectrometry in clinical laboratories, screening multiple analytes across the pathway in one simple assay became possible [8]. Here we describe a rapid HPLC-ESI-MS/MS assay for urinary pyrimidine metabolite determination. These five analytes belong to pyrimidine degradation pathway, uracil, thymine, dihydrouracil, dihydrothymine, and thymidine. While elevations of uracil and thymine are expected in pyrimidinuria (also called dihydropyrimidine dehydrogenase deficiency, OMIM 274270), elevations of above two compounds plus their dihydro-derivatives (dihydrouracil and dihydrothymine) are associated with dihydropyrimidinuria (also called dihydropyrimidinase deficiency, OMIM 222748) [9, 10]. Thymidine can be measured in both urine and plasma for the diagnosis of MNGIE (also called mitochondrial DNA depletion syndrome 1, OMIM 603041).

## 2 Materials

### 2.1 Samples

1.0–5.0 ml of randomly collected urine. No preservatives are required. Store the specimen frozen. Specimens may be stored for not more than 1 month. Specimens with a creatinine value of less than 0.1 mg/ml should be canceled.

### 2.2 Reagents

1. Running Buffer A and Sample Dilution Buffer: 2 mM ammonium acetate, 0.1 % formic acid. Store buffer at room temperature, stable for 1 year.
2. Running Buffer B: 2 mM ammonium acetate, 0.1 % formic acid in 100 % methanol. Mix well on stir plate. Store at room temperature. Stable for 1 year.

### 2.3 Standards and Calibrators

1. Stock Analyte Solutions: Uracil, thymine, dihydrothymine (MP Biomedicals, CA), dihydrouracil (Frinton Laboratories, NJ), and thymidine (Sigma Aldrich, MO).
2. Stock solutions of each analyte are made in running buffer A. Before mixture, concentrations of each analyte stock are determined by UV/VIS spectrophotometer according to their molar absorption coefficients at appropriate pH ranges.
3. Prepare a stock solution of each of the above compounds. Store in freezer (−80 °C). Stable for 2 years.

### 2.4 Quality Controls and Internal Standards

1. Internal standard mix: $^{13}$C-dihydrouracil, $D_4$-thymine, $D_3$-dihydrothymine, (Cambridge Isotope Laboratories, MA), $^{15}$N-uracil, and $D_4$-thymidine (CDN Isotopes, Canada).
2. Controls: Each run must contain two levels of spiked control. Pool urines for a volume of about 100 ml. Put 50 ml each into two 50 ml conical tubes and centrifuge at 3000 × *g* for 10 min. Transfer urine supernatants into new 50 ml conicals. The levels of endogenous pyrimidine in normal urine are very low and a small spike is used to create the low control range. Aliquot 1 ml to labeled microfuge tubes and store at −80 °C with the assay reagents.

### 2.5 Supplies and Equipment

1. Column: Atlantis T3, 5 μm particle, 2.1 × 100 mm (Waters, MA).
2. Alliance 2695 HPLC coupled with Quattro micro Tandem Mass Spectrometer (Waters, MA).

## 3 Methods

### 3.1 Stepwise Procedure

1. Allow urine samples to thaw completely and mix thoroughly. Samples may be maintained at room temperature while processing.
2. Prepare each urine sample as follows:
   (a) Sample volume: 100 μl
   (b) Internal standard mix: 20 μl

   Mix samples well and briefly centrifuge before loading to LCMS. Injection volume is 3 μl.
3. HPLC setting:
   (a) Flow rate: 0.2 ml/min
   (b) Gradient: 0–0.3 min, 97 % A; 0.3–2.3 min, 97–0 % A; 2.3–3.0 min, 100 % B; 3–4 min, 0–97 % A; 4–8 min, equilibrate with 97 % A.
4. TQD mass spectrometry setting:
   (a) Source: ESI+. MRM transitions for each analyte and other parameters, *see* Table 1.

**Table 1**
**Tandem mass spectrometry setup**

| Analyte | Transition | Dwell (s) | Cone (V) | Collision (eV) |
|---|---|---|---|---|
| Uracil | 112.8 > 70 | 0.25 | 24 | 14 |
| $^{15}N_2$-Uracil | 114.8 > 70.7 | 0.25 | 24 | 15 |
| Dihydrouracil | 114.8 > 72.9 | 0.25 | 25 | 11 |
| $^{13}C_4$,$^{15}$N-Dihydrouracil | 121.0 > 76.7 | 0.25 | 26 | 10 |
| Thymine | 127 > 110.2 | 0.25 | 26 | 13 |
| $D_4$-Thymine | 131 > 114.1 | 0.25 | 35 | 16 |
| Dihydrothymine | 129 > 69 | 0.25 | 22 | 14 |
| $D_9$-Dihydrothymine | 135 > 74 | 0.25 | 28 | 15 |
| Thymidine | 243.2 > 127.2 | 0.25 | 10 | 12 |
| $D_4$-Thymidine | 247.2 > 131.2 | 0.25 | 15 | 10 |

(b) Capillary: 2.9

(c) Extractor: 2.0

(d) Desolvation temperature: 400

### 3.2 Data Analysis

1. A sample of MRM chromatograms of purine metabolites is shown in Fig. 1. Normal reference ranges are listed in Table 2 and diagnostic markers for pyrimidine deficiencies in Table 3.
2. Quantlynx software (Waters, MA) was used to quantitate data (*see* **Notes 1–3**). Linear standard curves based on calibrator/internal standard responses vs. target concentration are established to quantitate unknown patient samples. Acceptable calibration curves have correlation coefficient ($R^2$) > 0.96 but preferable >0.99.

## 4 Notes

1. Urine pyrimidine values are normalized with creatine level of specimen. Diluted urine may cause artificial elevations of analyte concentrations. Extremely low urine samples with creating levels < 0.1 mg/ml are rejected and a replacement sample is required for appropriate measurement. Infantile urine samples are frequently contain low creatinine and canceled. To improve the chances of sample acceptability, 24 h randomly collected urine is recommended. In such cases, individual urine samples need to be pooled and frozen immediately after collection.

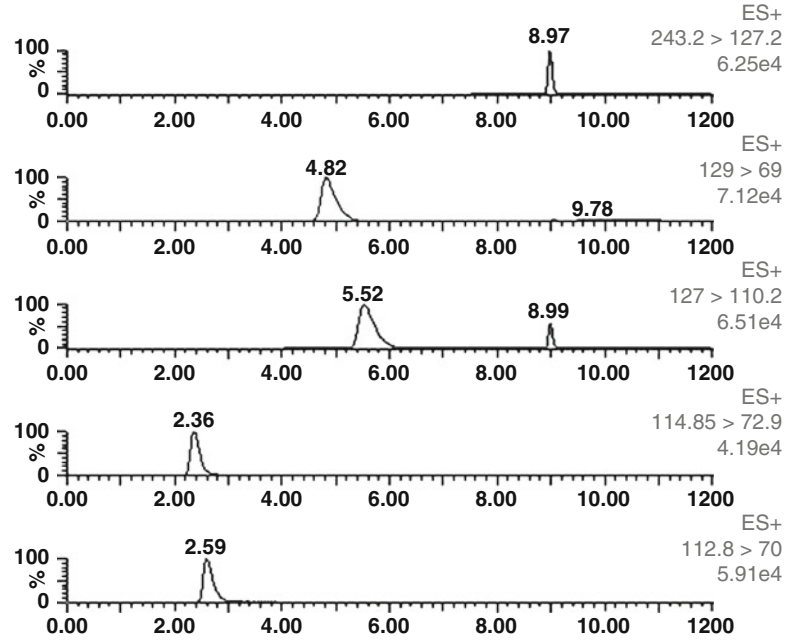



**Fig. 1** LCMS chromatograms of pyrimidine metabolites. From the *top*, thymidine, dihydrothymine, thymine, dihydrouracil, and uracil. Note that thymine elutes at 5.52 min. A contamination with the same 127 > 110.2 MRM is eluted later at 8.99 min

**Table 2**
**Normal reference ranges[a]**

| Analyte | Reference range |
|---|---|
| Uracil | 0–34 |
| Thymine | 0–1.3 |
| Thymidine | 0–0.2 |
| Dihydrouracil | 3–58 |
| Dihydrothymine | 0–1.3 |

[a]All values are expressed as mmol/mole creatinine

**Table 3**
**Diseases associated with elevated analyte concentrations**

| Disease | Metabolites present |
|---|---|
| Dihydropyrimidine dehydrogenase deficiency | Uracil, thymine |
| Dihydropyrimidinase deficiency | Dihydrouracil, dihydrothymine, uracil, thymine |
| MNGIE | Thymidine, deoxyuridine |

2. Occasionally urine samples may give low responses for some purine analytes. Signals of corresponding internal standards are usually low as well. This signal quenching phenomenon is most likely due to interferences by unknown medications or compounds in the urine sample. One quick solution is to dilute the urine specimen. This can help in many cases without further modification of LC settings. In rare cases in which dilution does not improve the signal intensity, a replacement specimen is required for accurate measurement.
3. It was found that an unidentified compound was eluting closely with dihydrouracil based on our experiences. This peak is only present in some urine samples and yields the same. It is important to 114.85 > 72.9 MRM transition. In order to accurately measure dihydrouracil, it is important to ensure that the LC gradient is optimized and able to clearly separate this contamination peak from real dihydrouracil peak (using isotopic internal standard as guidance).

## References


1. Nyhan WL (2005) Disorders of purine and pyrimidine metabolism. Mol Genet Metab 86(1–2):25–33
2. Jurecka A (2009) Inborn errors of purine and pyrimidine metabolism. J Inherit Metab Dis 32(2):247–263
3. Balasubramaniam S, Duley JA, Christodoulou J (2014) Inborn errors of pyrimidine metabolism: clinical update and therapy. J Inherit Metab Dis 37(5):687–698
4. Kelley RE, Andersson HC (2014) Disorders of purines and pyrimidines. Handb Clin Neurol 120:827–838
5. van Gennip AH, Bierau H, Nyhan WL (2006) Inborn error of purine and pyrimidine metabolism. In: Blau N, Hoffman G, Leonard J (eds) Physician's guide to the treatment and follow-up of metabolic diseases. Springer-Verlag, Berlin, Heidelberg, pp 245–255
6. van den Berghe G, Vencent M-F, Marie S (2012) Disorders of purine and pyrimidine metabolism. In: Saudubray JM, van den Berghe G, Walter JH (eds) Inborn metabolic diseases diagnosis and treatment. Springer-Verlag, Berlin, Heidelberg, pp 499–518
7. van Kuilenburg ABP, van Cruchten A, Aberling NGGM (2008) Screening for disorders of purine and pyrimidine metabolism using HPLC-electrospray tandem mass spectrometry. In: Blau N, Duran M, Gibson KM (eds) Laboratory guide to the methods in biochemical genetics. Springer-Verlag, Berlin, Heidelberg, pp 725–737
8. van Kuilenburg AB, Dobritzsch D, Meijer J, Meinsma R, Benoist JF, Assmann B, Schubert S, Hoffmann GF, Duran M, de Vries MC, Kurlemann G, Eyskens FJ, Greed L, Sass JO, Schwab KO, Sewell AC, Walter J, Hahn A, Zoetekouw L, Ribes A, Lind S, Hennekam RC (2010) Dihydropyrimidinase deficiency: phenotype, genotype and structural consequences in 17 patients. Biochim Biophys Acta 1802(7–8):639–648
9. van Kuilenburg AB, Meijer J, Dobritzsch D, Meinsma R, Duran M, Lohkamp B, Zoetekouw L, Abeling NG, van Tinteren HL, Bosch AM (2007) Clinical, biochemical and genetic findings in two siblings with a dihydropyrimidinase deficiency. Mol Genet Metab 91(2):157–164
10. Burton K (ed) (1974) Spectral data and pK values for purines, pyrimidines, nucleosides, and nucleotides. Data for biochemical research. Oxford University Press, London

# Chapter 26

# Quantitation of Plasma Renin Activity in Plasma Using Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

**J. Grace Van Der Gugten and Daniel T. Holmes**

## Abstract

Accurate determination of plasma renin activity (PRA) is essential for the development and maintenance of an effective screening program for primary aldosteronism (PA). PRA measurement can also be useful in the investigation of renal artery stenosis, syndrome of mineralocorticoid excess, Addison's disease, congenital adrenal hyperplasia, Bartter and Gitelman syndromes, and for inherited defects in the renin angiotensin aldosterone system (RAAS). We describe a semi-automated and simple method for the accurate and precise measurement of PRA from 500 μL of plasma (250 μL if blank subtraction is omitted, as discussed) using a liquid chromatography and tandem mass spectrometry (LC-MS/MS) method for angiotensin I (AngI) in 96-well format. After a 3 h AngI generation step at 37 °C in buffering conditions at pH 6, the reaction is quenched with 10 % formic acid containing AngI internal standard. Sample preparation then proceeds with offline solid phase extraction, two wash steps, and methanol elution followed by injection into the LC-MS/MS system. Quantitation is performed against a 7-point calibration linear curve prepared in buffer. The assay calibration range is 0.34–30.0 ng/mL which corresponds to PRA values of 0.11–10.0 ng/mL/h: much wider than was possible using traditional competitive antibody-based methods. Total precision in clinical production has been observed to be 5.8 to 5.0 % for Bio-Rad Hypertension Control materials having nominal PRA values ranging from 1.73 to 12.43 ng/mL/h. At AngI concentrations of 0.06 ng/L (corresponding to a PRA of 0.02 ng/mL/h), signal to noise ratios are 50:1 indicating that the limit of quantitation is well below the level required for clinical use.

**Key words** Primary aldosteronism, Mineralocorticoid hypertension, Secondary hypertension, Hypokalemia, Angiotensin I, Plasma renin activity, Mass spectrometry

## 1 Introduction

Renin (3.4.23.15) is an enzyme released into circulation by the juxtaglomerular apparatus of the nephron. Its action is to cleave the decapeptide angiotensinI (AngI, DRVYIHPFHL) from angiotensinogen, an α2-globulin secreted by the liver. Under the action of the angiotensin converting enzyme (ACE), which is primarily expressed in pulmonary vascular endothelium, AngI is converted to the octapeptide angiotensin II (AngII), a potent vasoconstrictor

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_26, 

that also upregulates the production of aldosterone at the level of the adrenal cortex. The principal action of aldosterone, as mediated by the mineralocorticoid receptor, is to cause the retention of sodium ions in exchange for potassium ions through expression of basolateral $K^+/Na^+$ channels in the renal collecting duct. This leads to the expansion of the plasma volume, the maintenance of blood pressure, and urinary wasting of $K^+$.

In pathological states, the adrenal production of aldosterone can become autonomous due to bilateral adrenal hyperplasia, aldosterone producing adenoma, and a number of other less frequent causes [1, 2]. The unregulated production of aldosterone leads to physiologically appropriate suppression of plasma renin activity (PRA) but pathological $Na^+$ retention and hypertension which may be accompanied by hypokalemia and metabolic alkalosis [1].

The accepted means of screening for PA is the determination of simultaneously collected plasma aldosterone and PRA and the calculation of the aldosterone to renin ratio (ARR). Ratios over a specific threshold, usually between 20 and 40 ng/dL:ng/mL/h depending on method-specific biases, represent a positive screen for PA in a clinically appropriate setting [2]. Some authors recommend additionally that plasma aldosterone be above a minimum threshold for an elevated ARR to be considered a positive screen for PA [3].

PRA determination is also useful in the diagnosis of hypertension caused by secondary (hyperreninemic) aldosteronism which is seen in renal artery stenosis (whether attributable to atherosclerosis or fibromuscular dysplasia) or rarely, renin producing tumors of the kidney. Other conditions where PRA determination may be diagnostically useful include primary adrenal insufficiency (Addison's disease), Bartter and Gitelman syndromes, and neonatal congenital or acquired neonatal pseudohypoaldosteronism.

PRA is determined by measuring the amount of AngI generated after incubation of plasma in appropriately buffered conditions (usually pH = 6) for a fixed time period which is at least 1 h, typically 3 h and occasionally 18 h if additional analytical sensitivity is desired [4]. In traditional assays, a "blank" specimen is also prepared by incubating an aliquot of plasma in identical buffering conditions but cooled on an ice-water bath so that renin is enzymatically inactive. The AngI concentration in the blank sample can then be used to correct for the presence of low-level AngI in the plasma at the time of (or inadvertently generated after) the collection. PRA is then calculated as:

$$\mathrm{PRA} = \frac{[\mathrm{AngI}]_{37^\circ\mathrm{C}} - [\mathrm{AngI}]_{\mathrm{blank}}}{\Delta t} \qquad (1)$$

where $[\mathrm{AngI}]_{37^\circ\mathrm{C}}$ represents the concentration of AngI after incubation at 37 °C, $[\mathrm{AngI}]_{\mathrm{blank}}$ represents the concentration of AngI after incubation on ice-water bath and $\Delta t$ is the duration of the incubation.

In this method, the AngI is generated using a 3 h incubation at pH = 6 where renin is approximately twofold more enzymatically active than at physiological pH [5]. Generated AngI is extracted from the plasma samples using solid phase extraction (SPE) and analyzed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). The implicit assumption is that the endogenous angiotensinogen in the specimen will remain at a sufficiently high concentration in the 3 h incubation so as not to be rate-limiting. Proteolytic degradation of AngI by ACE and nonspecific peptidases is inhibited during AngI generation by both ethylenediaminetetraacetic acid (EDTA) and phenylmethanesulfonylfluoride (PMSF) which are present in the generation buffer.

Analytically, AngI was traditionally determined by radioimmunoassay (RIA), but with the advent of LC-MS/MS instruments into routine clinical laboratories, several authors have published methods suitable for high-throughput environments [6–10]. In addition to the benefit of avoiding radiotracers used in RIA, LC-MS/MS has a linear relationship between analyte concentration and instrument response. This affords a very wide analytical range thereby permitting the direct analysis of specimens from neonates (and from other high-renin states) without pre-dilution which causes unpredictable and patient-specific effects on recovery [5]. LC-MS/MS also permits the use of ion ratios to verify the absence of analytical interferents and, by virtue of its analytical sensitivity and specificity, likely obviates the need for blank subtraction, provided that the generation step is adequately long [8, 10].

## 2 Materials

### 2.1 Samples

Plasma collected in EDTA tubes is the only acceptable sample type for this assay. Specimens should be collected into EDTA plasma tubes and centrifuged within 30 min (preferably within 10 min) and rapidly frozen until analysis [4] (*see* **Note 1**).

### 2.2 Solvents and Reagents

1. Bovine Serum Albumin, Fraction V, Omnipur® (EMD Millipore. Billerica, MA).
2. Phenylmethanesulfonylfluoride (PMSF), >98.5 % (Sigma-Aldrich St. Louis, MO).
3. 100 mM PMSF in methanol: Dissolve 0.174 g of PMSF into 10 mL of methanol. Store at 2–8 °C. Expected stability: >6 months.
4. Buffer A: 0.1 M Tris Base pH 6: Dissolve 12.11 g of Tris Base into 1000 mL of deionized water. Adjust to pH 6 with glacial acetic acid. Store at 2–8 °C. Expected stability: >6 months.
5. Working Buffer B: 1 % BSA (w/v) in Stock Buffer A: Dissolve 0.1 g of BSA into 10 mL of 0.1 M Tris Buffer pH 6. Prepare fresh for each batch.

6. Generation Buffer: Weigh 121.1 g of Tris Base + 74 g EDTA into a 1000 mL volumetric flask. Add DI water to about 900 mL. Sonicate for 30 min or until the Tris Base and EDTA are fully dissolved. Add DI water to the volume mark and mix well. Transfer to a labeled polypropylene storage container. Adjust to pH 5.45–5.50 with glacial acetic acid. Store at 2–8 °C. Expected stability: >6 months.
7. On the day of analysis, add 100 µL of the 100 mM PMSF solution to 10 mL of generation buffer.
8. Mobile Phase A: 0.2 % formic acid in water: Add 2 mL of formic acid to 1 L of DI water. Mix well.
9. Mobile Phase B: 0.2 % formic acid in methanol: Add 2 mL of formic acid to 1 L of methanol. Mix well.
10. 10 % Formic Acid in water: Add 50 mL of formic acid to 450 mL of DI water. Mix well. Store at room temperature. Expected stability: >3 months.
11. 5 % formic acid in water: Add 25 mL of formic acid to 475 mL of DI water. Mix well. Store at room temperature. Expected stability: >3 months.
12. 20 % methanol in water: Add 100 mL of methanol to 400 mL of DI water. Mix well. Store at room temperature. Expected stability: >3 months.
13. Lyphochek™ Hypertension Markers Control, Trilevel (Bio-Rad, Montreal QC, Canada).

### 2.3 Internal Standards and Standards

1. Primary standard: AngI: 3×10 µg (Proteochem, Loves Park, IL).
2. Stable isotopically labeled internal standard (SIS): AngI (D*R*VYIHPFHL) with isotopically labeled arginine residue ($^{13}C$,$^{15}N$) was synthesized by the University of Victoria Genome BC Proteomics Centre (*see* **Note 2**).
3. AngI stock solution (5000 ng/mL AngI in Working Buffer B): contents of 10 µg vials of AngI are dissolved in a total of exactly 2 mL of Working Buffer B to make a solution of 5000 ng/mL. Note that the dissolution process must be performed in 0.5 mL aliquots as the Proteochem vials are small in volume. Aliquot 500 µL of the 5000 ng/mL stock solution to microvials with lids, seal with parafilm, and store at –70 °C.
4. Angiotensin S7: Remove an aliquot of the 5000 ng/mL stock solution and allow it to thaw at room temperature. Label ten polystyrene 10×100 mm tubes appropriately (i.e., AngI S7 100 ng/mL, Date of Preparation). Add 1.96 mL of Working Buffer B to each tube. Aliquot 40 µL of the 5000 ng/mL stock solution to each of the ten tubes. Cap, Mix, and store at –70 °C until use.
5. AngI SIS Solutions:

(a) Stock solution, 1 mg/mL: dissolve 1 mg of AngI-SIS in DI water. Mix well to dissolve.

(b) Intermediate working solution, 10 μg/mL: dilute the 1 mg/mL Stock Solution 100-fold to a resulting concentration of 10 μg/mL: add 100 μL of the 1 mg/mL stock solution to 9.90 mL of DI water. Mix well. Aliquot 10× 1 mL to labeled cryovials and store at −70 °C.

(c) Working solution, 10 ng/mL in 10 % formic acid: Remove a vial of the 10 μg/mL Ang1-SIS Intermediate Working Solution and allow it to thaw. Mix well. Aliquot 0.5 mL into 500 mL of 10 % formic acid. The resulting concentration is 10 ng/mL. Store at 2–8 °C.

### 2.4 Calibrators and Controls

1. Controls used are Bio-Rad Hypertension controls Levels 1, 2, and 3. The target mean and standard deviations are set based on results of 20 analyses over 10 runs. Controls are run in duplicate with each analytical run.
2. An in-house patient pool is prepared approximately once yearly by pooling discarded anonymized previously analyzed patient plasma samples. The patient pool target value is set as per the Bio-Rad QC. The patient pool is run in duplicate with each analytical run.
3. Calibrators are prepared in-house using the AngI stock solution in 1 % BSA in Buffer A (Working Buffer B).

### 2.5 Analytical Equipment and Supplies

1. Strata-X 33u Polymeric Reversed Phase 96-Well Plate, 60 mg/well (Phenomenex, Torrance, CA).
2. Vacuum manifold or positive pressure manifold, installed in robotic liquid handler or manually controlled.
3. 2 mL 96 deep square well, V-bottom plates (Corning, Corning, NY).
4. Silicone cap mats with PTFE barrier for square well plates (Microliter Analytical Supplies, Suwanee GA).
5. 2 mL Nunc® 96 DeepWell™ round-bottom well plates (Thermo Scientific, Waltham, MA).
6. Nunc® cap mats for round bottom plates (Thermo Scientific, Waltham, MA).
7. API-5000 or API-5500 QTRAP® triple quadrupole mass spectrometer (AB SCIEX, Concord, ON) or other mass spectrometer capable of reaching the required limit of detection, equipped with appropriate software (e.g., Analyst®).
8. Shimadzu 20 AC LC System with pumps, column oven, degasser, autosampler.
9. Analytical column: 4u Proteo 90 Å, 50 × 2.0 mm (Phenomenex, Torrance, CA).
10. Guard column, C12, 4 × 2.0 mm (Phenomenex, Torrance, CA).

## 3 Methods

### 3.1 Stepwise Procedure

1. AngI S7 (100 ng/mL) is thawed at room temperature.
2. Prepare Working Buffer B.
3. Prepare calibration standards by hand or on robotic liquid handler (*see* **Note 3**). The calibration standards (*see* **Note 4**) are serial dilutions of S7 as follows:
   (a) S6: 420 µL of S7 is mixed with 980 µL of Working Buffer B—resulting concentration is 30.00 ng/mL.
   (b) S5: 420 µL of S6 is mixed with 980 µL of Working Buffer B—resulting concentration is 9.000 ng/mL.
   (c) S4: 420 µL of S5 is mixed with 980 µL of Working Buffer B—resulting concentration is 2.700 ng/mL.
   (d) S3: 700 µL of S4 is mixed with 700 µL of Working Buffer B—resulting concentration is 1.350 ng/mL.
   (e) S2: 700 µL of S3 is mixed with 700 µL of Working Buffer B—resulting concentration is 0.6750 ng/mL.
   (f) S1: 700 µL of S2 is mixed with 700 µL of Working Buffer B—resulting concentration is 0.3375 ng/mL.
   (g) Blank: Working Buffer B with no AngI added.
4. Prepare generation buffer as described above.
5. Samples and QCs are thawed in room temperature water bath for 5 min.
6. Samples and QCs are transferred to an ice bath to complete thawing.
7. Samples and QCs mixed and centrifuged for 5 min at 2100 × *g* at <5 °C.
8. 50 µL of generation buffer is added to 2 mL 96 deep square well plate.
9. 250 µL of calibrators, patient samples, and QCs are added to the appropriate wells of a 2 mL 96 deep square well plate.
10. The plate is sealed with a silicone cap mat and vortex-mixed briefly, then placed in 37 °C water bath for 3 h (*see* **Note 5**).
11. After 3 h, 300 µL of AngI SIS in 10 % formic acid is added to the incubation plate to stop generation of AngI. The plate is sealed with a new silicone cap mat and mixed briefly (*see* **Note 6**).
12. The AngI is extracted by solid phase extraction (SPE):
    (a) Condition step 1: 1 mL of methanol is added to each well of the SPE plate and vacuum is applied for 1 min (*see* **Note 7**).
    (b) Condition step 2: 1 mL of 5 % formic acid is added to each well of the SPE plate and vacuum is applied for 1 min.

(c) 600 μL of sample is added to each well and vacuum is applied for 1 min.

(d) Wash 1: 1 mL of 5 % formic acid is added to each well of the SPE plate and vacuum is applied for 1 min.

(e) Wash 2: 1 mL of 20 % methanol is added to each well of the SPE plate.

(f) Apply 10 min vacuum at 100 mbar to dry the SPE plate.

(g) Elute the AngI from the SPE plate to a 2 mL deep well round-bottom well plate with 250 μL of methanol. Apply vacuum for 2 min.

13. Seal the 2 mL deep well round plate with the appropriate cap mat and load on instrument.
14. Inject 20 μL of extract into the LC-MS/MS. Sample LC-MS/MS chromatograms for AngI and IS are shown in Fig. 1.

### 3.2 Analysis

1. HPLC gradient is provided in Table 1 and Fig. 2.
2. Instrument operating parameters are given in Table 2.
3. Data are analyzed using Analyst® Software (AB SCIEX, Concord, ON).
4. Standard curves are generated based on linear regression with $1/x^2$ weighting of the analyte/IS peak area ratio ($y$) versus analyte concentration ($x$) using the quantifying ions indicated in Table 2.
5. Run acceptability is based on control values falling within 2 standard deviations of the target value. Target values and SD are set based on the mean of 10 runs as described in Subheading 2.4, **item 1**.
6. An ion ratio limit of 15 % of the quantitation to qualifying ion is used to positively identify AngI peaks.

## 4 Notes

1. Ambient or refrigeration temperatures can facilitate the non-proteolytic activation of prorenin to renin by the unfolding of the pro-segment. Under normal circumstances, prorenin concentration is approximately tenfold higher than the concentration of renin but in the low renin state, which is of clinical interest in PA screening, this may increase to 100-fold [11]. Since about 2 % of prorenin is catalytically active [4], its contribution to PRA and direct renin immunoassays can be substantial and effort should be made to limit prorenin activation to prevent spurious overestimation of PRA. Further, samples left at ambient temperatures may begin to spontaneously generate AngI leading to substrate depletion. This alters substrate availability and enzyme kinetics leading to lower results. For these

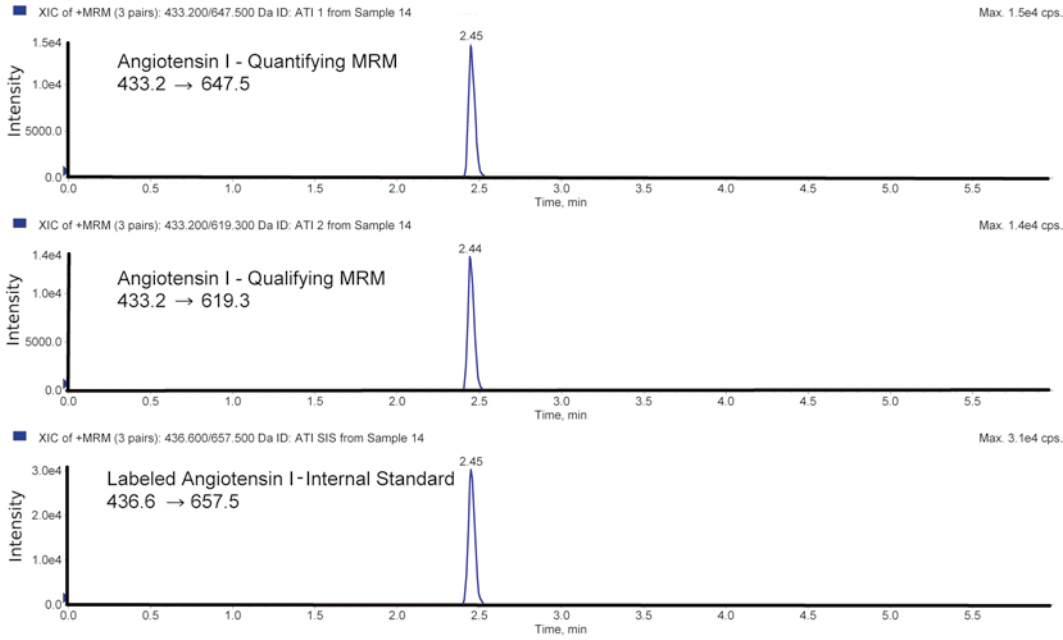



**Fig. 1** Representative chromatograms of the AngI quantifying multiple reaction monitoring (MRM) transition (433.2 → 647.5), qualifying MRM transition (433.2 → 619.3) and AngI SIS MRM transition (436.6 → 657.5). In the LC conditions provided the expected elution time of AngI is approximately 2.5 min

**Table 1**

**HPLC gradient. Optimized for Shimadzu LC20AD**

| **Column temp** | **55 °C** | |
|---|---|---|
| Flow rate | 0.50 mL/min | |
| Gradient | Time | Mobile phase A (%) |
| | 0.00 | 90 |
| | 0.50 | 90 |
| | 1.50 | 5 |
| | 3.50 | 5 |
| | 3.60 | 90 |
| | 6.00 | 90 |

reasons, we advise that specimens be immediately centrifuged, poured off and frozen at −20 °C or lower until analysis.

2. SIS AngI is commercially available from Anaspec (Fremont, CA). The $^{13}C$ and $^{15}N$ isotopic labeling of the Anaspec products (DR*VYI*HPFHL and DRVY*I*HPFHL) is different and so multiple reaction monitoring transitions of this product will differ from values shown in Table 2.

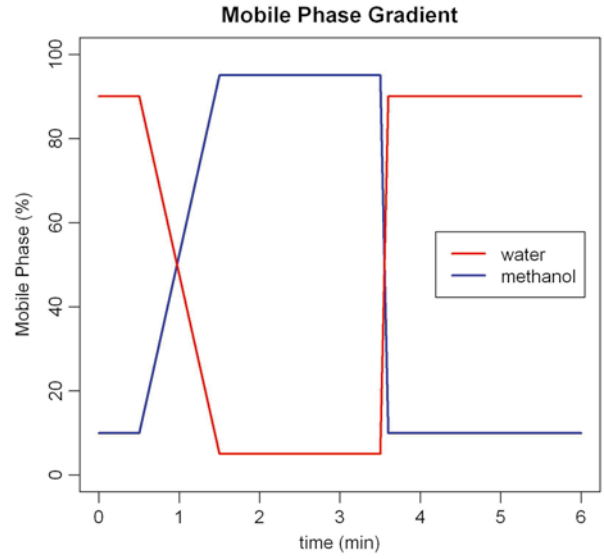



**Fig. 2** HPLC gradient

**Table 2**

**Optimized instrument settings for the AB SCIEX API-5500 QTRAP® and API-5000 triple quadrupole mass spectrometers in electrospray positive ion mode**

| | | | | | |
|---|---|---|---|---|---|
| Source parameters | | | | | |
| Curtain gas: | 35 µL/min | | | | |
| Source temperature: | 650 °C | | | | |
| Gas 1: | 35 µL/min | | | | |
| Gas 2: | 35 µL/min | | | | |
| CAD gas: | Medium/7* | | | | |
| IonSpray voltage: | 4000 V | | | | |
| Entrance potential | 10 V | | | | |
| Compound dependant parameters | | | | | |
| Compound name | Q1 Mass | Q3 Mass | Declustering potential (V) | Collision energy (V) | Cell exit potential |
| AngI—quantifier | 433.2 | 647.5 | 136 | 25 | 38 |
| AngI—qualifier | 433.2 | 619.3 | 120 | 30 | 30 |
| AngI SIS | 436.6 | 657.5 | 120 | 25 | 30 |

Precursor ion masses (i.e., *m/z* ratios) correspond to the $(M+3H)^{3+}$ ion of AngI. Product ions correspond to the singly charged $b_5$ and $a_5$ ions of (1–5)AngI (DRVYI) for the quantifier and qualifier respectively. All parameters are identical for both instruments except where *marked* (*asterisk*), which is specific to the API-5000. Tune settings may vary between instruments

3. Sample pipetting and subsequent vacuum or positive pressure extraction can be done manually using standard pipettes and stand-alone equipment. However, obtaining a robotic liquid handler such as a Hamilton STARlet (Hamilton Robotics Inc, Reno NV) represents a significant advantage when it comes to increasing throughput.
4. Although the material we have recommended for the preparation of AngI calibrators is pre-weighed, accuracy of the AngI calibration must be established by comparison against a standard reference material. A non-WHO reference material for AngI is available from National Institute for Biological Standards and Control (NIBSC, Hertfordshire, England) in vials containing either 2 μg (86/536) or 9 μg (71/328) of synthetic lyophilized AngI. This material can be used to check the accuracy of the calibrators or to reassign them. Readers are encouraged to review the Clinical Laboratory Standards Institute (CLSI) protocols C62-A [12] for mass spectrometry and EP32-R [13] for metrology and traceability for guidance.
5. To prepare a 96-well plate for blank (i.e., baseline AngI) subtraction for calculation using Eq. 1, prepare an identical plate as per Subheading 3.1 and incubate for 3 h on an ice-water bath instead of 37 °C or acidify immediately after pipetting with SIS/formic acid solution (Subheading 3.1, **step 10**) and analyze. In our laboratory, we have found that blank subtraction for LC-MS/MS-based PRA does not alter numerical result in any clinically meaningful way because the blanks AngI concentration is frequently below the limit of detection or very low relative to the AngI concentration in the generated sample. Users are encouraged to evaluate the value of blank subtraction in their own laboratories. In the absence of blank subtraction, Eq. 1 is modified to read: $\mathrm{PRA} = \frac{[\mathrm{AngI}]_{37°C}}{\Delta t}$.
6. Unintentional collection of specimens other than EDTA plasma (e.g., serum) will generally result in spuriously low or undetectable PRA results and will result in false positive screens for PA. There are numerous other analytical and preanalytical factors requiring careful consideration as part of a laboratory PA screening program [14].
7. Unless otherwise stated, at each step of Subheading 3.1 **step 12**, 200 mbar of vacuum is applied for 1 min. Users should ensure that all solvent/sample has passed through the SPE wells before releasing the vacuum. The SPE is performed using Phenomenex Strata-X 33u Polymeric Reversed Phase 96-Well Plate, 60 mg per well but an equivalent product may be substituted. SPE parameters (i.e., volumes, reagents used, vacuum pressure, and time) should be optimized in each laboratory. Although plate format is recommended, sample preparation could be easily modified for SPE cartridges. A positive pressure manifold can also be substituted for a vacuum manifold if desired.

## References


1. Young WF (2007) Primary aldosteronism: renaissance of a syndrome. Clin Endocrinol (Oxf) 66:607–618
2. Funder JW, Carey RM, Fardella C et al (2008) Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. J Clin Endocrinol Metab 93:3266–3328
3. Young WF (1997) Primary aldosteronism: update on diagnosis and treatment. Endocrinologist 7:213–221
4. Campbell DJ, Nussberger J, Stowasser M et al (2009) Activity assays and immunoassays for plasma renin and prorenin: information provided and precautions necessary for accurate measurement. Clin Chem 55:867–877
5. Sealey JE, Laragh JH (1975) Radioimmunoassay of plasma renin activity. Semin Nucl Med 5:189–202
6. Fredline VF, Kovacs EM, Taylor PJ et al (1999) Measurement of plasma renin activity with use of HPLC-electrospray-tandem mass spectrometry. Clin Chem 45:659–664
7. Bystrom CE, Salameh W, Reitz R et al (2010) Plasma renin activity by LC-MS/MS: development of a prototypical clinical assay reveals a subpopulation of human plasma samples with substantial peptidase activity. Clin Chem 56:1561–1569
8. Carter S, Owen LJ, Kerstens MN et al (2012) A liquid chromatography tandem mass spectrometry assay for plasma renin activity using online solid-phase extraction. Ann Clin Biochem 49:570–579
9. Owen LJ, Adaway J, Morris K et al (2014) A widely applicable plasma renin activity assay by LC-MS/MS with offline solid phase extraction. Ann Clin Biochem 51:409–411
10. Popp R, Malmström D, Chambers AG et al (2014) An automated assay for the clinical measurement of plasma renin activity by immuno-MALDI (iMALDI). Biochim Biophys Acta 1854:547–558
11. Sealey JE, Gordon RD, Mantero F (2005) Plasma renin and aldosterone measurements in low renin hypertensive states. Trends Endocrinol Metab 16:86–91
12. Clarke W, Molinaro RJ, Bachmann LM et al (2014) C62-A: liquid chromatography-tandem mass spectrometry methods; approved guideline. Clinical and Laboratory Standards Institute. 34:1–71
13. Salit ML, Ciesiolka T, Greenberg N et al (2006) EP-32-R: metrological traceability and its implementation; a report. Clinical and Laboratory Standards Institute. 26:1–43
14. Rehan M, Raizman JE, Cavalier E et al (2015) Laboratory challenges in primary aldosteronism screening and diagnosis. Clin Biochem 48:377–387

# Chapter 27

# Quantitation of S-Adenosylmethionine and S-Adenosylhomocysteine in Plasma Using Liquid Chromatography-Electrospray Tandem Mass Spectrometry

**Erland Arning and Teodoro Bottiglieri**

## Abstract

We describe a simple stable isotope dilution method for accurate determination of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in plasma as a diagnostic test. SAM and SAH are key metabolic intermediates of methionine metabolism and the methylation cycle. Determination of SAM and SAH in plasma was performed by high performance liquid chromatography coupled with electrospray positive ionization tandem mass spectrometry (HPLC-ESI-MS/MS). Calibrators (SAM and SAH) and internal standards ($^2H_3$-SAM and $^2H_4$-SAH) were included in each analytical run for calibration. Sample preparation involved combining 20 μL sample with 180 μL of internal standard solution consisting of heavy isotope labeled internal standards in mobile phase A and filtering by ultracentrifugation through a 10 kd MW cutoff membrane. Sample filtrate (3 μL) was injected by a Shimadzu Nexera LC System interfaced with a 5500 QTRAP® (AB Sciex). Chromatographic separation was achieved on a 250 mm × 2.0 mm EA:faast column from Phenomenex. Samples were eluted at a flow rate of 0.20 mL/min with a binary gradient with a total run time of 10 min. The source operated in positive ion mode at an ion spray voltage of +5000 V. SAM and SAH resolved by a gradient to 100 % methanol with retention times of 6.0 and 5.7 min, respectively. The observed $m/z$ values of the fragment ions were $m/z$ 399 → 250 for SAM, $m/z$ 385 → 136 for SAH, $m/z$ 402 → 250 for $^2H_3$-SAM, $m/z$ 203 → 46. The calibration curve was linear over the ranges of 12.5–5000 nmol/L for SAM and SAH.

**Key words** S-adenosylmethionine, S-adenosylhomocysteine, Methylation, Mass spectrometry

## 1 Introduction

S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) are key metabolic intermediates of methionine metabolism and the methylation cycle. SAM is directly involved in the transfer of a methyl group (CH3) to numerous methyltransferase reactions that involve critical pathways required for normal cell function. These include methylation of DNA, RNA, phospholipids, proteins, and various amino acids and other small molecules. The by-product of all methyltransferase reactions is SAH which is rapidly metabolized

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_27, 

to homocysteine. SAH is a competitive inhibitor of SAM-dependent methyltransferase reactions. Measuring the concentration of SAM and SAH in tissues and biological fluids has become important to determine the SAM/SAH ratio, also commonly known as the "methylation index." The pivotal role that methylation plays in cell metabolism has led to many studies in which the SAM/SAH ratio has been used as a measure of methyltransferase activity [1]. Measuring blood SAM and SAH concentrations is also useful in diagnosing inherited metabolic disorders involved in methionine metabolism. Although these disorders are rare, the determination of SAM and SAH combined with other amino acid analysis can differentiate several metabolic disorders characterized by hypermethioninemia [2]. SAM is also available as an over-the-counter dietary supplement [3]. The method described is also useful in determining blood levels of SAM and SAH in clinical trials following acute or chronic oral supplementation.

## 2 Materials

### 2.1 Samples

Human heparinized or EDTA plasma is preferred; however serum is acceptable—Specimen drawn any time during the day will be acceptable. No patient preparation is required. Specimen should be placed on wet ice following collection and separated by centrifugation within 30 min. Separated plasma should be transferred to a cryovial and stored at −80 °C until time of testing.

### 2.2 Solvents and Reagents

1. Mobile Phase A (4 mM ammonium acetate/0.1 % formic acid/0.1 % heptafluorobutyric acid in HPLC-grade water): In a hood, add 308 mg ammonium acetate, 1 mL of formic acid, and 1 mL of heptafluorobutyric acid to a 1 L volumetric flask, bring to volume with water, and mix. Stable at room temperature, 18–24 °C, up to 3 months.
2. Mobile Phase B (0.1 % formic acid in methanol): In a hood, add 1 mL of formic acid to a 1 L volumetric flask, bring to volume with methanol, and mix. Stable at room temperature, 18–24 °C, up to 3 months.

### 2.3 Internal Standards and Standards

1. Primary standards: SAM ((S-5′-adenosyl)-L-methionine p-toluenesulfonate salt) and SAH ((S-5′-adenosyl)-L-homocysteine) (Sigma).
2. Primary internal standards (I.S.): SAM-$D_3$ ($^2H_3$-S-adenosylmethionine) (CDN Isotopes) and SAH-$D_4$ ($^2H_4$-S-adenosylhomocysteine) (Cayman Chemical).
3. SAM Standard Stock Solution (1 mM): Add 57.1 mg SAM to 100 mL volumetric flask, bring to volume with 0.1 M HCl,

and mix. Store in 150 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).

4. SAH Standard Stock Solution (1 mM): Add 38.4 mg SAH to 100 mL volumetric flask, bring to volume with 0.1 M HCl, and mix. Store in 150 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).
5. SAM-$D_3$ I.S. Stock Solution (1 mM): Add 10.9 mg SAM-$D_3$ to 10 mL volumetric flask, bring to volume with 0.1 M HCl, and mix. Store in 200 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).
6. SAH-$D_4$ I.S. Stock Solution (1 mM): Add 38.8 mg SAH-$D_4$ to 100 mL volumetric flask, bring to volume with 0.1 M HCl, and mix. Store in 200 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).
7. I.S. Working Solution (SAM-$D_3$ and SAH-$D_4$ prepared in mobile phase A): SAM-$D_3$ requires purification following preparation of 1 mM stock solution (*see* **Note 2**). Once SAM-$D_3$ is purified, add 25 µL of 1 mM SAH-$D_3$ stock solution for every 50 mL of SAM-$D_3$ in mobile phase A. Store in 50 mL aliquots at −80 °C for up to 4 years. 50 mL of SAM-$D_3$ and SAH-$D_3$ in mobile phase A is stable for 3 months at 2–8 °C after thawing for use.

### 2.4 Calibrators and Controls

1. Calibrators: SAM/SAH Working Standard Curve, dilute stock solutions of SAM and SAH as follows:
   (a) Dilution A (100 µM): Add 100 µL of 1 mM SAM and 100 µL of 1 mM SAH stock solution to 800 µL of water and mix well by vortex.
   (b) Dilution B (10 µM): Add 100 µL Dilution A to 900 µL of water and mix well by vortex.
   (c) Dilution C (1 µM): Add 100 µL Dilution B to 900 µL of water and mix well by vortex.
   (d) Working Standard Curve (25–400 nM): Add 400 µL of Dilution C to 600 µL of water and mix well by vortex. Perform four additional serial dilutions by adding 500 µL of previous standard to 500 µL of water. This will provide a calibration curve of (400, 200, 100, 50, 25 nM). Working standard curve may be stored in the refrigerator at 4 °C for up to 8 h (*see* **Note 3**).
2. Control: SAM/SAH Low Control (SAM = 70–120 nM and SAH = 20–60 nM target value):
   (a) Prepare 10 mL pooled plasma.
   (b) Assay pooled plasma to quantitate the native concentration of SAM and SAH.

(c) Spike or dilute pooled plasma with diluted stock standard or water to obtain a final concentration of 70–120 nM SAM and 20–60 nM SAH. Store in 80 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).

3. Control: SAM/SAH High Control (SAM = 200–400 nM and SAH = 100–200 nM target value):
   (a) Prepare 10 mL pooled plasma.
   (b) Assay pooled plasma to quantitate the native concentration of SAM and SAH.
   (c) Spike or dilute pooled plasma with diluted stock standard or water to obtain a final concentration of 200–400 nM SAM and 100–200 nM SAH. Store in 80 µL aliquots at −80 °C for up to 4 years (*see* **Note 1**).

### 2.5 Analytical Equipment and Supplies

#### 2.5.1 Analytical Equipment

1. Shimadzu Nexera liquid chromatograph system with AB Sciex 5500QTRAP® with Analyst software.
2. Analytical Column: Phenomenex EA:faast, 3 µm, 250 × 2 mm.
3. Guard Column: Phenomenex Security Guard, 5 µm, 4 × 3 mm.
4. Microcentrifugal Filter Units: Amicon Ultra 0.5 mL, 10 kDa NMWL (Millipore).
5. 1.5 mL microcentrifuge tubes.

#### 2.5.2 Clean-up Equipment for SAM-$D_3$

1. SAM-$D_3$ Clean-up Column: Phenomenex Sphereclone-ODS(2) 250 × 4.6 mm 5 µm.
2. Shimadzu LC-10 AD HPLC Pump.
3. ESA Model 542 HPLC Autosampler.
4. UV Detector.
5. 1.5 mL microcentrifuge tubes.

## 3 Methods

### 3.1 Sample Preparation

1. To labeled 1.5 mL microcentrifugal units, pipette 40 µL plasma (calibrators, controls, patient plasma).
2. Add 160 µL of SAM/SAH I.S. Working Solution.
3. Cap and vortex mix tubes at maximum speed for 3 s.
4. Centrifuge for 20 min at 14,000 × *g*.
5. Transfer 150 µL filtrate into corresponding work list position in 96-well microtiter plate and cover with silicone cover.
6. Place completed 96-well microtiter plate onto refrigerated autosampler (4 °C).

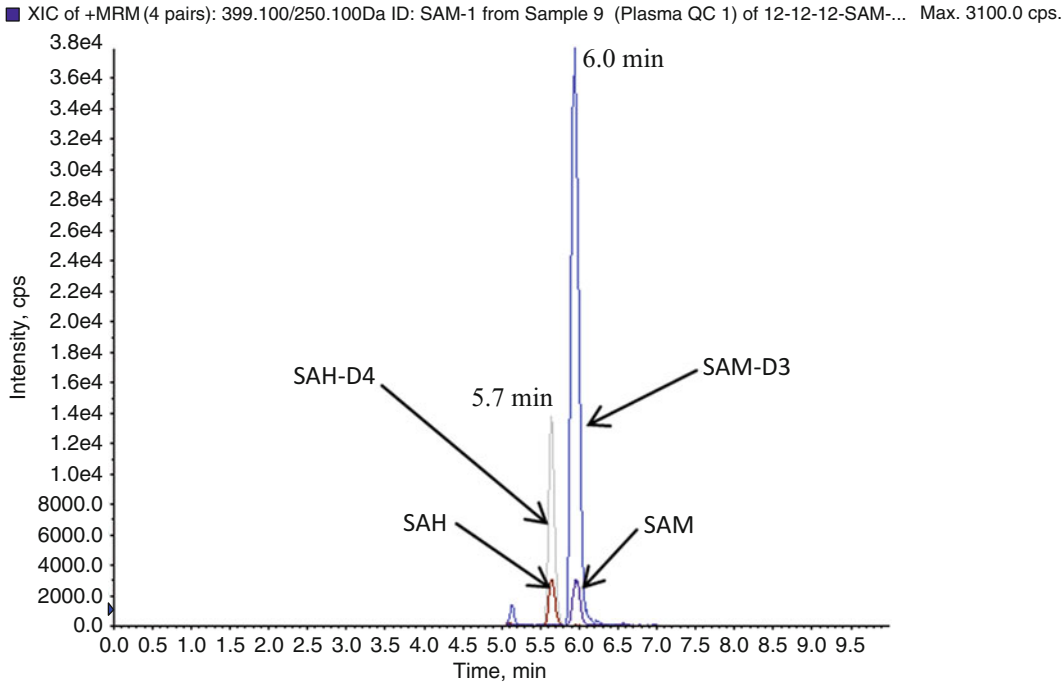



**Fig. 1** HPLC-ESI-MS/MS ion chromatogram of SAM (*m/z* 399.1 > 250.1), SAH (*m/z* 385.1 > 136.1), SAM-$D_3$ (*m/z* 402.1 > 250.1) and SAH-$D_4$ (*m/z* 389.1 > 138.1). Concentration of SAM and SAH is 95 nM and 41 nM, respectively

7. Inject 4 μL of sample onto HPLC-ESI-MS/MS. Representative HPLC-ESI-MS/MS ion chromatograms for SAM/SAH and I.S. are shown in Fig. 1 (*see* **Note 4**).

### 3.2 Data Analysis

1. Instrumental operating parameters are given in Tables 1 and 2.
2. Data are analyzed using Analyst software (AB Sciex).
3. Standard curves are generated based on linear regression of the analyte/I.S. peak-area ratio ($y$) versus analyte concentration ($x$) using the primary ions indicated in Table 1.2.
4. Acceptability of each run is confirmed if the calculated control concentrations fall within two standard deviations of the target values. Inter-day precision was evaluated by repeated analysis of bi-level QC material analyzed in duplicate over a period of 20 different days.
5. Liquid chromatography retention time window limits for SAM and SAM-$D_3$ are set at 6.0 (±0.2) min; SAH and SAH-$D_4$ 5.7 (±0.2) min.
6. The assay has a lower limit of quantitation of 1 nM for both SAM and SAH, with precisions of <6 % over the entire range.

**Table 1**
**HPLC-ESI-MS/MS operating conditions**

| | | |
|---|---|---|
| (a) HPLC (SAM and SAH)[a] | | |
| Column temp. | 40 °C | |
| Flow rate | 0.20 mL/min | |
| Gradient | Time (min) | Mobile phase A (%) |
| | 0 | 75 |
| | 7 | 0 |
| | 7.1 | 75 |
| | 10 | Stop |
| (b) MS/MS tune settings[b] | | |
| Entrance Potential (V) | 10 | |
| Curtain gas (psi) | 20 | |
| CAD gas | Medium | |
| Ion spray (V) | 5000 | |
| Temp (°C) | 700 | |
| GS 1 (psi) | 40 | |
| GS 2 (psi) | 50 | |
| Resolution Q1 and Q3 | Unit | |

[a]Optimized for Shimadzu Nexera liquid chromatography system equipped with Phenomenex EZ:faast, 3 μm, 250 × 2 mm analytical column; Mobile phase A: 4 mM ammonium acetate, 0.1 % formic acid and 0.1 % heptafluorobutyric acid in water; Mobile phase B: 0.1 % formic acid in methanol
[b]Optimized for AB Sciex 5500QTRAP®. Tune settings may vary slightly between instruments

**Table 2**
**HPLC-ESI-MS/MS operating conditions**

| | MRM transition | | | | | |
|---|---|---|---|---|---|---|
| **Compound** | **Q1 (*m/z*)** | **Q3 (*m/z*)** | **Dwell time (ms)** | **DP (V)** | **CE (V)** | **CXP (V)** |
| SAM | 399.1[a] | 250.1[a,b] | 150 | 31 | 21 | 18 |
| SAH | 385.1[a] | 136.1[a,b] | 150 | 35 | 24 | 10 |
| SAM-$D_3$ | 402.1[a] | 250.1[a,b] | 150 | 31 | 21 | 18 |
| SAH-$D_4$ | 389.1[a] | 138.1[a,b] | 150 | 35 | 24 | 10 |

[a]Optimized *m/z* may change based on tuning parameters and instrument used
[b]Primary ions for SAM and SAH quantification

The reference range used is based on plasma levels from healthy controls ($n = 12$); SAM = 33–95 nmol/L and SAH = 13–28 nmol/L. This reference range is comparable to other reference ranges available in the literature. *See* **Note 5** for information regarding ion suppression studies.

## 4 Notes

1. Individual sets of SAM and SAH Standard Stock Solution and controls can be pre-aliquoted and frozen until use in each analytical run. For each set pipette 80 μL of stock standard/control solution into 1.5 mL microfuge tubes and freeze at −80 °C until use. Thaw completely before use. Stable for 4 years at −80 °C.
2. SAM-$D_3$ from CDN isotopes contains unlabeled SAH, which requires purification prior to use as an internal standard. A 200 μM SAM-$D_3$ is prepared from the 1 mM stock solution by combining 200 μL of SAM-$D_3$ and 800 μL water. SAM-$D_3$ (20 μL injection) is resolved from SAH on a Phenomenex Sphereclone-ODS(2) 250×4.6 mm 5 μm HPLC column (room temp) by an isocratic mobile phase consisting of 0.05 % formic acid in 10 % methanol at a flow rate of 1.0 mL/min. An injection is set for every 3 min with UV detection at 257 nm with continuous signal collection viewed by chromatographic software. The eluent containing SAM-$D_3$ is collected manually as it immediately exits the UV detector (care should be taken to only collect SAM-$D_3$ peak). SAH may be injected on its own or in combination with SAM-$D_3$ to verify elution profile. For a representative chromatogram of SAM-$D_3$ and SAH *see* Fig. 2. The number of injections collected depends on the volume of internal standard being prepared. Once injections are complete, add enough mobile phase A to fill 50 mL conical vial and mix well. Analyze the purified SAM-$D_3$ by the LC-MS/MS method described above to determine the amount of SAM-$D_3$ collected. Target value is a peak height of at least $4 \times 10^{c4}$. (This value may be dependent on the mass spectrometer and background for SAM-$D_3$ MRM) Purified SAM-$D_3$ will require further dilution with mobile phase A to achieve this concentration. Also verify that unlabeled SAH is not present in the internal standard. Multiple 50 mL conical vials can be prepared and stored at −80 °C for up to 4 years.
3. A new standard curve (SAM and SAH) should be prepared with each analytical run to optimize method performance.
4. The controls are analyzed at the beginning of analysis, every five unknowns, and at the end of the assay as analysis verification.
5. Ion suppression effects were evaluated by sample infusion method. No significant interferences or ion suppression was identified.

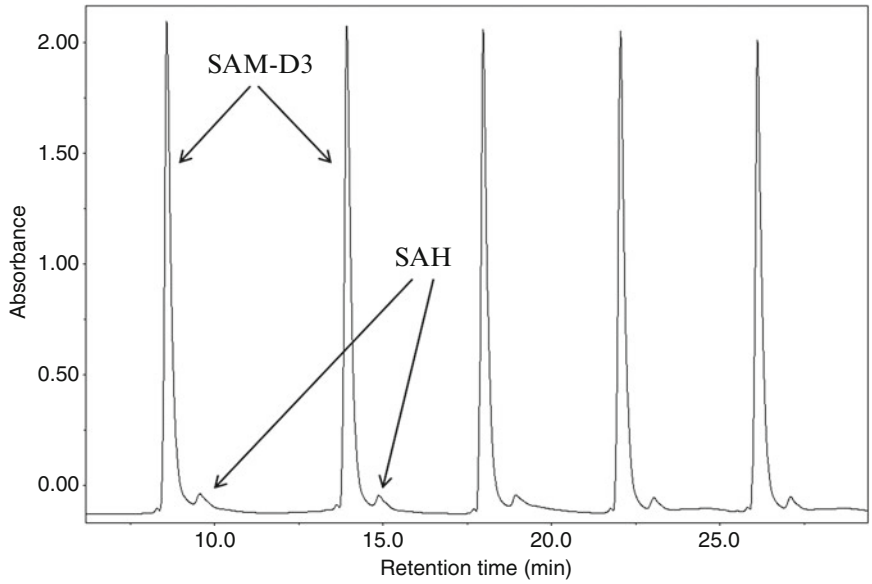



**Fig. 2** HPLC-UV chromatogram of D3-SAM. Multiple injections of 200 μM SAM-$D_3$ within same chromatographic run

## References

1. Cantoni GL (1985) The role of S-adenosylhomocysteine in the biological utilization of S-adenosylmethionine. Prog Clin Biol Res 198:47–65
2. Mudd SH (2011) Hypermethioninemias of genetic and non-genetic origin: a review. Am J Med Genet C Semin Med Genet 157C(1): 3–32
3. Bottiglieri T (2002) S-Adenosyl-L-methionine (SAMe): from the bench to the bedside—molecular basis of a pleiotropic molecule. Am J Clin Nutr 76(5):1151S–1157S

# Chapter 28

# A Simple, High-Throughput Method for Analysis of Ceramide, Glucosylceramide, and Ceramide Trihexoside in Dried Blood Spots by LC/MS/MS

**Wei-Lien Chuang, Joshua Pacheco, and Kate Zhang**

## Abstract

A unique monophasic extraction system coupled with LC/MS/MS to reduce matrix effects for sphingolipid analysis was developed. A solvent mixture of methanol, acetonitrile, and water was identified to simultaneously extract multiple sphingolipids with broad polarity range. To reduce matrix effects, the targeted sphingolipids were analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). The extraction solvent was used as an isocratic mobile phase in chromatographic separation to eliminate solvent exchange steps and enable high-throughput multiple lipid assay. The assay is linear for ceramide from 0.6 to 9 μg/mL with bias <15 %. The intra-assay coefficient of variation is less than 10 % for concentrations from 1.2 to 9 μg/mL, and less than 25 % for concentrations below 1.2 μg/mL. For glucosylceramide and ceramide trihexoside the linear range is 0.05–3 μg/mL with biases <10 % and <20 %, respectively. The intra-assay coefficient of variation for these analytes is less than 10 % at concentrations from 0.4 to 3 μg/mL, and less than 25 % for concentrations below 0.4 μg/mL.

**Key words** Sphingolipids, Dried blood spot, Monophasic lipid extraction, Quantitation, Liquid chromatography, Mass spectrometry

## 1 Introduction

Sphingolipids (SPLs) are a complex class of molecules that are found in essentially all animals, plants, and fungi and some prokaryotic organisms and viruses [1]. Remarkably, SPLs account for over 30 % of the total lipid in the plasma membrane [1, 2]. In addition, SPLs are commonly believed to protect the cell surface against harmful environmental factors by forming a mechanically stable and chemically resistant outer leaflet of the plasma membrane lipid bilayer. Certain complex glycosphingolipids have been found to be involved in specific functions, such as cell recognition and signaling [3–6]. Recently, relatively simple sphingolipid metabolites, such as ceramide (Cer) and sphingosine-1-phosphate, have been shown to be important mediators in the signaling cascades involved in apoptosis,

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_28, 

proliferation, and stress responses [7]. These discoveries raised the interest in understanding the sphingolipid (SPL) metabolic pathway and the exact lipid compositions of distinct subcellular membrane systems. It is known that Cer, glucosylceramide (GL-1, GluCer), ceramide trihexoside (CTH, GL-3, or GB3), gangliosides (GM1, GM2, and GM3), and galactosylceramide (GalCer) are involved in the sphingolipid metabolic pathway, and abnormal metabolism is associated with lysosomal storage diseases (LSDs) such as Gaucher, Fabry, Tay-Sachs, and Krabbe [8]. Simultaneous monitoring of multiple lipids may be helpful in understanding the disease mechanism.

Advances in mass spectrometric technology have provided great specificity and sensitivity in the characterization and quantification of SPLs in lipid extracts compared to conventional analytical technologies, such as high performance liquid chromatography, thin-layer chromatography, and gas chromatography [9]. A variety of sample preparation procedures, ionization models, and instrument designs have been developed to analyze SPLs by MS technology [10–12]. Among them, the extraction process is considered as one of the most critical steps in sample preparation for lipid analysis. Although the gold standard chloroform-methanol biphasic lipid extraction, known as the Bligh & Dyer method, proves to be a robust and sensitive procedure, the process is tedious and time consuming [13]. Recently, two monophasic extraction methods have been reported to address these issues. The first utilizes a chloroform/methanol/water solvent mixture and direct infusion nano-ESI-high resolution/accurate MS for the lipidome analysis in rat retinal samples [14]. The second utilizes an isopropanol/water/ethyl acetate mixture to simultaneously extract several classes of SPLs and is followed by quantitative LC/MS/MS analysis [15]. Compared to the Bligh & Dyer biphasic lipid extraction method, both monophasic strategies increase assay throughput and minimize the assay variance due to lipid loss in the biphasic interface. However, ion suppression effects caused by abundant co-extracted phospholipids need to be carefully evaluated and reduced.

The aim of the present study was to develop a simple, sensitive and chloroform-free LC/MS/MS technique for multiple SPL profiling and quantitation using human dried blood spots. We identified a monophasic lipid extraction mixture containing methanol/acetonitrile/water (80/15/5 %). This optimum ratio of solvents makes it possible to extract all targeted SPLs with a wide hydrophobicity range including Cer, GL-1, GL-3, GM1, GM2, and GM3. Chromatographic separation was utilized to minimize ion suppression effects due to interference from unwanted lipids and biomolecules [16] (Fig. 1). The strategic use of the extraction solvent as the mobile phase eliminated the need for a solvent exchange step before sample injection. This newly established method has demonstrated comparable precision and accuracy to traditional sphingolipid assays and can also be used to monitor lipid profile in research and clinical applications.

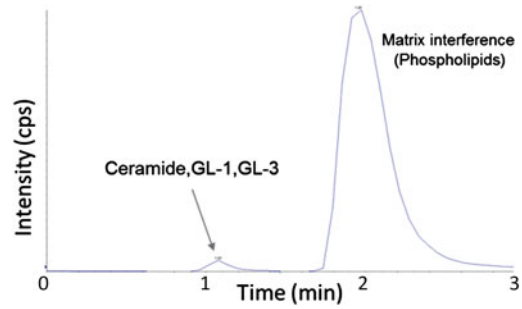



**Fig. 1** Typical elution profile for sphingolipids analysis by LC/MS/MS

## 2 Materials and Equipment

### 2.1 Samples

Venous blood is drawn into Vacutainer® tubes containing EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ), shipped on cold packs overnight, and held at 4 °C upon arrival. The blood samples are usually spotted within 24 h of collection.

### 2.2 Solvents and Reagents

1. Acetonitrile, Chloroform, Methanol, and Water are purchased from Burdick & Jackson (Morristown, NJ).
2. Trifluoroacetic acid is purchased from EMD Millipore (Billerica, MA).
3. Dimethyl sulfoxide is purchased from Sigma (St. Louis, MO).
4. Sample extraction solution (methanol/acetonitrile/water, 80/15/5 %, v/v/v): To make 1 L of solution, combine 800 mL methanol, 150 mL acetonitrile, 50 mL water in a 1 L glass bottle. Mix on a stir plate for 30 min prior to use.
5. HPLC mobile phase (methanol/acetonitrile/water (80/15/5 %) with 0.5 % trifluoroacetic acid): Combine 800 mL methanol, 150 mL acetonitrile, 50 mL water, and 5 mL trifluoroacetic acid in a 1 L glass bottle. Mix on a stir plate for 30 min prior to use.
6. 2:1 Chloroform/methanol: Combine 600 mL chloroform and 300 mL methanol in a 1 L glass bottle. Mix on a stir plate for 30 min prior to use.

### 2.3 Standards and Internal Standards

1. Glucocerebrosides (#1521), N-Octadecanoyl-D35-psychosine (#1914), Ceramide trihexosides (#1067), N-Heptadecanoyl ceramide trihexoside (#1523), and Ceramides (#1056) are purchased from Matreya, LLC (Pleasant Gap, PA).
2. C17-Ceramide (#860517) is purchased from Avanti Polar Lipids (Alabaster, AL).

### 2.4 Analytical Equipment and Supplies

1. Whatman 903 protein saver cards and MiniPax® absorbent packets are purchased from Sigma-Aldrich (St. Louis, MO).
2. Rocking platform shaker and Multi-Tube Vortexer are purchased from VWR (Radnor, PA).
3. Hot pocket column heater is purchased from Thermo Scientific (Waltham, MA).
4. Ultra silica column (#9100552) is purchased from Restek (Bellefonte, PA).
5. API5000 mass spectrometer is from AB SCIEX (Framingham, MA).
6. HTC-PAL autosampler is from Leap Technologies (Carrboro, NC).
7. 1200 series binary pump and automated degasser are from Agilent (Santa Clara, CA).

### 2.5 Standard Solutions Preparation

1. Individual standard stock solution preparation:
   (a) Standard stock solutions A (1 mg/mL): Weight 5 mg each standard into individual vials and dissolve each in 5 mL 2:1 chloroform/methanol solution. Vortex solutions for 5 min, sonicate for 10 min and then vortex an additional 5 min.
   (b) Standard stock solutions B (10 μg/mL): 100 μL of stock solution A is diluted in 9.9 mL 2:1 chloroform/methanol solution. Vortex solutions for 5 min
2. Standard mixture solution preparation (2.4 μg/mL Cer, 0.8 μg/mL GL-1, 0.8 μg/mL GL-3): From the Stock B solutions for each standard, 2.4 mL of Cer, 800 μL of GL-3, and 800 μL GL-1 are mixed in a vial and dried under nitrogen. The dried vial is then reconstituted in 10 mL of 2:1 chloroform/methanol. Vortex solution for 5 min, sonicate for 10 min, and then vortex an additional 5 min.

### 2.6 Internal Standards Solutions Preparation

1. Individual internal standard stock solution preparation:
   (a) Internal standard stock solutions A (1 mg/mL): Weight 5 mg each internal standard into individual vials and dissolve each in 5 mL 2:1 chloroform/methanol solution. Vortex solutions for 5 min, sonicate for 10 min, and then vortex an additional 5 min.
   (b) Internal standard stock solutions B (10 μg/mL): 100 μL of internal standard stock solution A is diluted in 9.9 mL 2:1 chloroform/methanol solution. Vortex solutions for 5 min.
2. Internal standard mixture solution preparation (3.75 μg/mL C17-Cer, 1.25 μg/mL N-Octadecanoyl-D35-psychosine, 1.25 μg/mL N-Heptadecanoyl ceramide trihexoside):

From the Stock B solutions for each internal standard, 3.75 mL of C17-Cer, 1.25 mL of N-Octadecanoyl-D35-psychosine, and

1.25 mL of N-Heptadecanoyl ceramide trihexoside are mixed in a vial and dried under nitrogen. The dried vial is then reconstituted in 10 mL of 2:1 chloroform/methanol. Vortex solution for 5 min, sonicate for 10 min, and then vortex an additional 5 min.

### 2.7 Preparation of Calibrators

Using standard mixture solution and internal standard mixture solution, pipette the volume shown (Table 1) into 1 mL total recovery vials to create calibrators 1–10. Dry calibrators under nitrogen and store at −20 °C. Reconstitute each calibrator in 200 μL pooled DBS extract (preparation shown below). Vortex the calibrator vials for 5 min, sonicate for 10 min, and vortex an additional 5 min.

### 2.8 Preparation of Control Samples

1. Using the standard stock solutions B and internal standard working solution mixture, pipette the volume (Table 2) into 4 mL glass vials to create low, medium, and high controls. Dry controls under nitrogen. Reconstitute each control in 4 mL pooled DBS extract (preparation shown below). Vortex the calibrator vials for 5 min, sonicate for 10 min, and vortex an additional 5 min. It's important to point out that actual QC level should be the combination of spiked and endogenous lipid.
2. 200 μL aliquots of each control are pipetted in total recovery vials, dried under $N_2$, and stored at −20 °C.
3. On the day of analysis, reconstitute an aliquot of each control in 200 μL of sample extraction solution. Vortex 5 min, sonicate 10 min, and vortex an additional 5 min.

**Table 1**
**Preparation of calibration standards**

| Calibration standards | Theoretical Cer/GL-1/GL-3 (ng/mL) | Standard mixture solution (μL) | Internal standard mixture solution (μL) |
|---|---|---|---|
| 1 | 0 | 0 | 40 |
| 2 | 150/50/50 | 12.5 | 40 |
| 3 | 300/100/100 | 25 | 40 |
| 4 | 600/200/200 | 50 | 40 |
| 5 | 1200/400/400 | 100 | 40 |
| 6 | 2400/800/800 | 200 | 40 |
| 7 | 3600/1200/1200 | 300 | 40 |
| 8 | 4800/1600/1600 | 400 | 40 |
| 9 | 6000/2000/2000 | 500 | 40 |
| 10 | 9000/3000/3000 | 750 | 40 |

**Table 2**
**Preparation of controls**

| Control | Spiked Cer/GL-1/GL-3 (ng/mL) | Cer stock solution B (μL) | GL-1 stock solution B (μL) | GL-3 stock solution B (μL) | Internal standard mixture solution (μL) |
|---|---|---|---|---|---|
| Endogenous level | 0/0/0 | 0 | 0 | 0 | 800 |
| Low | 240/80/80 | 96 | 32 | 32 | 800 |
| Medium | 1200/400/400 | 480 | 160 | 160 | 800 |
| High | 4800/1600/1600 | 1920 | 640 | 640 | 800 |

## 3 Methods and Procedures

### 3.1 DBS Preparation

Bring blood samples to room temperature for 10 min. Place on rocking platform shaker and gentle mix for 1 min prior to spotting (excessive rocking can lyse blood cells in the sample). Fold back the outer flap of Whatman 903 Protein Saver Cards so that the blood will soak only the inner filter paper. Spot 75 μL of blood in each printed circle with a single application of blood (do not layer blood). Allow to dry at room temperature at least 4 h. Place the cards inside a bag with absorbent packets and store at −20 °C.

### 3.2 DBS Extract Preparation

1. Punch a 3 mm (1/8 in.) disc from a 75 μL DBS and place into a microcentrifuge tube.
2. Pipet 200 μL of the sample extraction solution (methanol/acetonitrile/water, 80/15/5 %, v/v/v, *see* Subheading 2.2, **item 4**) into each tube.
3. Cap and vortex the tubes for 30 min in multi-tube vortexer.
4. Sonicate the tubes in a bath sonicator at room temperature for 10 min.
5. Centrifuge the tubes for 5 min at 16,200 × *g* in a microcentrifuge.
6. Transfer the supernatant to an autosampler vial.

### 3.3 LC/MS/MS Analysis

1. HPLC operating parameters:
   (a) Isocratic separation is done using an ultra silica column (5 μm, 100 Å, 50 × 2.1 mm) for a 5 min run at a flow rate of 150 μL/min. The mobile phase is methanol/acetonitrile/water (80/15/5 %) with 0.5 % trifluoroacetic acid (*see* **Note 1**).

(b) The column is held at 60 °C during the run.

(c) There is a three injection sequence to analyze each sample.

- The first injection is 10 μL of extracted sample.
- Second, inject 30 μL dimethyl sulfoxide to clear any remaining phospholipids from the column.
- Finally, a 30 μL injection of mobile phase re-equilibrates the system prior to injection of the next sample.

2. Mass spectrometer operating parameters are given in Tables 3, 4, and 5 (*see* **Notes 2** and **3**).
3. Data analysis
   (a) Samples are analyzed using Analyst Software version 1.5.
   (b) Peak integrations are performed on the TIC for each analyte, in the retention window from 0.7 to 1.4 min.
   (c) Standard curves are based on linear regression ($1/x$ weighted) of the analyte/IS peak area ratio ($y$) versus analyte concentration with typical correlation coefficients for the standard curve of $R^2 > 0.99$.
   (d) Sample concentrations are determined by interpolation of the peak area ratio from the calibration curve slope. This value is then multiplied by the assay dilution factor 62.5 (shown below) to determine the concentration of analyte in whole blood.

$$\text{Dilution factor} = \text{sample extraction solution volume}\left(200\ \mu\text{L}\right) / \text{amount of blood in 3mm punch}\left(3.2\ \mu\text{L}\right) = 62.5.$$

**Table 3**
**Source parameters in mass spectrometry analysis**

| Global source settings | |
|---|---|
| Collision gas (CAD) | 8 |
| Curtain gas (CUR) | 30 psi |
| Nebulizer gas (GSI) | 40 psi |
| Auxiliary gas (GS2) | 40 psi |
| Ion spray voltage (IS) | 5300 V |
| Temperature (TEM) | 300 °C |
| Interface heater (ihe) | On |

**Table 4**

**Internal standard MRM parameters in mass spectrometry analysis**

| C17-Ceramide | | | | | |
|---|---|---|---|---|---|
| Q1 Mass (Da) | Q3 Mass (Da) | DP (V) | EP (V) | CE (V) | CXP (V) |
| 534.2 | 264.4 | 84 | 15 | 30 | 15 |
| N-Octadecanoyl-D35-psychosine | | | | | |
| Q1 Mass (Da) | Q3 Mass (Da) | DP (V) | EP (V) | CE (V) | CXP (V) |
| 763.9 | 583.7 | 90 | 10 | 28 | 9 |
| N-Heptadecanoyl ceramide trihexoside | | | | | |
| Q1 Mass (Da) | Q3 Mass (Da) | DP (V) | EP (V) | CE (V) | CXP (V) |
| 1038.9 | 264.3 | 120 | 10 | 72 | 22 |

## 4 Notes

1. The current simple chromatographic method does not separate stereoisomers such as GL-1 and GalCer. Extra separation is recommended.
2. The current LC method only prevents major matrix effects by phospholipids and SPM interference, but not by in-source fragmentation between sphingolipids eluted at similar retention times due to their structural similarities. Declustering potential (DP) optimization should consider maximizing sensitivity and minimizing in-source fragmentation during the tuning process. For example, GL-1 could significantly contribute to Cer level when the DP for Cer was set for maximum sensitivity. Such interference should be evaluated quantitatively for samples used in the studies.
3. Potential interference from matrix crosstalk could occur for compounds with similar MRM transitions. For example, the MRM transition of C24:0-OH GL-3 (1152.9/264.2) could not be completely distinguished from the C16:0 GM3 transition (1153.7/264.3) due to instrument resolution limitations. A two-dimensional chromatographic separation, a more specific MRM transition, or high resolution MS could address the issues.

**Table 5**

**Analyte MRM parameters in mass spectrometry analysis**

| Ceramides | | | | | |
|---|---|---|---|---|---|
| Q1 Mass (Da) | Q3 Mass (Da) | DP (V) | EP (V) | CE (V) | CXP (V) |
| 520.5 | 264.4 | 84 | 15 | 30 | 15 |
| 548.5 | 264.4 | 84 | 15 | 30 | 15 |
| 564.5 | 264.4 | 84 | 15 | 30 | 15 |
| 576.6 | 264.4 | 84 | 15 | 30 | 15 |
| 604.6 | 264.4 | 84 | 15 | 30 | 15 |
| 620.6 | 264.4 | 84 | 15 | 30 | 15 |
| 630.6 | 264.4 | 84 | 15 | 30 | 15 |
| 632.6 | 264.4 | 84 | 15 | 30 | 15 |
| 684.6 | 264.4 | 84 | 15 | 30 | 15 |
| Glucosylceramides | | | | | |
| Q1 Mass (Da) | Q3 Mass (Da) | DP (V) | EP (V) | CE (V) | CXP (V) |
| 700.7 | 520.5 | 90 | 10 | 28 | 9 |
| 728.7 | 548.5 | 90 | 10 | 28 | 9 |
| 756.7 | 576.5 | 90 | 10 | 28 | 9 |
| 782.8 | 602.6 | 90 | 10 | 28 | 9 |
| 784.8 | 604.6 | 90 | 10 | 28 | 9 |
| 798.8 | 618.6 | 90 | 10 | 28 | 9 |
| 810.8 | 630.6 | 90 | 10 | 28 | 9 |
| 812.8 | 632.6 | 90 | 10 | 28 | 9 |
| Ceramide trihexosides | | | | | |
| Q1 Mass (Da) | Q3 Mass (Da) | DP (V) | EP (V) | CE (V) | CXP (V) |
| 1024.9 | 264.3 | 120 | 10 | 72 | 22 |
| 1052.9 | 264.3 | 120 | 10 | 72 | 22 |
| 1080.9 | 264.3 | 120 | 10 | 72 | 22 |
| 1106.9 | 264.3 | 120 | 10 | 72 | 22 |
| 1108.9 | 264.3 | 120 | 10 | 72 | 22 |
| 1134.9 | 264.3 | 120 | 10 | 72 | 22 |
| 1136.9 | 264.3 | 120 | 10 | 72 | 22 |
| 1152.9 | 264.3 | 120 | 10 | 72 | 22 |
| 1164.9 | 264.3 | 120 | 10 | 72 | 22 |

## References


1. Merrill AH Jr, Wang MD, Park M, Sullards MC (2007) (Glyco)sphingolipidology: an amazing challenge and opportunity for systems biology. Trends Biochem Sci 32:457–468
2. Patton JL, Lester RL (1991) The phosphoinositol sphingolipids of Saccharomyces cerevisiae are highly localized in the plasma membrane. J Bacteriol 173:3101–3108
3. Bielawski J, Pierce JS, Snider J, Rembiesa B, Szulc ZM, Bielawska A (2009) Comprehensive quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. Methods Mol Biol 579:443–467
4. Hannun YA, Obeid LM (2002) The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. J Biol Chem 277:25847–25850
5. Sastry PS (1985) Lipids of nervous tissue: composition and metabolism. Prog Lipid Res 24:69–176
6. Vos JP, Lopes-Cardozo M, Gadella BM (1994) Metabolic and functional aspects of sulfogalactolipids. Biochim Biophys Acta 1211:125–149

7. Giussani P, Tringali C, Riboni L, Viani P, Venerando B (2014) Sphingolipids: key regulators of apoptosis and pivotal players in cancer drug resistance. Int J Mol Sci 15: 4356–4392
8. Platt FM (2014) Sphingolipid lysosomal storage disorders. Nature 510:68–75
9. Ivleva VB, Sapp LM, O'Connor PB, Costello CE (2005) Ganglioside analysis by thin-layer chromatography matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometry. J Am Soc Mass Spectrom 16:1552–1560
10. Gu M, Kerwin JL, Watts JD, Aebersold R (1997) Ceramide profiling of complex lipid mixtures by electrospray ionization mass spectrometry. Anal Biochem 244:347–356
11. Liebisch G, Drobnik W, Reil M, Trumbach B, Arnecke R, Olgemoller B, Roscher A, Schmitz G (1999) Quantitative measurement of different ceramide species from crude cellular extracts by electrospray ionization tandem mass spectrometry (ESI-MS/MS). J Lipid Res 40:1539–1546
12. Mano N, Oda Y, Yamada K, Asakawa N, Katayama K (1997) Simultaneous quantitative determination method for sphingolipid metabolites by liquid chromatography/ionspray ionization tandem mass spectrometry. Anal Biochem 244:291–300
13. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
14. Lydic TA, Busik JV, Reid GE (2014) A monophasic extraction strategy for the simultaneous lipidome analysis of polar and nonpolar retina lipids. J Lipid Res 55:1797–1809
15. Bielawski J, Szulc ZM, Hannun YA, Bielawska A (2006) Simultaneous quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. Methods 39:82–91
16. Little JL, Wempe MF, Buchanan CM (2006) Liquid chromatography-mass spectrometry/mass spectrometry method development for drug metabolism studies: examining lipid matrix ionization effects in plasma. J Chromatogr B Analyt Technol Biomed Life Sci 833:219–230

# Chapter 29

# Quantification of Dehydroepiandrosterone, 11-Deoxycortisol, 17-Hydroxyprogesterone, and Testosterone by Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS)

**Ada Munar, Clint Frazee, and Uttam Garg**

## Abstract

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders due to enzymatic defects in the biosynthetic pathway of cortisol and/or aldosterone. The analysis of cortisol, 17-hydroxyprogesterone (OHPG), dehydroepiandrosterone (DHEA), 11-deoxycortisol, and testosterone is generally performed in the diagnosis and/or follow-up of CAH. Cortisol is generally analyzed by immunoassays whereas other hormones are preferably assayed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). A multiple reaction monitoring, positive mode atmospheric pressure chemical ionization, LC/MS/MS method is described for the simultaneous quantification of 17-hydroxyprogesterone, DHEA, 11-deoxycortisol, and testosterone. Stable-isotope labeled internal standards are added to serum samples and steroids are extracted by liquid-liquid extraction using methyl tert-butyl ether. The extract is evaporated under stream of nitrogen and the residue is reconstituted in methanol and analyzed by LC/MS/MS.

**Key words** Dehydroepiandrosterone, 11-Deoxycortisol, 17-Hydroxyprogesterone and testosterone, Tandem mass spectrometry (LC/MS/MS)

## 1 Introduction

Congenital adrenal hyperplasia (CAH) is a family of autosomal recessive disorders involving defect in cortisol synthesis in the adrenal cortex. This results in hyperplasia of the adrenal glands due to hypersecretion of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH). More than 90 % of CAH cases are due to deficiency of 21-hydroxylase that results in blockage of cortisol production and excessive androgen production. Depending on the severity of 21-hydroxylase deficiency, there is variable degrees of glucocorticoid and mineralocorticoid deficiency, and excess of sex steroids. This results in salt-wasting crises in the newborns and may result in incorrect gender assignment of

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis*: *Methods and Protocols*, Methods in Molecular Biology vol. 1378, DOI 10.1007/978-1-4939-3182-8_29, © Springer Science+Business Media New York 2016

virilized females [1, 2]. Newborn screening for CAH is performed to initiate early treatment that includes glucocorticoid and mineralocorticoid replacement therapy [3, 4].

Various steroid hormones are measured in the diagnosis and follow-up of CAH. Immunoassays are available for most CAH-related steroids; however, they suffer from cross-reactivity [5, 6]. GC-MS methods are also available for steroid analysis but are cumbersome because they involve extraction and derivatization steps [6–9]. In recent years, LC/MS/MS has become a preferred method for steroid analysis [6, 10–12]. Here we describe LC/MS/MS method for simultaneous measurement of 17-hydroxyprogesterone, DHEA, 11-deoxycortisol, and testosterone.

## 2 Materials

### 2.1 Samples

Serum from plain red or red gel is an acceptable sample. Store serum in freezer (<–20 °C) until analysis.

### 2.2 Solvents and Reagents

1. *tert*-Butyl methyl ether, HPLC grade.
2. Mobile phase A: water, HPLC grade.
3. Mobile phase B: methanol, HPLC grade.
4. Serum (Golden West Biologicals, CA) (*see* **Note 1**).

### 2.3 Internal Standards and Standards

1. 1 mg/mL stock standards: 17- Hydroxyprogesterone, 11-Deoxycortisol, Dehydroepiandrosterone, and Testosterone (Cerilliant, Round Rock TX).
2. 10 μg/mL primary combo standards: Prepared by transferring 100 μL of each stock to a 10 mL volumetric flask and diluting with methanol. Stable for 1 year at –20 °C.
3. 1 μg/mL secondary standards: Prepared by transferring 1 mL of primary standards to a 10 mL volumetric flask and diluting with methanol. Stable for 1 year at –20 °C.
4. 0.1 μg/mL tertiary standards: Prepared by transferring 1 mL of secondary standards to a 10 mL volumetric flask and diluting with methanol. Stable for 1 year at –20 °C.
5. 100 μg/mL primary internal standards: 17-Hydroxyprogesterone-*D8*, 11-Deoxycortisol-*D5*, Dehydroepiandrosterone-D5, and Testosterone-*D3* (Cerilliant, Round Rock TX).
6. 10 μg/mL secondary internal standards: Prepared by transferring 1.0 mL of each primary internal standard to a 10 mL volumetric flask and diluting with methanol. Stable for 1 year at –20 °C.

7. 100 ng/mL tertiary internal standards: Prepared by transferring 100 µL of secondary internal standard to a 10 mL volumetric flask and diluting with methanol. Stable for 1 year at −20 °C.
8. 10 ng/mL working internal standards: Prepared by transferring 1 mL of tertiary internal standard to a 10 mL volumetric flask and diluting with methanol. Stable for 1 year at −20 °C.

### 2.4 Calibrators and Controls

1. Calibrators: Prepare calibrators 1–7 according to Table 1.
2. Quality Controls: Prepare controls 1–3 according to Table 2 (*see* **Note 2**).
3. Add appropriate amount of standards to 10 mL volumetric flask and qs to 10 mL with ultra-low steroids serum.

### 2.5 Analytical Equipment and Supplies

1. AB Sciex LC/MS/MS 4000Q TRAP (Foster City, CA).
2. Shimadzu Prominence HPLC system with autosampler, two pumps, and degasser (Lenexa, KS).
3. Autosampler vials with caps.
4. Disposable culture tubes 13 × 100 mm.
5. Disposable culture tubes 10 × 75 mm.
6. Analytical column: Kinetex C18, 100 × 3 mm, 2.6 µm, 100 A (Phenomenex).
7. Guard column: Pinnacle, C18, 10 mm × 4 mm × 5 µm (Restek, Bellefonte, PA).

**Table 1**
**Preparation of calibrators using ultra-low steroids serum**

| Calibrator | Tertiary combo standards (µL) | Secondary combo standards (µL) | Serum volume | Final conc. (ng/dL) |
|---|---|---|---|---|
| Blank | | | 10 mL | |
| 1 | 10 µL | | qs to 10 mL | 10 |
| 2 | 20 µL | | qs to 10 mL | 20 |
| 3 | 50 µL | | qs to 10 mL | 50 |
| 4 | | 20 | qs to 10 mL | 200 |
| 5 | | 100 | qs to 10 mL | 1000 |
| 6 | | 200 | qs to 10 mL | 2000 |
| 7 (Testosterone only) | | | | 5 |

Note: prepare calibrator 7 (5 ng/dL) by diluting calibrator 1 using blank serum to 1:2

**Table 2**
**Preparation of quality controls using ultra-low steroids serum**

| QC | Tertiary combo standards (μL) | Secondary combo standards (μL) | Serum volume | Final conc. (ng/dL) |
|---|---|---|---|---|
| 1 | 50 μL | | qs to 10 mL | 50 |
| 2 | | 100 μL | qs to 10 mL | 1000 |
| 3 | | 160 μL | qs to 10 mL | 1600 |

## 3 Methods

### 3.1 Stepwise Procedure

1. Pipette 300 μL of each calibrator, control, and sample to the appropriately labeled 10 × 75 mm glass tubes.
2. Add 50 μL working internal standard to each tube.
3. Add 1.5 mL methyl tert-Butyl Ether (MTBE). Vortex and rock the tubes for 15 min.
4. Centrifuge the tubes for 2000 × *g* for 10 min.
5. Transfer the upper organic layer to a 13 × 100 mm.
6. Evaporate the extract to dryness under stream of nitrogen at 45 °C.
7. Reconstitute with 100 μL of methanol and vortex briefly.
8. Transfer reconstituted samples to the autosampler vials and inject 20 μL into LC/MS/MS for analysis.

### 3.2 Instrument Operating Conditions

Instrument's operating conditions are given on Table 3.

### 3.3 Data Analysis

1. Data are collected and analyzed using Analyst 1.5.1 software (AB Sciex, Foster City, CA).
2. Calibration curves are constructed from peak area ratios of MRM of calibrators and internal standards versus concentration.
3. A typical calibration curve has a correlation ($r^2$) > 0.99.
4. Compound-specific parameters for each analytes are given in Table 4.
5. Quality control samples are evaluated with each run. The run is considered acceptable if calculated concentrations of controls are within the ±20 % of target values.
6. Samples with results greater than upper limit of linearity should be diluted with blank serum.
7. A typical ion chromatogram for various steroids is shown in Fig. 1.

**Table 3**
**Instruments operating conditions**

| (a) HPLC | | |
|---|---|---|
| Time (min) | Mobile phase A (%) | Mobile phase B (%) |
| 0.1 | 90 | 10 |
| 2 | 90 | 10 |
| 3 | 60 | 40 |
| 13 | 5 | 95 |
| 15 | 90 | 10 |
| Column temperature—50 °C. Flow rate—0.55 mL/min | | |
| (b) MS/MS parameters | | |
| Curtain gas | 25 psi | |
| Source temperature | 500 °C | |
| Collision gas (CAD) | High | |
| Ion source gas 1 (GS1) | 50 psi | |

**Table 4**
**Compound-specific parameters**

| Analyte | Q1 mass (amu) | Q3 mass (amu) | Qualifier ion | Declustering potential (DP) | Collision energy (CE) | Cell exit potential (CXP) |
|---|---|---|---|---|---|---|
| 17-OHP | 331.3 | 97.1 | 109.2 | 80 | 38 | 17 |
| 17-OHP *D8* | 339.2 | 100.0 | | 80 | 40 | 17 |
| DHEA | 271.2 | 213.1 | 253.2 | 56 | 32 | 10 |
| DHEA-*D5* | 276.2 | 258.3 | | 61 | 21 | 16 |
| 11-Deoxycortisol | 347.1 | 97.0 | 109.2 | 71 | 37 | 6 |
| 11-Deoxycortisol *D5* | 352.2 | 100.0 | | 66 | 41 | 6 |
| Testosterone | 288.9 | 97.1 | 109.1 | 36 | 35 | 16 |
| Testosterone-*D3* | 292.4 | 97.1 | | 61 | 35 | 16 |

## 4 Notes

1. Validate serum before use for calibrators and quality controls to ensure the absence of each analyte.
2. When possible, calibrators and controls should be prepared from different lot of stock solution on separate days.

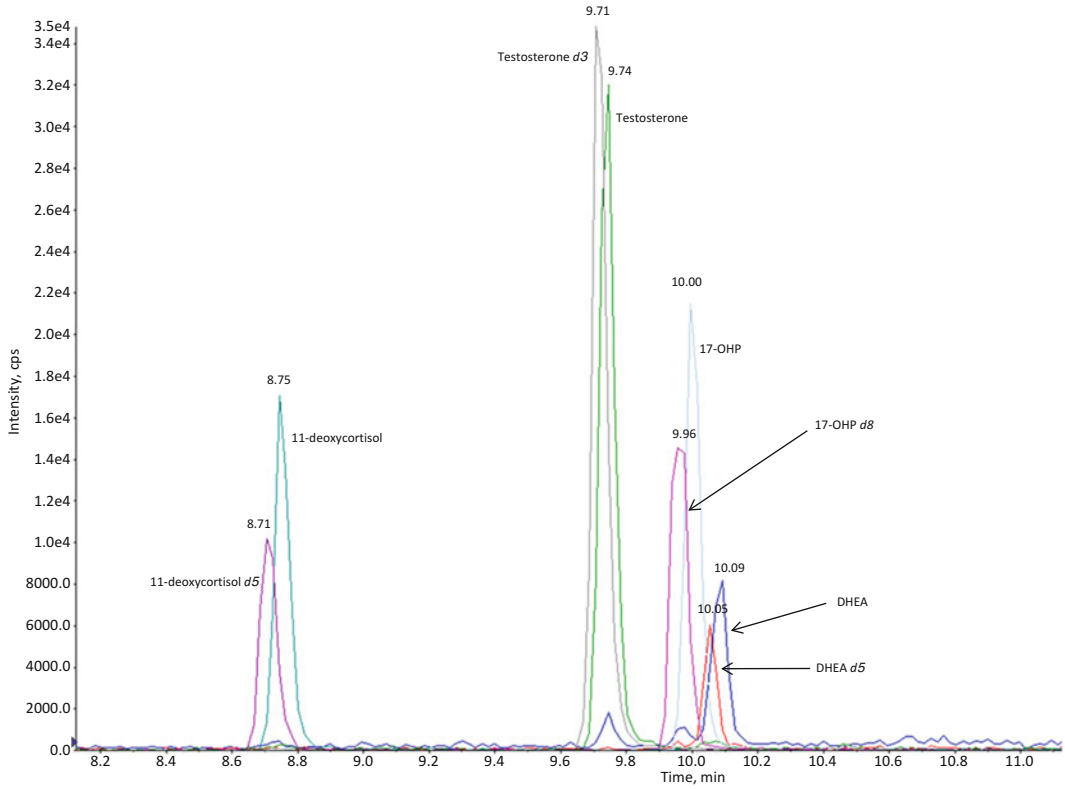



**Fig. 1** Ion chromatogram for various steroids

## References


1. Shaw AM (2010) 21-hydroxylase deficiency congenital adrenal hyperplasia. Neonatal Netw 29:191–196
2. White PC, Bachega TA (2012) Congenital adrenal hyperplasia due to 21 hydroxylase deficiency: from birth to adulthood. Semin Reprod Med 30:400–409
3. Heather NL, Seneviratne SN, Webster D, Derraik JG, Jefferies C, Carll J, Jiang Y, Cutfield WS, Hofman PL (2015) Newborn screening for congenital adrenal hyperplasia in New Zealand, 1994-2013. J Clin Endocrinol Metab 100:1002–1008
4. White PC (2013) Optimizing newborn screening for congenital adrenal hyperplasia. J Pediatr 163:10–12
5. Taieb J, Benattar C, Birr AS, Lindenbaum A (2002) Limitations of steroid determination by direct immunoassay. Clin Chem 48:583–585
6. Taylor AE, Keevil B, Huhtaniemi I (2015) Mass spectrometry and immunoassay; how to measure steroid hormones today and tomorrow. Eur J Endocrinol 173(2):D1–D12
7. Abdel-Khalik J, Bjorklund E, Hansen M (2013) Simultaneous determination of endogenous steroid hormones in human and animal plasma and serum by liquid or gas chromatography coupled to tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 928:58–77
8. Bulska E, Gorczyca D, Zalewska I, Pokrywka A, Kwiatkowska D (2015) Analytical approach for the determination of steroid profile of humans by gas chromatography isotope ratio mass spectrometry aimed at distinguishing between endogenous and exogenous steroids. J Pharm Biomed Anal 106:159–166
9. McDonald JG, Matthew S, Auchus RJ (2011) Steroid profiling by gas chromatography-mass spectrometry and high performance liquid chromatography-mass spectrometry for adrenal diseases. Horm Cancer 2:324–332
10. Fanelli F, Belluomo I, Di Lallo VD, Cuomo G, De Iasio R, Baccini M, Casadio E, Casetta B, Vicennati V, Gambineri A et al (2011) Serum steroid profiling by isotopic dilution-liquid chromatography-mass spectrometry: comparison

with current immunoassays and reference intervals in healthy adults. Steroids 76:244–253
11. Kulle AE, Welzel M, Holterhus PM, Riepe FG (2011) Principles and clinical applications of liquid chromatography-tandem mass spectrometry for the determination of adrenal and gonadal steroid hormones. J Endocrinol Invest 34:702–708
12. Stanczyk FZ, Clarke NJ (2010) Advantages and challenges of mass spectrometry assays for steroid hormones. J Steroid Biochem Mol Biol 121:491–495

# Chapter 30

# Urinary Succinylacetone Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

**Hongjie Chen and Chunli Yu**

## Abstract

Succinylacetone (SA) is used for the diagnosis and monitoring of patients with tyrosinemia type I (Tyr I). SA is exclusively elevated in blood and urine of patients with Tyr I. As urinary SA concentration is much higher than blood, SA is usually tested in urine samples. Urinary SA quantitation by gas chromatography mass spectrometry (GC-MS) is described in this chapter. The urine sample in the amount of 1 μmol creatinine is used for testing. 3,4,5,6,7-$^{13}$C5-succinylacetone ($^{13}$C5-SA) is used as an internal standard (IS). SA and $^{13}$C5-SA are oximated and extracted from urine with organic solvents, and then derivatized to form trimethylsilane (TMS) derivatives. TMS derivatives of SA and $^{13}$C5-SA are detected and quantified by GC-MS using selective ion monitoring (SIM). The assay is linear from 0.05 to 450 mmol/mol creatinine to cover the broad range of urinary SA concentrations.

**Key words** Succinylacetone, Tyrosinemia I, Gas chromatography, Mass spectrometry

## 1 Introduction

Tyrosinemia type I (Tyr I), also called hepatorenal tyrosinemia, is the most severe form of tyrosinemia (OMIM 276700) caused by a deficiency of fumarylacetoacetate hydrolase (FAH; EC 3.7.1.2), the enzyme catalyzing the conversion of fumarylacetoacetate (FAA) into fumarate and acetoacetate in the last step of tyrosine degradation pathway [1, 2]. Liver, renal tubules and peripheral nerves are primarily affected in Tyr I with variable clinical manifestations including acute liver failure, liver dysfunction, cirrhosis, hepatocellular carcinoma, renal Fanconi syndrome, and porphyria-like neurologic crises [1, 2]. The biochemical features of elevated tyrosine and elevated urinary excretions of tyrosine metabolites 4-hydroxyphenyllactate, 4-hydroxyphenylpyruvate and 4-hydroxyphenylacetate are similar to other tyrosinemias. However, the accumulations of intermediary metabolites prior to the block

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_30, 

are unique for this metabolic disorder. These metabolites include FAA, maleylacetoacetate (MAA), succinylacetoacetate (SAA) condensed from FAA and MAA, and succinylacetone (SA) spontaneously decarboxylated from SAA. All of these metabolites are highly toxic that lead to the symptoms of Tyr I [3]. Only SA is detected and exclusively elevated in blood and urine of patients with Tyr I; therefore it is clinically used as a diagnostic marker for this disease [3]. SA is also used as a surrogate marker for monitoring the efficacy of the NTBC therapy [4]. The elevation of δ-aminolevulinic acid (ALA) is the other unique biochemical feature for Tyr I resulting from the inhibitory effect of ALA dehydratase by SA [5].

SA is commonly tested in the urine specimen in the clinical laboratories as the urinary concentration is much higher than blood. In newborn screening (NBS) laboratories, SA is routinely analyzed from dried blood spot (DBS) specimen for screening Tyr I.

SA or 4, 6-dioxohepatanoic acid can be analyzed by either gas chromatography-mass spectrometry (GC-MS) methods [6–8] or liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods [9–11]. The two keto groups in SA structure make it difficult to measure directly by any analytical method. Several LC-MS/MS methods have been reported involving various derivatizations of keto group (s) to hydrozone, butyl esters or hydrazides followed by mass spectrometry measurement [9–11]. The analytical sensitivity has been greatly improved so that even the SA in DBS extract can be accurately measured [9, 11]. GC/MS methods have been used to analyze SA in clinical laboratories for decades. SA in the sample is treated with hydroxylamine to form 3(5)-methyl-5(3)-isoxazole propionate and extracted using liquid-liquid extraction with organic solvents and derivatized with trimethylsilane (TMS) or other derivative agents prior to GC/MS analysis [6–8]. GC-MS methods are still valid, sensitive, and accurate for urinary SA quantitation.

In this chapter, the protocol of urinary SA analysis by GC-MS is described in detail. Urine sample containing 1 μmol creatinine is used for the analysis. 3,4,5,6,7-$^{13}$C5-succinylacetone is added to each sample as internal standard (IS). After oximation reaction with hydroxylamine hydrochloride, SA is extracted from the sample using ethyl acetate/ethyl ether mixture (1:1 v/v), and derivatized with BSTFA plus 1 % TMCS at 80 °C. The TMS derivative of 3(5)-methyl-5(3)-isoxazole propionate isomers are separated and shown as doublet peaks in GC-MS chromatogram [6]. Chromatographic retention time and mass spectra under selective ion monitoring (SIM) mode are used for the identification and quantitation of SA.

## 2 Materials

### 2.1 Samples

Random urine collection without addition of preservatives (*see* **Note 1**). Samples are stable for 3 months at –20 °C. Positive samples need to be kept at –80 °C for long-term storage.

### 2.2 Reagents and Buffer

1. 5 % Hydroxylamine hydrochloride: Dissolve 25 g hydroxylamine hydrochloride in 500 mL of deionized water. Store at room temperature, protected from light for up to 1 year.
2. 5 *N* Sodium hydroxide (NaOH): Mix 100 mL of 10 *N* NaOH with 100 mL of deionized water. Store at room temperature for up to 1 year.
3. 5 *N* Hydrochloride (HCl) saturated with sodium chloride (NaCl): Mix 150 mL of 12 *N* HCl with 210 mL deionized water and 24 g NaCl. If you cannot see salt crystals at the bottom of the reagent container, add additional NaCl until no more will dissolve. Store at room temperature for up to 1 year.
4. Ethyl acetate/ethyl ether extraction mixture (1:1 v/v): Mix 450 mL of ethyl acetate and 450 mL of ethyl ether. Store at room temperature, protected from light for up to 1 month.

### 2.3 Standards and Calibrators

1. 5 mM SA stock solution for calibration: Dissolve 7.9 mg SA in 10 mL deionized water. Store at –80 °C indefinitely.
2. 500 µM SA working solution for calibration: Dilute 0.2 mL of 5 mM SA stock solution with 2.0 mL of deionized water. Prepare fresh and discard after calibration.
3. Prepare calibrators according to Table 1.

### 2.4 Internal Standards

1. 2.5 mM 3,4,5,6,7-$^{13}$C5-succinylacetone ($^{13}$C5-SA) internal standard stock solution: Dissolve 10 mg $^{13}$C5-SA in 24.4 mL deionized water. Store at –80 °C indefinitely.

**Table 1**

**Preparation of calibrators**

| | 500 µM succinylacetone (µL) | Water (µL) | Succinylacetone (nmol) |
|---|---|---|---|
| Cal 1 | 0 | 1500 | 0 |
| Cal 2 | 25 | 1475 | 12.5 |
| Cal 3 | 50 | 1450 | 25 |
| Cal 4 | 75 | 1425 | 37.5 |
| Cal 5 | 100 | 1400 | 50 |
| Cal 6 | 200 | 1300 | 100 |

2. 250 μM 3,4,5,6,7-$^{13}$C5-succinylacetone ($^{13}$C5-SA) internal standard working solution: Dilute 5 mL of 2.5 mM $^{13}$C5-SA stock solution with 45 mL of deionized water. Aliquot into 15 mL tubes, store at −80 °C. Keep one aliquot at 4 °C, protected from light for up to 3 months.

### 2.5 Quality Controls

1. Number and frequency: 2 levels of quality controls (QC) are run with every analytical batch.
2. Preparation and storage of QC materials

   Level 1 or QCL: 25 mL commercially purchased drug free human urine sample pool.

   Then succinylacetone concentration of 100 mmol/mol creatinine is equal to $N/1130$. Calculation is as follows:

   (a) Measure the blank urine creatinine level in mg/mL as $N$ mg/mL or $N/113$ mol/L.

   (b) Then succinylacetone concentration of 100 mmol/mol creatinine is equal to $N/1130$.

   Add $(N/1130) \times 158.15 \times 25$ mg succinylacetone to 25 mL blank urine. Calculate sample volume (containing 1 μmol creatinine) for one assay, make small aliquots with a bit more than you need for one assay and store at −80 °C until exhausted.
3. Validation and certification of QC lot: Target values for new control lot are established from 20 assays performed with the current controls. The acceptable range is defined as mean ± 2 SD.
4. Maintaining QC Lot: QC tracking lot must be created and maintained by person routinely performing test. When inventory is low (<30 aliquots), new QC lot needs to be prepared and validated prior to clinical use.

### 2.6 Supplies

1. 15 mL (16 × 125 mm) screw-cap glass tubes.
2. 10 mL (16 × 100 mm) screw-cap glass tubes.
3. Transfer pipettes.
4. Auto sampler vials with caps (VWR 9301-0978 and 5182-0550).
5. Nitrogen gas.
6. Helium gas.
7. GC column: Agilent HP-5 column with dimensions of 25 m × 0.20 mm × 0.33 μm.

### 2.7 Equipment

1. Agilent 7890 Gas Chromatography/5975 Mass Spectrometry (GC/MS) system with autosampler.
2. Glas-Col Evaporator.

3. Single-tube Vortex.
4. Multi-tube Vortex.
5. Eppendorf 5702 centrifuge.

## 3 Methods

### 3.1 Stepwise Procedure

1. Remove patient samples and quality control samples from freezer to thaw at room temperature. Set the temperature of the evaporator to 70 °C.
2. Prepare the worksheet: enter the creatinine values of the samples and calculate the sample volumes as equivalent to 1 μmol creatinine (0.113/creatinine in mg/mL).
3. Label one set of 15 mL glass tubes and two sets of 10 mL glass tubes (blank, QCL, QCH, patient samples) in numerical order as in the worksheet.
4. Pipette samples into labeled 15 mL tubes as per the volume calculated in the worksheet.
5. Add water to each sample as per calculations in the worksheet to bring the final volume up to 1.5 mL.
6. Add 100 μL 250 μM $^{13}$C5-SA internal standard (25 nmol) to all samples.
7. Start oximation reaction by adding:
   (a)400 μL 5 *N* NaOH
   (b) 800 μL 5 % hydroxylamine hydrochloride
   Cap the tubes, vortex each tube for 5 s and incubate at 70 °C for 30 min.
8. Remove the tubes from the heating block and allow tubes to cool for 5 min.
9. Add 800 μL NaCl-saturated 5 *N* HCl to each sample.
10. Add 6 mL extraction mixture (ethyl acetate/ethyl ether, 1:1 v/v) and cap the tubes.
11. Vortex for 10 min using multi-tube vortex (setting = 7.5) to extract the organic acids.
12. Allow samples to settle for 1 min to separate organic and aqueous layers.
13. Transfer organic (upper) layer of each sample to the first set of labeled 10 mL glass tubes with a transfer pipette. Leave a bit at the interface to avoid taking aqueous layer (*see* **Note 2**).
14. Add one medium scoop of sodium sulfate (~1 g) using a scoopula and vortex the tubes with bench-top vortex to absorb any moisture.

15. Centrifuge at 1500 × *g* for 3 min.
16. Decant the organic layer of each sample to the second set of labeled 10 mL glass tubes.
17. Place tubes containing extracted organic acids on heating block to evaporate liquid and dry under a steady stream of nitrogen for approximately 10 min at 70 °C (set $N_2$ pressure at 10 psi) (*see* **Note 3**).
18. Add 200 µL BSTFA/1 % TMCS to the dried extract, cap tubes, and vortex.
19. Place tubes in 80 °C heating block for 30 min for derivatization.
20. Allow samples to cool for 5 min and transfer to labeled sample vials for GC-MS analysis.

### 3.2 Instrument Operating Conditions

*See* Table 2 for instrument's operating conditions.

### 3.3 Data Analysis

1. The classic biochemical features of Tyr I are represented in the total ion chromatography (TIC) of urine organic acids profile from a Tyr I infant prior to NTBC treatment (Fig. 1a). The electron impact (EI) ionization mass spectra of TMS derivatives of oximated SA is shown in Fig. 1b.
2. The extracted ion chromatogram (EIC) mass spectrum of 212 and 217 from GC-MS selected ion chromatogram of TMS derivatives of oximated SA and $^{13}$C5-SA are shown as doublet peaks (Fig. 2). The ions used for identification and quantitation are listed in Table 3.
3. ChemStation Enhanced Data Analysis software is used for data analysis. The peak area ratio of SA/internal standard is used for constructing the calibration curve and calculating SA concentration in controls and clinical samples (*see* **Note 4**).
4. The calibration curves are accepted if correlation coefficient ($r^2$) > 0.99 and bias of each calibration level is within 15 % or 20 % at LLOQ and ULOQ.
5. Typical intra- and inter-assay imprecision is < 10 %.
6. The assay is linear from 0.05 to 450 nmol. Samples with a result above 450 nmol must be diluted and repeated.
7. Quality control: The run is considered acceptable if both QC values are within 2SD of the mean or one QC value is outside of 2SD but within 3SD while the second QC value is within 2SD.

**Table 2**

**GC operating conditions**

| | |
|---|---|
| *Inlet settings* | |
| Mode: splitless | Inlet temperature: 250 °C |
| Pressure: 19.59 psi | Total flow: 52.7 mL/min |
| Septum purge flow: 3 mL/min | Gas saver: 20.0 mL/min after 2 min |
| Purge flow to split vent: 49 mL/min at 1 min | Gas type: Helium |
| *Column* | |
| Mode: constant flow | Initial flow: 1 mL/min |
| Initial pressure: 19.6 psi | Holdup time: 1 min |
| *Oven temperature program* | |
| Initial temperature: 80 °C | Ramp 1: 15 °C/min from 80 °C to 200 °C |
| Ramp 2: 32 °C/min from 200 °C to 300 °C | Run time: 11.125 min |
| *Mass spectrometer* | |
| Tune file: atune.u | Acquisition mode: SIM |
| Solvent delay: 6 min | EMV mode: gain factor (1.00) |
| SA SIM: 212.10, 138.10; dwell: 100 ms | $^{13}$C5-SA SIM: 217.10, 142.10; Dwell: 100 ms |
| MS quad: 150 °C; MS source: 230 °C | Detector: off at 9 min |

## 4 Notes

1. Random urine sample is collected without addition of preservatives and saved at −20 °C till analysis.
2. Be careful not to transfer any remaining aqueous layer by leaving a bit of the organic (upper) layer of each sample at the interface.
3. Make sure the tubes containing extracted organic acids are dry before adding the derivatizing reagents.
4. Integrate the doublet peaks for peak area quantitation.

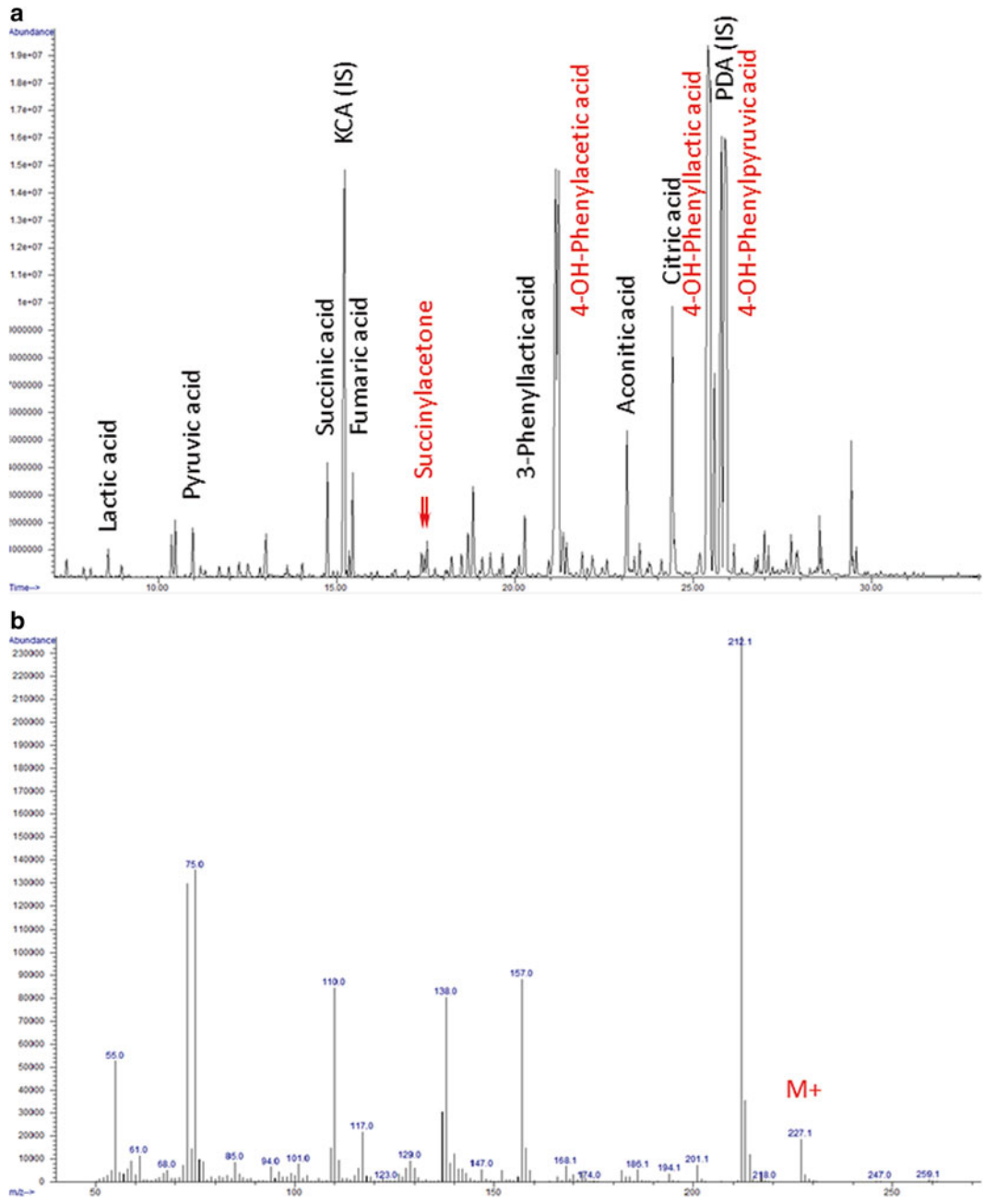



**Fig. 1** Representative urine organic acid profile of a 7-day-old male infant with tyrosinemia type I (Tyr I) prior to initiation of treatment. (**a**) Total ion chromatogram (TIC). α-Ketocaproic acid (KCA) and pentadecanoic acid (PDA) are used as internal standards (IS). Elevations of succinylacetone (SA), 4-hydroxyphenyllactic, 4-hydroxyphenylpyruvic, and 4-hydroxyphenylacetic acids (*marked in red texts*) are typical features of Tyr I. (**b**) Electron impact (EI) mass spectrum of mono-TMS derivatives of SA, represented as 3(5)-methyl-5(3)-isoxazole propionate isomers, derivative compounds from SA oximation. *M*+ molecular ion

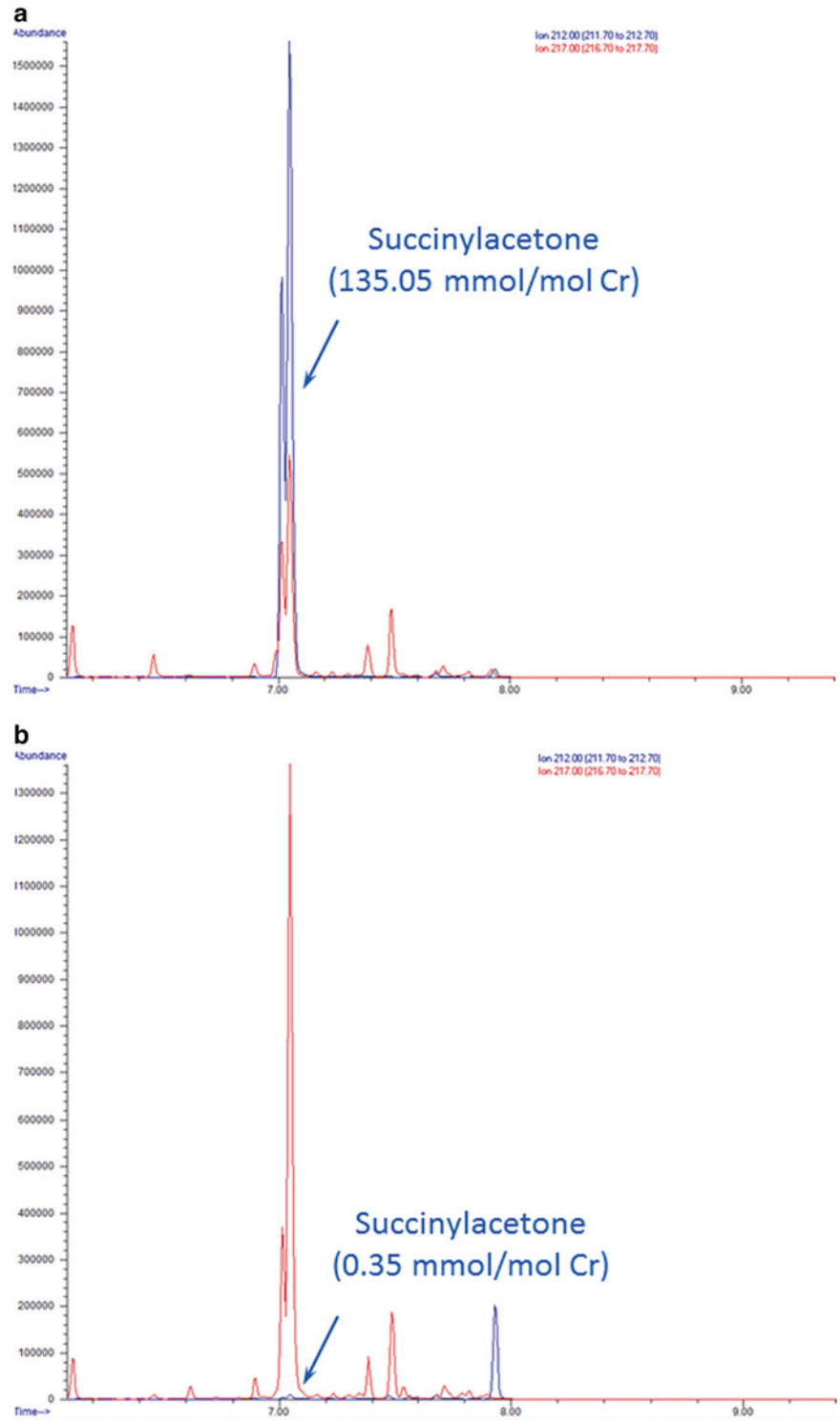



**Fig. 2** Extracted ion chromatogram (EIC) of succinylacetone (SA) and internal standard (IS) from the urines of a tyrosinemia type I (Tyr I) patient before (**a**) and after (**b**) treatment. SA (*m/z* of 212) is *marked in blue trace*. IS (*m/z* of 217) is marked in *red trace*. Both SA and IS are shown in doublet peaks representing 3(5)-methyl-5(3)-isoxazole propionate isomers. SA amount in the sample is visually reflected by the relevant abundance to the IS

**Table 3**

**Quantitation and qualifying ions for succinylacetone**

| Analyte | Quantitation ion | Qualifier ion |
|---|---|---|
| Succinylacetone | 212 | 138 |
| 3,4,5,6,7-$^{13}$C5-succinylacetone | 217 | 142 |

## References


1. de Laet C, Dionisi-Vici C, Leonard JV, McKiernan P, Mitchell G, Monti L, de Baulny HO, Pintos-Morell G, Spiekerkotter U (2013) Recommendations for the management of tyrosinaemia type 1. Orphanet J Rare Dis 8:8
2. Barnby E (2014) Tyrosinemia type 1: an overview of nursing care. Pediatr Nurs 40:61–68, 90
3. Lindblad B, Lindstedt S, Steen G (1977) Enzymic defects in hereditary tyrosinemia. Proc Natl Acad Sci U S A 74:4641–4645
4. Santra S, Baumann U (2008) Experience of nitisinone for the pharmacological treatment of hereditary tyrosinaemia type 1. Expert Opin Pharmacother 9:1229–1236
5. Sassa S, Kappas A (1983) Hereditary tyrosinemia and the heme biosynthetic pathway. Profound inhibition of delta-aminolevulinic acid dehydratase activity by succinylacetone. J Clin Invest 71:625–634
6. Tuchman M, Whitley CB, Ramnaraine ML, Bowers LD, Fregien KD, Krivit W (1984) Determination of urinary succinylacetone by capillary gas chromatography. J Chromatogr Sci 22:211–215
7. Jakobs C, Dorland L, Wikkerink B, Kok RM, de Jong AP, Wadman SK (1988) Stable isotope dilution analysis of succinylacetone using electron capture negative ion mass fragmentography: an accurate approach to the pre- and neonatal diagnosis of hereditary tyrosinemia type I. Clin Chim Acta 171:223–231
8. Schierbeek H, Berger R (1989) Determination of succinylacetone and succinylacetoacetate in physiological samples as the common product 5(3)-methyl-3(5)-isoxazole propionic-acid using an isotope-dilution method and mass-spectrometry. Clin Chim Acta 184:243–250
9. Allard P, Grenier A, Korson MS, Zytkovicz TH (2004) Newborn screening for hepatorenal tyrosinemia by tandem mass spectrometry: analysis of succinylacetone extracted from dried blood spots. Clin Biochem 37:1010–1015
10. Rashed MS, Al-Ahaidib LY, Al-Dirbashi OY, Al Amoudi M, Al-Sayed MM, Rahbeeni Z, Al-Hassnan Z, Al-Dbaas A, Al-Owain M, Ni Luanaigh M (2005) Tandem mass spectrometric assay of succinylacetone in urine for the diagnosis of hepatorenal tyrosinemia. Anal Biochem 339:310–317
11. Al-Dirbashi OY, Rashed MS, Jacob M, Al-Ahaideb LY, Al-Amoudi M, Rahbeeni Z, Al-Sayed MM, Al-Hassnan Z, Al-Owain M, Al-Zeidan H (2008) Improved method to determine succinylacetone in dried blood spots for diagnosis of tyrosinemia type 1 using UPLC-MS/MS. Biomed Chromatogr 22:1181–1185

# Chapter 31

# Quantification of 1,25-Dihydroxyvitamin D2 and D3 in Serum Using Liquid Chromatography-Tandem Mass Spectrometry

**Jonathon Mahlow, Dustin R. Bunch, and Sihe Wang**

## Abstract

1,25-Dihydroxyvitamin D is the active form of vitamin D and plays a critical role in the maintenance of calcium and phosphorous metabolism of the human body. Measurement of 1,25-dihydroxyvitamin D in serum can aid in clinical diagnosis and/or management of renal disease, sarcoidosis, and rare inherited diseases. We present here an effective and accurate method for measuring 1,25-dihydroxyvitamin D3 and 1,25-dihydroxyvitamin D2 by high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) after immunoaffinity extraction. The MS/MS is operated in multiple reaction mode with positive electrospray. Quantification is based on peak area ratios of the analytes to respective deuterated internal standards. This method offered a linear range from 4.0 to 160.0 pg/mL with analytical recovery of 89.9–115.5 % for both 1,25-dihydroxyvitamin D3 and 1,25-dihydroxyvitamin D2.

**Key words** 1,25-Dihydroxyvitamin D, Liquid chromatography, Mass spectrometry, Serum, Quantification

## 1 Introduction

There are two common forms of vitamin D, cholecalciferol (D3) and ergocalciferol (D2). D3 is obtained through biosynthesis from the 7-dehydrocholesterol precursor in the skin upon exposure to ultraviolet (UV) irradiation and from the diet. D2 is mainly derived from plant sources or manufactured by irradiation of ergosterol produced by yeasts [1]. D3 and D2 share the same metabolic pathway and are quickly converted into 25-hydroxyvitamin D [25(OH)D] by the liver followed by conversion primarily in kidney to the biologically active form 1α,25-dihydroxyvitamin D [1,25(OH)2D]. 1,25(OH)2D production is tightly regulated by parathyroid hormone, phosphorus and calcium [2]. In circulation, the majority of 1,25(OH)2D is tightly bound to vitamin D-binding protein (VDP) and nonspecifically to albumin. Serum 1,25(OH)2D measurement is useful for evaluating patients with chronic renal failure, hypo-

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_31, 

parathyroidism, hyperphosphatemia, hypomagnesemia, rickets, and granulomatous diseases [3].

1,25(OH)2D circulates at extremely low concentrations [normal range: 15–60 pg/mL] [4], and historically measured by radioreceptor assay (RRA) or radio-immunoassay (RIA). Both RRA and RIA require extensive sample preparation to remove interfering substances, such as cross-reactive vitamin D metabolites. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered a more accurate technology for measuring steroid-related compounds [5]. Direct LC-MS/MS analysis of 1,25(OH)2D is challenging due to the low concentrations in serum and lack of ionizable polar groups which leads to low ionization efficiencies. Attempts to increase ionization efficiency have been reported. 4-Phenyl-1,2, 4-triazoline-3,5-dione (PTAD), a Cookson-type reagent, is used to derivatize 1,25(OH)2D [6, 7] but can require long chromatography time (~27 min) [7]. Other approaches include adduct formation with ammonia [8] and lithium [9]. In this LC-MS/MS method, 1,25(OH)2D is quantified using the lithium adducts after one-step immunoaffinity extraction that efficiently removes interferences, as well as matrix effects, present in patient serum [10].

## 2 Materials

### 2.1 Samples

Serum is an acceptable sample for this procedure. Samples are stable for 24 h at room temperature, 1 week refrigerated, and 3 months frozen at −60 °C or below.

### 2.2 Solvents and Reagents (See Notes 1 and 2)

1. 50 mM lithium acetate dihydrate: Dissolve 0.79 g lithium acetate dihydrate in water and bring to a volume of 100 mL. Mix until completely dissolved. Rinse vacuum filtration apparatus with methanol and then water and allow to dryness. Filter the solution with 0.45 μm aqueous filter paper (stable for 2 weeks at room temperature).
2. 0.5 mM lithium acetate: Using a volumetric pipette, add 10 mL of 50 mM lithium acetate to a 1000 mL Class A volumetric flask. Bring to volume with water (stable for 2 weeks at room temperature).
3. 0.5 mM lithium acetate in methanol: Using a volumetric pipette, add 20 mL of 50 mM lithium acetate to a 2000 mL Class A volumetric flask. Bring to volume with methanol (stable for 1 month at room temperature).
4. 0.5 mM lithium acetate in 70:30 (methanol:water): Measure 1400 mL of 0.5 mM lithium acetate in methanol and pour into a 2000 mL bottle. Using a clean dry graduated cylinder, measure 600 mL of 0.5 mM lithium acetate in water and pour into the bottle. Mix by inversion (stable for 1 month at room temperature).

5. 7:2:1 (V:V:V) acetonitrile:isopropanol:acetone: Using a graduated cylinder, combine 700 mL acetonitrile, 200 mL isopropanol and 100 mL acetone in a 1000 mL bottle (stable for 1 year at room temperature).
6. 0.1 % formic acid in water: Add approximately 900 mL water to a 1000 mL Class A volumetric flask. Add 1 mL formic acid to the flask and bring to volume with water (stable for 1 month at room temperature).
7. Reconstitution solution (70:30, methanol:water): Using a graduated cylinder measure 75 mL water and pour into a 250 mL bottle. Then measure 175 mL methanol and pour into the bottle (stable for 1 year at room temperature).
8. 0.1 % glacial acetic acid in water:methanol (80:20): Using a graduated cylinder, add 800 mL water and 200 mL methanol to a 1000 mL bottle. Using a pipette, add 1 mL glacial acetic acid to the bottle and mix well (stable for 1 month at room temperature).
9. SeraCon II CD Human Serum from SeraCare Life Sciences, Milford, MA.
10. 1,25(OH)2D ImmunoTube (ImmundiagnostikAG, Bensheim, Germany).

### 2.3 Standards

1. Primary standards: 1α,25-Dihydroxyvitamin D3 (5 μg/mL); 1α,25-dihydroxyvitamin D2 (5 μg/mL) from Cerilliant Corporation, Round Rock, TX. Break one ampule of 1,25(OH)2D3 and one ampule of 1,25(OH)2D2 and transfer entire contents (~1 mL) into separate 1.5 mL labeled glass screw-top vials (stable until expiration on certificate of analysis at −60 °C).
2. Combined substock of 1,25(OH)2D3 and 1,25(OH)2D2 (10 ng/mL): Using the verified concentrations of the 1,25(OH)2D3 and 1,25(OH)2D2 stock standard solutions (as found on the Certificate of Analysis), calculate the volume of each needed to make 25 mL of substock standard. Then using a Hamilton syringe add the calculated volumes of each calibrator stock solution into the same 25 mL Class A volumetric flask. Bring to volume with 2-propanol (stable for 1 year at −20 °C).

*Example:*

Verified conc. stock 1,25(OH)2D3 = 4.866 μg/mL = 4866 ng/mL.

Desired conc. substock 1,25(OH)2D3 standard = 10 ng/mL.

Desired volume of working 1,25(OH)2D3 standard = 25 mL = 25,000 μL:

$$\text{Amt. of } 1,25(\text{OH})2\text{D3 std.} = \frac{10\text{ng}/\text{mL} \times 25{,}000\mu\text{L}}{4866\text{ng}/\text{mL}} = 51.4\mu\text{L}.$$

### 2.4 Internal Standards

1. 1α,25-Dihydroxyvitamin D3 (26,26,26,27,27,27-d6), Medical Isotopes, Pelham, NH.
2. 1α,25-Dihydroxyvitamin D3 (26,26,26,27,27,27-d6), Medical Isotopes, Pelham, NH.
3. Preparation of individual standard stocks (20 μg/mL): Check the vial label to ensure the content is approximately 1 mg. Add 0.5 mL 2-propanol to each vial then quantitatively transfer the contents of the individual d6-1,25(OH)2D2 and d6-1,25(OH)2D3 standard vials to separate 50 mL Class A volumetric flasks. Repeat 2-propanol transfer step 3 times to ensure complete transfer of material. Bring to volume with 2-propanol (stable for 2 years when stored at <−60 °C).
4. Preparation of individual internal standard substock (200 ng/mL): Using a Class A volumetric pipette, add 1 mL of each 20 μg/mL stock internal standard into separate 100 mL Class A volumetric flasks. Bring to volume with 2-propanol (stable for 2 years when stored at <−60 °C).
5. Preparation of combined working internal standard (d6-1,25(OH)2D3 and d6-1,25(OH)2D2): Using a Class A volumetric pipette add 1.1 mL of the d6-1,25(OH)2D3 200 ng/mL substock internal standard and 2 mL of the d6-1,25(OH)2D2 200 ng/mL substock internal standard into the same 100 mL Class A volumetric flask. Bring to volume with 70:30 methanol:water (stable for 1 year when stored at <−20 °C). This recipe will change based on the concentration of the original internal stock vial.
6. Preparation of injection standard (test mix): In a 100 mL Class A volumetric flask, add the following using a Hamilton glass syringe:
   (a) d6-1,25(OH)2D3 internal standard substock (50 μL; 200 ng/mL d6-1,25(OH)2D3).
   (b) d6-1,25(OH)2D2 internal standard substock (25 μL; 200 ng/mL d6-1,25(OH)2D2).
   (c) Combined substock calibration standard (500 μL; 10 ng/mL).
   (d) Bring to volume with 70:30 methanol:water (stable for 3 months when stored at <−20 °C).

### 2.5 Calibrators and Controls

1. Calibrators: Standard 5 Solution (160 pg/mL) [Note: Defrost and pool at least 300 mL of aliquoted SeraCon II for this step.]: Using a Hamilton syringe, add 1600 μL of the 10 ng/mL Combined substock to a 100 mL class A volumetric flask. Bring to volume with SeraCon II. (Note: Allow this solution to rest >1 min before further dilution.)

2. Cal 5 is serially diluted with volumetric pipettes into 125 mL bottles to prepare the rest of the working standards as shown in Table 1.
3. All working standards are aliquoted (0.6 mL) into labeled clear glass screw top 2 mL vials (stable for 6 months when stored at < −60 °C).

### 2.6 Analytical Equipment and Supplies

1. Onyx monolithic C18 columns (100 × 3.0 mm).
2. UV/visible spectrophotometer.
3. Savant SpeedVac.
4. Vortex genie mixer.
5. TLX2 Autosampler and HPLC (Thermo Fisher Scientific).
6. Liquid chromatography parameters include:
   (a) Mobile phase A: 0.5 mM lithium acetate in 70:30 methanol:water.
   (b) Mobile phase B: 0.5 mM lithium acetate in methanol.
   (c) Mobile phase C: 0.1 % glacial acetic acid in 80:20 water:methanol.
   (d) Injection volume: 40 µL.
   (e) Elution gradients, *see* Table 2.

**Table 1**
**Preparation of calibrators**

| To make calibrator | Use source solution | Volume of source solution (mL) | Volume of SeraCon II | Final concentration (pg/mL) |
|---|---|---|---|---|
| Cal 4 | Cal 5 | 50 | 50 | 80 |
| Cal 3 | Cal 4 | 25 | 75 | 20 |
| Cal 2 | Cal 3 | 50 | 50 | 10 |
| Cal 1 | Cal 2 | 50 | 50 | 5 |

**Table 2**
**Elution gradients**

| Method time (min) | Duration (s) | Flow rate (mL/min) | Step/ramp | Mobile phase A% | Mobile phase B% |
|---|---|---|---|---|---|
| 0 | 30 | 0.5 | Step | 100 | NA |
| 0.5 | 300 | 0.5 | Ramp | NA | 100 |
| 5.5 | 90 | 0.5 | Step | NA | 100 |
| 7 | 180 | 0.5 | Step | 100 | NA |

7. Thermo TSQ Vantage Tandem Mass Spectrometer (Thermo Fisher Scientific) parameters include H-ESI Probe settings as follows:
   (a) Spray voltage: 500 V.
   (b) Vaporizer temp: 325 °C.
   (c) Polarity: positive.
   (d) Sheath gas pressure: 45 psi.
   (e) Aux gas pressure: 5.0 psi.
   (f) Capillary temperature: 300 °C.
   (g) Skimmer offset: 0 units.
   (h) Sweep gas pressure: 5 psi.
   (i) S-lens: 120 V.
   (j) Collision pressure: 1.4 mtorr.
   (k) Collision energy: 25 eV.

## 3 Methods

### 3.1 Stepwise Procedures

1. Bring calibrators, controls, and samples to room temperature and mix thoroughly.
2. Perform daily maintenance and quality checks. Run test mix sample to assess system performance. If necessary, change ion transfer tube (*see* **Note 3**).
3. Acceptable system performance: Peak areas for 1,25(OH)2D3 and 1,25(OH)2D2 for three injections must be at least 70 % of the running average. Retention time must not deviate more than 0.2 min from the running average. If the performance is not considered acceptable by the above criteria, up to two further injections of the test mix may be done before troubleshooting becomes necessary.
4. Label one set of ImmunoTubes, one set of injection vials, and two sets of 12 × 75 mm glass tubes for each standard, control, and patient sample.
   (a) Blank
   (b) Calibration Standard 1
   (c) Calibration Standard 2
   (d) Calibration Standard 3
   (e) Calibration Standard 4
   (f) Calibration Standard 5
   (g) Low or high control
   (h) Patient

(i) Patient
(j) Patient
(k) High or low control (alternate level from beginning of batch)
(l) Blank
(m) TestMix_End

5. Place each ImmunoTube into one of its respective 12 × 75 mm tubes.
6. Uncap each ImmunoTube and ensure that each cap is coordinated to the tube from which it was originally removed. (Note: Do not remove caps until ready to pipette samples.)
7. Pipette 500 µL standard, control, and patient sample into its respective ImmunoTube.
8. Using a re-pipettor, add 25 µL working internal standard to each ImmunoTube.
9. Cap ImmunoTubes (ensuring that each cap is returned to its original tube) and invert tubes two times before placing nested samples on bench top tube rocker at room temperature for 60–90 min.
10. Remove the nested tubes from the bench top tube rocker.
11. Centrifuge tubes at 850 × *g* for 1 min in Silencer.
12. Remove nested tubes from centrifuge.
13. Snap off and discard the breakaway plug at the bottom of each ImmunoTube then remove the cap of each sample and place back into its respective glass tube.
14. Centrifuge tubes at 850 × *g* for 1 min in Silencer.
15. Using a re-pipettor, add 500 µL of water.
16. Centrifuge tubes at 850 × *g* for 1 min in Silencer.
17. Using a re-pipettor, add 500 µL of water.
18. Centrifuge tubes at 850 × *g* for 1 min in Silencer.
19. Using a re-pipettor, add 500 µL of water.
20. Centrifuge tubes at 1330 × *g* for 2 min in Silencer.
21. Remove nested tubes from centrifuge.
22. Transfer ImmunoTubes to the set of clean, labeled 12 × 75 mm glass tubes.
23. Using a re-pipettor, add 400 µL of 95 % ethanol to each extraction tube. *Do not allow any of this solvent to contact the outside of the ImmunoTubes.*
24. Wait for at least 1 min to provide time for the alcohol to facilitate release of the analyte from the beads.
25. Centrifuge tubes at 850 × *g* for 1 min in Silencer.

26. Remove nested samples from centrifuge.
27. Remove ImmunoTubes from 12 × 75 mm elution tubes and discard.
28. Place the glass tubes containing the eluate into a Savant SpeedVac set to medium heat and evaporate to complete dryness (~30 min).
29. Using a re-pipettor, add 100 µL of reconstitution solution (70:30 methanol:water) to each tube.
30. Vortex each tube for a minimum of 5 s to facilitate complete reconstitution.
31. Centrifuge in Silencer at 850 × *g* for at least 1 min.
32. Carefully remove the tubes from the centrifuge.
33. Using a 100 µL pipette, transfer the samples to their respective injection vials.
34. Cap vials and store at 2–8 °C or in refrigerated autosampler stack until analysis.

### 3.2 Analysis

1. Instrument operating parameters are given in Subheading 2.6 and an example of chromatography in Fig. 1.
2. Data are analyzed using LCQuan Software (Thermo Scientific).
3. Chromatograms are integrated and standard curves generated from the relative peak area ratio of each respective ion (d6-1,25(OH)2D3, 1,25(OH)2D3, d6-1,25(OH)2D2, 1,25(OH)2D2).
4. Concentration is based on the peak area ratio of the standard to the internal standard and the equation of the calibration curve.
5. Reference range: 1,25(OH)2D (Total) = 15.0–60.0 pg/mL.
6. The assay is thought to be linear over the following ranges:

   1,25(OH)2D2 = 4.0–160.0 pg/mL

   1,25(OH)2D3 = 4.0–160.0 pg/mL

## 4 Notes

1. Water—must meet Clinical Laboratory Standards Institute—Clinical Laboratory Reagent Water criteria (CLSI CLRW).
2. All reagents must be prepared using chromatography quality reagents, and stored in glassware specifically designated for LC-MS/MS only. General laboratory glassware contains contamination from detergents and must not be used. Only use bottles with Teflon-lined caps; do not use parafilm.

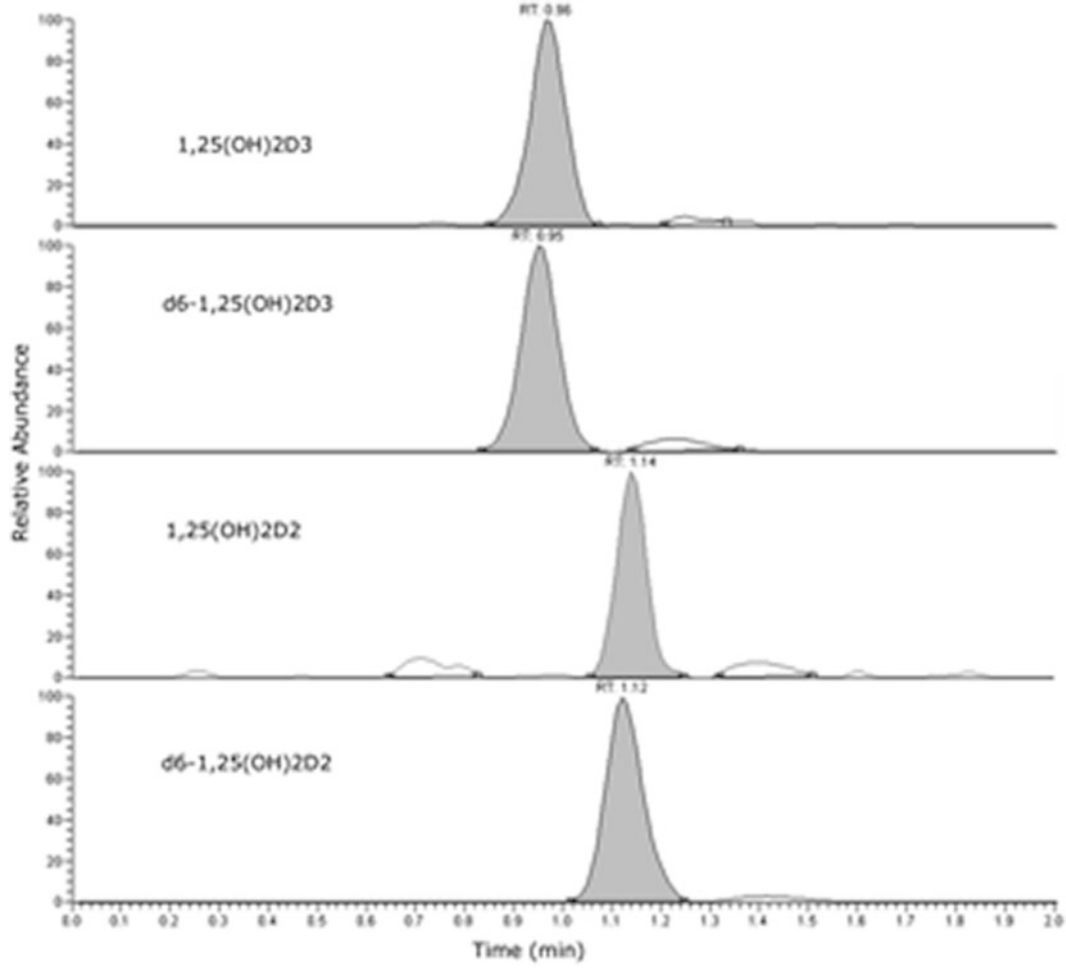



**Fig. 1** 1,25(OH)2D patient chromatogram

3. Cleaning ion transfer tubes (ITTs).
   (a) ITTs for 1,25(OH)2D testing must be changed prior to submitting a batch if the analytical system has been used for another project since the last 1,25(OH)2D batch.
   (b) To clean ITTs for Thermo Vantage.
      - Record ITT ID numbers.
      - Soak in glacial acetic acid (Fisher) ~24 h.
      - Rinse with methanol.
      - Sonicate in methanol for 15 min.
      - Dry.

## References


1. Wang S (2009) Epidemiology of vitamin D in health and disease. Nutr Res Rev 22:188–203
2. DeLuca HF (2008) Evolution of our understanding of vitamin D. Nutr Rev 66:S73–S87
3. El-Khoury JM, Reineks EZ, Wang S (2011) Progress of liquid chromatography-mass spectrometry in measurement of vitamin D metabolites and analogues. Clin Biochem 44:66–76
4. Burtis CA, Ashwood ER, Burns DE (2006) Tietz textbook of clinical chemistry and molecular diagnostics, 4th edn. W.B. Saunders, Philadelphia
5. Carter GD (2009) 25-Hydroxyvitamin D assays: the quest for accuracy. Clin Chem 55:1300–1302
6. Aronov PA, Hall LM, Dettmer K, Stephensen CB, Hammock BD (2008) Metabolic profiling of major vitamin D metabolites using Diels-Alder derivatization and ultra-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 391:1917–1930
7. Duan X, Weinstock-Guttman B, Wang H, Bang E, Li J, Ramanathan M, Qu J (2010) Ultrasensitive quantification of serum vitamin D metabolites using selective solid-phase extraction coupled to microflow liquid chromatography and isotope-dilution mass spectrometry. Anal Chem 82:2488–2497
8. Kissmeyer AM, Sonne K (2001) Sensitive analysis of 1alpha,25-dihydroxyvitamin D3 in biological fluids by liquid chromatography-tandem mass spectrometry. J Chromatogr A 935:93–103
9. Casetta B, Jans I, Billen J, Vanderschueren D, Bouillon R (2010) Development of a method for the quantification of 1alpha,25(OH)2-vitamin D3 in serum by liquid chromatography tandem mass spectrometry without derivatization. Eur J Mass Spectrom (Chichester, Eng) 16:81–9
10. Yuan C, Kosewick J, He X, Kozak M, Wang S (2011) Sensitive measurement of serum 1alpha,25-dihydroxyvitamin D by liquid chromatography/tandem mass spectrometry after removing interference with immunoaffinity extraction. Rapid Commun Mass Spectrom 25:1241–1249

# Chapter 32

# High-Throughput Serum 25-Hydroxy Vitamin D Testing with Automated Sample Preparation

**Judy Stone**

## Abstract

Serum from bar-coded tubes, and then internal standard, are pipetted to 96-well plates with an 8-channel automated liquid handler (ALH). The first precipitation reagent (methanol:$ZnSO_4$) is added and mixed with the 8-channel ALH. A second protein precipitating agent, 1 % formic acid in acetonitrile, is added and mixed with a 96-channel ALH. After a 4-min delay for larger precipitates to settle to the bottom of the plate, the upper 36 % of the precipitate/supernatant mix is transferred with the 96-channel ALH to a Sigma Hybrid SPE® plate and vacuumed through for removal of phospholipids and precipitated proteins. The filtrate is collected in a second 96-well plate (collection plate) which is foil-sealed, placed in the autosampler (ALS), and injected into a multiplexed LC-MS/MS system running AB Sciex Cliquid® and MPX® software. Two Shimadzu LC stacks, with multiplex timing controlled by MPX® software, inject alternately to one AB Sciex API-5000 MS/MS using positive atmospheric pressure chemical ionization (APCI) and a 1.87 min water/acetonitrile LC gradient with a 2.1 × 20 mm, 2.7 μm, C18 fused core particle column (Sigma Ascentis Express). LC-MS/MS through put is ~44 samples/h/LC-MS/MS system with dual-LC channel multiplexing. Plate maps are transferred electronically from the ALH and reformatted into LC-MS/MS sample table format using the Data Innovations LLC (DI) Instrument Manager middleware application. Before collection plates are loaded into the ALS, the plate bar code is manually scanned to download the sample table from the DI middleware to the LC-MS/MS. After acquisition—LC-MS/MS data is analyzed with AB Sciex Multiquant® software using customized queries, and then results are transferred electronically via a DI interface to the LIS. 2500 samples/day can be extracted by two analysts using four ALHs in 4–6 h. LC-MS/MS analysis of those samples on three dual-channel LC multiplexed LC-MS/MS systems requires 19–21 h and data analysis can be done by two analysts in 4–6 h.

**Key words** 25-Hydroxy vitamin D, Automated liquid handling, LC multiplexing, Automated data management, High-throughput

## 1 Introduction

This method and the associated automation processes described were developed at TPMG Kaiser Regional Laboratories in Richmond, CA, in response to rapidly increasing 25-hydroxy vitamin D test volumes and to dissatisfaction with the performance and cost of automated immunoassay testing for this analyte [1].

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis*: *Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_32, © Springer Science+Business Media New York 2016

Although LC-MS/MS technology had been in use at the Regional Laboratories for several years, all options in place for sample preparation and result entry to the LIS at that time were manual, with maximum batch sizes of 100–150 samples/day.

In order to cope with test volumes for 25-hydroxy vitamin D rising to 2500 samples/day, we chose to automate sample preparation using 96-well plates, ALHs, and serum protein precipitation combined with phospholipid removal [2, 3]. Although multiplexing solutions were available to increase throughput of the LC-MS/MS with up to four LC streams per mass spectrometer, we opted for the AB Sciex-Shimadzu MPX® multiplexing system with somewhat slower but less complex two LC streams per mass spectrometer [4]. Additionally, AB Sciex MPX® multiplexing software integrates well with AB Sciex Cliquid® batch control and reporting software, an application that had already been deployed at the Regional Laboratories with good staff satisfaction. Familiarity of staff with Shimadzu LC components was another factor that favored the choice of MPX®. The clinical laboratory middleware application, Instrument Manager, was well established and in wide use at Regional Laboratories, providing an opportunity to collaborate with AB Sciex, Hamilton Co., and DI as they developed an array of solutions to automate information transfer between instruments and with the Laboratory Information System (LIS) via the Instrument Manager application [5]. Guiding principles for automation design were (a) modular components, (b) verifying plate ID and orientation by scanning the plate barcode following every plate relocation, (c) no manual sample sorting, and (d) no manual information entry or transfer.

This consolidated system for automated LC-MS/MS sample and information handling proved to be robust, with excellent precision (between day coefficients of variation [CVs] between 3.5 and 7.5 %), better than expected maintenance-free intervals for the mass spectrometers (no cleaning other than scheduled 6-month preventative maintenance), a very low frequency of repeat testing, no failures in turnaround time, and good performance in external quality assurance schemes (proficiency testing). The automated data review/information management infrastructure has been successfully applied within the Regional Laboratories to other LC-MS/MS methods and across different models of AB Sciex mass spectrometers.

## 2 Materials

### 2.1 Reagents and Buffers

1. Mobile phase A (LC-MS-grade $H_2O$).
2. Mobile phase B (LC-MS-grade acetonitrile).
3. Load mobile phase (70:30 mobile phase A:mobile phase B).

4. Needle wash and wash B (LC-MS-grade methanol).
5. Wash C (LC-MS-grade acetonitrile).
6. 90:10 (vol:vol) Methanol:100 mg/mL ZnSO4 (first precipitation reagent).
7. Acetonitrile, 1 % formic acid (second precipitation reagent).
8. 25-Hydroxy vitamin D2 in ethanol, 50 µg/mL and 25-hydroxy vitamin D3 in ethanol, 100 µg/mL.
9. Unextracted standard mix for system suitability testing (SST)—6.3 ng/mL of 25-hydroxy vitamin D2, 25-hydroxy vitamin D2-d6, 25-hydroxy vitamin D3, and 25-hydroxy vitamin D3-d3 in 75:42:30:2.8 of acetonitrile 1 % formic acid:$H_2O$:methanol:100 mg/mL $ZnSO_4$.
10. Orange G serum pool for ALH liquid handler volume verification [6].

### 2.2 Calibrators/ Calibration

1. Calibrators are custom manufactured by Perkin-Elmer, supplied in sufficient volume to last for at least 6 months and traceable to the NIST Standard Reference Material (SRM) 972 for 25-hydroxy vitamin D. The calibration matrix is double charcoal stripped, delipidized serum with 25-hydroxy vitamin D2 and D3 concentrations <1 ng/mL. Calibrators are supplied ready to use, frozen, at 25 mL/vial, six vials/set. As needed, a set of six vials are thawed, mixed thoroughly, and 250 µL aliquots are transferred to barcoded tubes. Calibrator aliquots in sealed tubes are validated as stable for at least 3 months at −15 to −30 °C. Concentrations of 25-hydroxy vitamin D3 and 25-hydroxy vitamin D2 have target spiking values within ±20 % of:

*Barcode label concentration (ng/mL):*

STD_01 5.

STD_02 30.

STD_03 60.

STD_04 90.

STD_05 120.

STD_06 150.

### 2.3 Internal Standard and Quality Controls

1. Primary internal standards.
   (a) Deuterated 25-hydroxy vitamin D3 (26,26,26,27,27,27-d6) (25-hydroxy VitD3-d6).
   (b) Deuterated 25-hydroxy vitamin D2 (d3) (25-hydroxy VitD2-d3).
2. Working mixed internal standard: 160 ng/mL each of 25-hydroxy VitD2-d3 and of 25-hydroxy VitD3-d6 in 50:50 (vol:vol) methanol:water (*see* **Note 1**).

3. Quality controls.
   (a) A commercial, lyophilized, human serum-based, trilevel control is used. The manufacturer's instructions for reconstitution are followed.
   (b) After reconstitution, 250 µL aliquots are created in barcoded tubes, capped and have been validated as stable for up to 25 days at 2–8 °C.

### 2.4 Supplies

1. Orange G dye (for ALH volume verification).
2. Double-charcoal-stripped serum, VD-DC Mass Spec Gold from Golden West Biologics.
3. ALH tips (8-channel- Hamilton CORE® tips, 10–300 µL, carbonized for liquid-level sense, NTR [stacked] tips and 96 channel—clear, 200 µL, wide-bore tips [wide-bore to prevent clogging with precipitate]).
4. 2.1 × 20 mm, 2.7 µm, Ascentis Express C18 LC column (fused core particle).
5. Column Saver Pre-filter, 0.5 µm, from Mac-Mod Analytical, Chadds Ford, PA.
6. 50 mg/96-well Hybrid SPE® plates from Sigma-Aldrich.
7. 96-Well, 1.0 mL (half-height), conical bottom, polypropylene sample and collection plates.
8. 96-Well, flat-bottom, clear, polystyrene micro-titer plates for liquid handler volume verification.
9. Foil plate seals, 125 × 78 mm individual sheets, blue stripe indicates top side.
10. 13 × 75 mm polypropylene test tubes.
11. Barcode labels for plates—CTD Thermal label. 11/4″ × 2″, 2000/roll. Barcode labels for plates are printed with thermal transfer on label stock that is resistant to solvent fumes. If the wrong label stock and printer technology is used—the barcodes will become blurred, the background turns gray, and the barcode cannot be scanned reliably.

### 2.5 Equipment

1. For tube to plate step: Hamilton MicroLab STARlet 8-channel liquid handler, with Venus software, autoload option, barcode reader (for plates and tubes), grippers for moving plates, liquid-level sensing, and total aspiration and dispense monitoring (TADM) for clot detection. The deck is configured for one rack containing three plate stacker positions and two plate-filling stations, one rack of NTR (stacked) tips, one 3-position reagent container rack, and 30 sample racks for 13 mm diameter tubes.

2. For protein crash and filtration steps: Perkin-Elmer Zephyr 96-channel liquid handler with Maestro software, a gripper for moving labware, a barcode reader, a vacuum module and vacuum pump, and 12 deck positions for SBS footprint labware. The barcode reader was relocated to the right side of the instrument in order to read plate barcodes that have been placed on the side of the plate between the H12 and A12 wells (as required for the Hamilton STARlet alH barcode reader).
3. Zebra barcode label printers.
4. Manual heat sealers for applying foil seals to 96-well plates. Sealing protocol is 170 °C for 3 s.
5. Two Shimadzu Prominence LC stacks (two streams) per mass spectrometer are comprised of the following components:
   (a) Five binary pumps (two Pump As, two Pump Bs, one Loading pump).
   (b) Two temperature-controlled autosamplers.
   (c) Two temperature-controlled rack changers (permits storage of up to 12 plates/rack changer for automated introduction to the associated autosampler).
   (d) One temperature-controlled column oven (Nexera model instead of Prominence—*see* **Note 2**), holds two columns, one for each stream.
   (e) Two loading valves and one stream select valve (controls flows for LC multiplexing), one divert valve (directs LC flow to waste except for a 22 s window for peak elution from each stream).
   (f) PEEK tubing: Use of fused core column particle architecture and a short column length (2 cm) results in good LC efficiency (narrow, 3–5 s, LC peak widths) and LC pressures <4500 psi (310 bar) despite high flow rates and a 2.7 μm particle diameter. As pressures are routinely <4500 psi, user-friendly PEEK tubing, instead of stainless steel tubing, can be used to connect LC components and columns.
6. AB Sciex API5000 with Turboflow ion source and APCI probe.

## 3 Methods

### *3.1 Stepwise Extraction Procedure*

#### *3.1.1 Extraction Tube to Plate*

Using the STARlet 8-channel liquid handler for tube to plate pipetting and first crash solvent (two plate walk away capacity, consumables and samples for up to five plates can be placed on the deck before restocking is needed):

Load Samples, Reagents, and Consumables

1. Up to 9 barcoded 1 mL, conical bottom, polypropylene, 96-well plates are loaded in a defined order in the plate stacker rack on the STARlet deck (*see* **Note 3**).
2. Reagent containers of mixed working internal standard and of methanol:$ZnSO_4$ precipitation reagent are loaded on the deck. The internal standard container is capped to reduce evaporation with a custom lid designed for automated handling by one of the pipettor channels. The stability of the internal standard in the reservoir is validated for up to 12 plates when using the custom lid.
3. An empty reagent container is loaded for storage of the internal standard container lid while the internal standard solution is being pipetted.
4. Stacked NTR tips are loaded on the deck.
5. Up to 15 sample racks, each holding 32, 13 mm diameter ($13 \times 75$ mm or $13 \times 100$ mm) sample tubes, are placed on the autoload tray in front of the deck. Blanks, calibrators, and quality control (QC) materials are placed in rack positions defined differently for each of the plates extracted on the same liquid handler (*see* **Note 4**). Patient samples are placed randomly, in the order received, in rack positions between the predefined calibrator, blank, and QC rack positions. Minimum volume of serum in the sample tube for accurate pipetting is 250 μL.

Initiate Pipetting

1. The analyst starts the run with the Venus software and the STARlet scans sample tube barcodes (accession numbers) as the first 3 sample racks (96 samples) are drawn into the liquid handler deck using the autoload function.
2. Using the grippers the robot presents the first plate from the stacker to the barcode reader and then places it on the rear filling station.
3. The robot pipets serum from sample tubes, eight at a time, to all 96 wells. A fresh tip is used for each sample. Used tips are shucked to a waste bag off the deck (*see* **Note 5**).
4. The internal standard reservoir lid is removed by one of the pipet channels and stored on the empty reagent container.
5. Internal standard is pipetted into eight wells at a time, and mixed with serum by repeated aspiration/dispense cycles of the tips. A fresh tip is used for each well.
6. The lid is placed back on the internal standard reservoir.
7. The first precipitation reagent (90:10 methanol:$ZnSO_4$) is added to eight wells at a time and mixed with the tips. A fresh tip is used for each well.

8. The robot uses the grippers to move the filled plate to the front filling station. While the 8-channel liquid handler is filling the next plate, the analyst can pick up and move the first filled plate to the 96-channel liquid handler for further processing.
9. The sample accession numbers, matched to plate well locations (e.g., A1, H12), are associated with the plate barcode and written to a plate map file on the DI server as well as to a duplicate .txt file on the STARlet C drive (*see* **Note 6**).
10. The robot will use the grippers to present the next plate from the stacker to the barcode reader, place it on the back filling station, and begin pipetting the next set of 96 samples (*see* **step 6**).
11. Once a filled sample plate is removed from the 8-channel liquid handler, the plate barcode is manually scanned to print two replicate barcode labels from the Zebra barcode label printer (*see* **Note 7**).

### *3.1.2 Extraction Protein and Phospholipid Removal*

Using the Zephyr 96-channel liquid handler for the second crash reagent and filtration to remove proteins and phospholipids (two plate walk away capacity):

#### Load Reagents and Consumables

1. Three racks of wide-bore tips, a reagent container of precipitating reagent #2, and two Hybrid SPE® plates are placed on the deck of the 96-channel liquid handler.
2. A filled sample plate and empty collection plate with matching barcodes are placed on the deck of the liquid handler. A second set (second filled sample plate and empty collection plate with matching barcodes) can be added to the deck before or during processing of the first plate.

#### Initiate Pipetting

1. The extraction is started with the Maestro software.
2. The sample plate is picked up by the robot grippers, presented to the barcode reader for scanning, and returned to the original deck position.
3. The robot grippers move the empty collection (injection) plate to the barcode reader and the barcode label is compared to the sample plate barcode. If the barcodes match, the collection plate is placed in the well of the vacuum module. If not matched—an error message appears on the screen and the method aborts.
4. The robot grippers place the collar of the vacuum module over the collection plate in the well of the vacuum module.
5. The robot grippers place the first Hybrid SPE® plate on top of the collar.

6. The 96-channel pipettor transfers 1 % formic acid in acetonitrile (precipitating reagent #2) from the reservoir to all wells of the first sample plate. The acetonitrile tips are returned to the tip box. They are clean (no contact with samples) and can be reused (*see* **Note 8**).
7. The robot picks up a new set of wide-bore tips (sample tips #1), mixes the serum and precipitating reagents by multiple aspiration/dispense cycles of the tips, and then pauses above the plate for 4 min to let the heavier clumps of precipitate settle out (*see* **Note 9**).
8. The robot transfers 36 % of the supernatant from the sample plate 1 to the Hybrid SPE® plate on the vacuum module. Sample tips #1 are returned to the tip box (for discard).
9. The vacuum pump is turned on and the gantry of the 96 pipet head presses down on the Hybrid SPE® plate to insure a good seal and effective vacuum.
10. Precipitated proteins and phospholipids stay in the plate bed while serum supernatant is pulled through the plate by vacuum into collection plate 1.
11. The vacuum pump is turned off and the Hybrid SPE® plate and collar are disassembled by the robot.
12. Collection plate 1 is moved to the front of the deck, where it can be picked up by the analyst and a foil cover heat-sealed into place so the plate can be loaded into the LC-MS/MS. The same process continues without pause for sample and collection Plates 2.

### *3.2 Stepwise LC-MS/MS Analysis*

1. After performing daily LC-MS/MS maintenance (*see* **Note 10**) including a system suitability test (SST), the sample tables for two plates are downloaded from Instrument Manager to Cliquid-MPX® using the Instrument Manager ABSciex LIMS Compatibility driver. The plate barcodes are scanned into the respective fields for Stream 1 and Stream 2 in the Import Sample List File screen. The Cliquid-MPX® software interweaves the two sample tables in an injection queue such that samples are introduced alternately from Stream 1 and Stream 2 with multiplexed timing controlled by MPX®. Subsequent sets of two sample tables and two plates can then be introduced at any time, up to a maximum of 12 plates/rack changer or 24 plates/LC-MS/MS.
2. AB Sciex recommends, and validation testing confirmed, that a minimum of 30 min at operating APCI source temperature (300 °C) is necessary before starting a run to obtain optimal signal-to-noise (S/N) and signal stability. If the SST completes before the first two plates are ready to submit to the

LC-MS/MS queue, the APCI source is maintained at operating temperature by alternating discard injections from Stream 1 and Stream 2 until the plates are ready for injection.

3. Extracted plates are snapped into racks that then fit in to the Rack Changer drawers, three racks per drawer (*see* **Note 11**). While the system is actively injecting samples, opening the rack changer drawers to introduce plates is carefully timed around the autosampler inject cycle and movement of the rack changer robot to avoid instrument control errors/robot crashes that could stop the run/damage the robot.
4. Two multiplexed plates take about 4.4 h to finish injecting. In order to verify acceptable calibration and QC without having to wait until the first two multiplexed plates are finished injecting—the 11 wells at the beginning of the first plate on Stream 1 and on Stream 2 that contain blanks, calibrators, and QCs are submitted as two separate batches and the remaining patient samples in the first two multiplexed plates are submitted as a second set of two separate batches. As soon as they are completed, the batch with calibrators and QC can be analyzed with Multiquant® data analysis software and if any problems are identified, the run can be stopped with less than 30 min of run time lost.
5. During batch submission there is an option to print a results report. Instead, a simple list of samples per plate by injection order, associated with well, plate, stream, and LC-MS/MS system identifiers, is selected to print once the injection of the two plates is finished. No chromatographic peaks (extracted ion chromatograms—XICs), no peak information, no calibration information and no calculated results are included in the Sample List report because all data review is done on the screen, none from a printed report.
6. The final batches submitted for each stream are (a) ten injections from each stream of 50:50 methanol:water with APCI source temperature at 550 °C (source clean up/bake out method/sequence) followed by (b) methanol (wash B) and (c) acetonitrile (wash C) washes of the system prior to going to standby (all source and LC temperatures and gas and LC flows off). Without the source bake out routine after every overnight run—there is a progressive drop off in S/N to unacceptable levels after several weeks of running hundreds of samples/day. Including the source bakeout after every run is an effective preventative maintenance step, otherwise the only means to regain former sensitivity after exposing the source to several thousand extracted serum samples appears to be the replacement of all APCI source components.

### 3.3 Data Analysis

1. Stand-alone workstations (not connected to an LC-MS/MS instrument) running AB Sciex Analyst® and Multiquant® software are used for data analysis. Analyst® and Multiquant® can be configured to analyze data files that reside on another workstation (e.g., the PC controlling an LC-MSMS) through a local area network (LAN).
2. To create a Multiquant® result table (QSession)—calibrator data files from the appropriate plate (*see* **step 7** below) and all patient and QC files from a single plate are selected. Each plate/QSession is considered a separate batch that is either accepted or rejected for reporting based on LC-MS/MS data review and QC sample review. A data review checklist is completed and signed for each plate.
3. In Multiquant® the SignalFinder (no smoothing) integration algorithm is selected and a customized query flags failures for four quality parameters that characterize the acceptability of LC-MS/MS peaks.
4. The scanned plate barcode from the sample table is displayed in a custom field of the Multiquant® result table (QSession). The first step in data analysis is to verify that the plate barcode displayed in the QSession matches the data file names and the Sample List report file name.
5. Internal standard peaks (16/screen) are reviewed in MultiQuant Peak Review. Manual integration is rarely needed and must be approved by a supervisor. A metric plot of injection number versus internal standard peak areas is created with a drag to select the internal standard peak area columns in the result table and a click on the metric plot icon. Outliers of internal standard peak area (missed integration or no peak) are immediately obvious from the plot. The plot is interactive and the peak integration for a questionable point in the plot can be reviewed with one click.
6. Analyte peaks are reviewed and if necessary, integration is corrected.
7. Calibration curves are reviewed. Acceptance criteria for calibrations are $R^2 > 0.995$, % accuracy at the lower limit of quantitation (LLOQ) within ±20 %, % accuracy for all other calibrators within ±15 %. Minimum acceptable S/N at the LLOQ is 10, typically it is 40–50. The calibration scheme is:
   (a) Each LC stream is calibrated separately.
   (b) If there are more than two plates/stream—the first and last plates on each stream contain calibrators.
   (c) The default calibration protocol is use of calibrators from the first plate on a stream for plates in the first half of the run on that stream and use of calibrators from the last plate

on a stream for plates in the latter half of the run on that stream.

(d) Calibrator exclusion rules are as follows:

- A single calibrator outside the accuracy ranges can be excluded as long as QC are within range after exclusion. No more than one standard is excluded.
- Exclusion of STD_01 (LLOQ) as an alternative to batch failure is acceptable only if STD_01 from another plate on either stream of the same instrument, same day can be substituted with acceptable accuracy. Acceptable accuracy for patient and QC samples using calibrators from the first versus the last plate of the same stream or from another stream on the same instrument was validated in-house for up to 5 plates/stream (10 plates/instrument) representing ~22 h of continuous injections.
- If STD_06 is excluded—all samples > than the STD_05 concentration are not reported and are re-extracted.

8. The query adds a custom column to the result table (QSession) and flags samples with a comment in blue (reportable once correct integration is verified) or red (*not* reportable if correct integration is verified) text in the custom column under the conditions below. Integration is reviewed a second time for all (blue or red) flagged peaks/samples.

(a) An MRM qualifier/quantifier peak area ratio error is defined as a ratio more than ±25 % different from that of the average ratio for the calibrators. Samples with a 25-hydroxy vitamin D2 or D3 concentration > the LLOQ of 5 ng/mL and an MRM ratio error will generate a flag in red text (*not* reportable if correct integration is verified). Sample concentrations < the LLOQ and with an MRM ratio error will generate a blue text flag (reportable once correct integration is verified). The target range of ±25 % was derived from consensus guidelines and validated in-house [7].

(b) A relative retention time (RRT) error occurs when the RRt is outside the range of 1.00 to 1.06. Sample concentrations above the LLOQ and with an RRT error will generate a flag in red text (*not* reportable if correct integration is verified). Sample concentrations < the LLOQ and with an RRT error will generate a blue text flag (reportable once correct integration is verified). The acceptable range was derived from the in-house validation.

(c) A Peak Width error occurs when the peak width is outside the range of 0.02–0.12 min (1.2–7.2 s). Sample concentrations with a peak width error will generate a flag

in red text (review integration for bizarre peak shape, interfering peaks). The target range was established and validated in-house. Most peak widths are in the range of 0.03–0.08 min—but very small peaks may be <2 s and very large peaks may be greater than 5 s.

(d) An internal standard (I.S.) peak area error occurs when the I.S. peak area is outside the range of 50–150 % of the average I.S. peak areas for the calibrators. All samples with I.S. peak area errors will generate a flag in red text (*not* reportable if correct integration is verified). The target range was validated in-house.

9. QC results are reviewed for acceptability. Stream specific QC results are recorded automatically in the BioRad Laboratories Unity RealTime software when a finalized QSession file is uploaded to Instrument Manager.
10. The QSession file is saved with the plate barcode as the file name and a pdf report file (report by sample format) is created and stored electronically. No QSession reports are printed.
11. Interface: Results are uploaded from MultiQuant® to the Instrument Manager middleware in batch mode, one plate at a time, using an XML export template, the Instrument Manager ABSciex LIMS Compatibility driver, selection of "LIMS Transfer" from the File menu, and a single click on "Transfer." Instrument Manager rules prevent results with red failure flags (*see* **step 8** above) from being transmitted to the LIS (results are suppressed). Results with no flags or blue flags are automatically transmitted to the LIS for verification. Results with a total 25-hydroxy vitamin D (sum of D2 + D3) of < 5 ng/mL (repeated for confirmation) or >150 ng/mL (repeated on dilution) are also suppressed in Instrument Manager. Results for patients < 12 months of age are suppressed so the sample can be analyzed with a different method that does not detect the C3-epimer of 25-hydroxy vitamin D3 [8]. An Instrument Manager Exception Printer is configured to print a list of samples that have suppressed results and their associated failure flag(s) and sample locations whenever a batch (plate) is transmitted from MultiQuant® to Instrument Manager.

## 3.4 Instrument Operating Conditions

Loading pump flow rate: 1 mL/min

Injection volume: 20 µL

### 3.4.1 LC Parameters

Autosampler cooling temperature set point: 12 °C

Column oven set point: 40 °C

**Table 1**
**LC gradient**

| Elapsed time (min) | % Mobile phase B | Flow rate (mL/min) |
|---|---|---|
| 0 | 30 | 1.0 |
| 0.900 | 80 | 1.0 |
| 0.901 | 92 | 1.0 |
| 1.150 | 92 | 1.0 |
| 1.160 | 95 | 1.0 |
| 1.170 | 95 | 1.7 |
| 1.460 | 95 | 1.7 |
| 1.470 | 30 | 1.5 |
| 1.550 | 30 | 1.0 |
| 1.870 | 30 | 1.0 |

*See* Table 1 (LC Gradient)

### *3.4.2 Mass Spectrometer Settings*

Acquisition window

| | | |
|---|---|---|
| Start time: | 0.86 min | |
| End time: | 1.23 min | |
| Divert valve setting | Total time (min) | Position |
| 1 | 0.89 | B-Source |
| 2 | 1.20 | A-Waste |

Ionization—positive mode, APCI
Source parameters:

| | |
|---|---|
| Collision gas | 6.00 |
| Curtain gas | 35.00 |
| Gas 1 | 60.00 |
| Gas 2 | 60.00 |
| Temperature | 300 |
| Nebulizer current | 5.00 |

**Table 2**
**MRM parameters**

| Analyte | Q1 *m/z* | Q3 *m/z* | Dwell time (ms) | Purpose |
|---|---|---|---|---|
| 25-Hydroxy vitamin D3 (-$H_2O$) | 383.3 | 211.3 | 15 | D3 Quantifier |
| 25-Hydroxy vitamin D3 (-$H_2O$) | 383.3 | 229.2 | 15 | D3 Qualifier |
| 25-Hydroxy vitamin D3-d6 (-$H_2O$) | 389.3 | 211.3 | 15 | D3 Internal Standard |
| 25-Hydroxy vitamin D2 (-$H_2O$) | 395.3 | 269.2 | 15 | D2 Quantifier |
| 25-Hydroxy vitamin D2 (-$H_2O$) | 395.3 | 211.3 | 15 | D2 Qualifier |
| 25-Hydroxy vitamin D2-d3 (-$H_2O$) | 398.3 | 272.3 | 15 | D2 Internal Standard |

*See* Table 2—(MRM parameters)

### 3.5 Liquid Handler Volume Verification

Pipetting precision and accuracy for the liquid handlers was checked monthly using Orange G dye and a microplate reader [6]. Orange G was added to three different matrices: (a) pooled fresh serum, (b) 50:50 methanol:water, and (c) acetonitrile with 1 % (vol/vol) formic acid to verify pipetting performance for the serum, internal standard, and second precipitation reagent steps, respectively.

### 3.6 Preventative Maintenance Intervals

LC autosampler and pump preventative maintenance by the vendor (PM) was originally scheduled for once/year, but was eventually increased to four times/year. The stream select, loading, and divert valves did not have a scheduled PM; eventually a PM twice/year was scheduled. ALH PM was originally scheduled for twice/year, eventually increased to four times/year. MS/MS PM was scheduled for twice/year and no change was necessary.

## 4 Notes

1. Methanol concentrations <50 % for the working internal standard matrix were associated with decreased internal standard recovery (lower peak areas) as compared to methanol concentrations between 50 and 100 %. The goal for the working internal standard matrix was to minimize precipitation of serum proteins when adding internal standard to serum and to reduce the evaporation rate from the internal standard container (residing on the ALH deck for several hours) by using the lowest feasible methanol concentration. To further minimize evaporation of the working internal standard during the 4–6 h necessary to process 10–15 plates, Hamilton Co. designed and manufactured a custom lid for the internal standard container that could be removed and replaced as

needed by one of the 8 pipettor channels on the Hamilton STARlet model ALH.

We initially tested 25-hydroxy vitamin D2 labeled with six instead of with three deuteriums ($M+H=419\ m/z$) as an internal standard, but found that loss of water ($-18\ m/z$) in the source produced a precursor ion with the same mass as unlabeled 25-hydroxy vitamin D3 ($401\ m/z$). Adjusting source temperature and gas flows reduced, but did not eliminate signal at $401\ m/z$, so 25-hydroxy vitamin D2-d3 ($M+H-H_2O=398$) is used instead.

2. The default configuration for the AB Sciex-MPX®—Shimadzu dual-multiplexing LC stack is with a Prominence model column oven. Substitution of the Nexera model column oven (CTO-30A), with a height of 8.3 in. as compared to the 16.3 in. height of the Prominence column oven, allows all seven LC components to be stacked in three columns rather than four columns. The shorter distance between LC components leads to a decrease in length of the PEEK LC tubing connecting the components and therefore a decrease in extra-column dead volume, yielding higher efficiency (narrower peaks, lower quantitation limits) and the capability to run faster gradients (Fig. 1). The Nexera oven was redefined in the oven firmware as a Prominence model, in order to communicate correctly with the other LC components and AB Sciex software.
3. A standardized naming structure and extraction order for plate barcodes facilitates information transfer and allows predesignation of the intended location for each plate on one of the three LC-MS/MS systems. The plate barcode is also the file name for the plate map. The barcode includes the date of extraction (MMDDYY) and specifies location of the plates to be tested on each LC-MS/MS. The LC-MS/MS system is A, B or C; the Stream Number on that LC-MS/MS is S1 or S2; and the Plate Position within the rack changer for that stream is PP01 through PP12. For example: 051211_B_S1_PP02 would be Plate Position 2 on Stream 1, LC-MS/MS B, extracted on 5/12/11. The order for parallel processing of plates on the two STARlet liquid handlers (defined in the SOP by the order in which barcoded plates are loaded in the plate stackers) is optimized to complete extraction of two start-up plates (one plate for each stream is needed in order to start the run) for all three LC-MS/MS systems in the shortest possible time. As the step that limits throughput for the process is the LC-MS/MS (4–6 h to extract all samples but 16–22 h to inject all samples)—starting all LC-MS/MS runs as early as possible is critical to successful workflow. The advantage of designating the plate locations before extraction, as well as the order in which plates are extracted, is the capability to optimize and then rigorously stan-

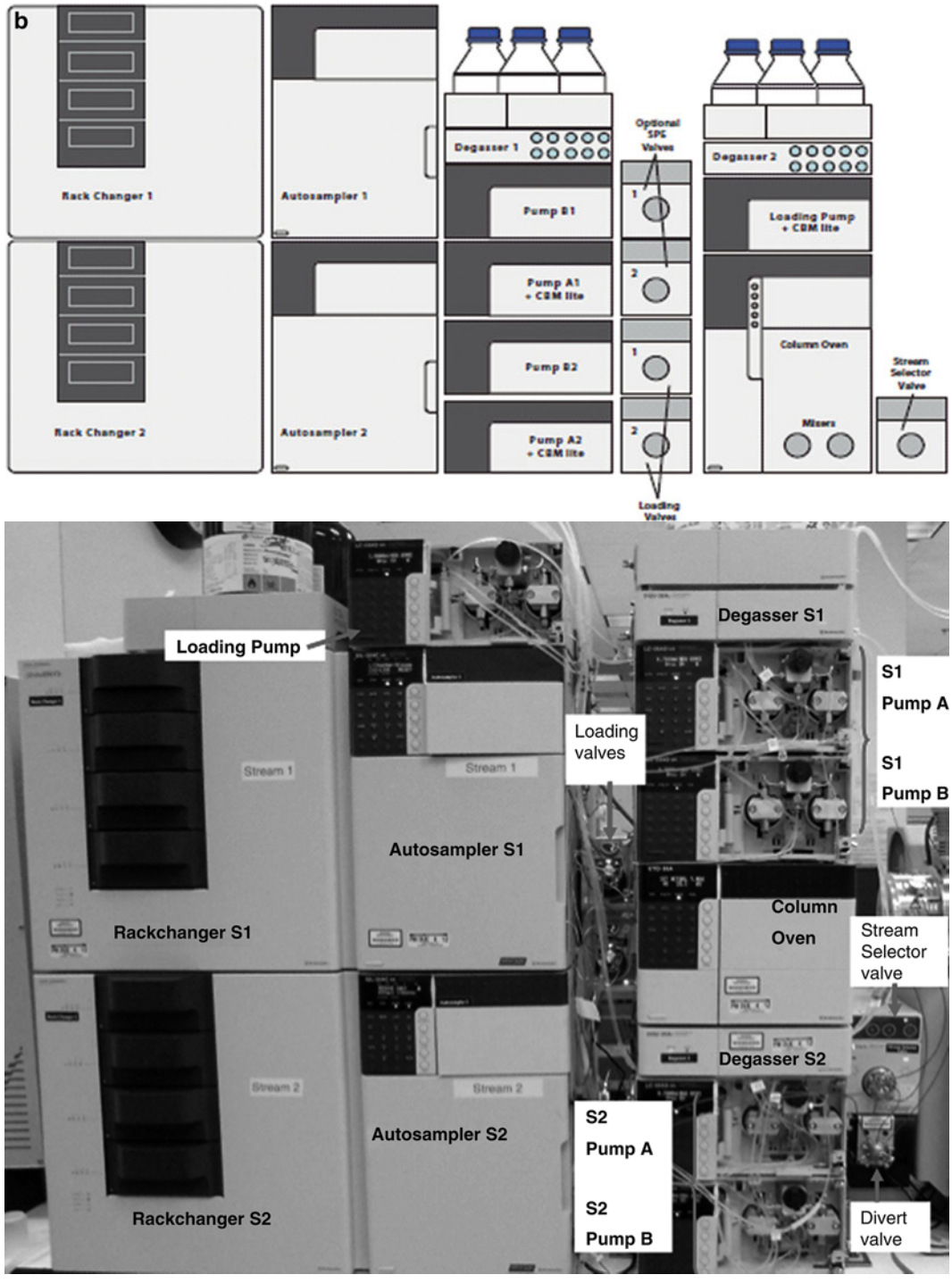



**Fig. 1** (**a**) Shimadzu MPX® LC stacks with modified configuration, using the Nexera model column oven (8.3″ high). (**b**) Shimadzu MPX® LC stacks with default configuration, using the Prominence model column oven (16.3″ high)

dardize the extraction and LC-MS/MS batch submission workflows for the best throughput. The disadvantage is the need to relabel plates, rename plate map files, and retransmit plate maps from the liquid handler to the DI middleware if a plate has to be relocated because an LC stream is out of service.

4. The first and last plate for each LC stream contains a set of six calibrators and blanks. Calibrator tubes are recognized as standards by the various software applications through the use of a prefix (STD_) in the barcode label. All three levels of QC material are placed on every plate but in wells/in an order unique to each plate extracted on each liquid handler and defined in the procedure. QC tubes are recognized as quality control samples by the various software applications through the use of a prefix (QC_) in the barcode label. As a precaution against sample misidentification—the expected versus observed location/order of QC results are checked during data analysis to verify that the correct plate was placed with the correct orientation in the correct plate position of the rack changer on the correct LC-MS/MS system.
5. The carbonized pipet tips move down into the tube seeking the liquid surface with capacitance level sensing and once detected, descend several millimeters below the meniscus before aspirating. The 8 pipet channels can adjust to different heights, as needed, for pipetting from levels of serum that vary between tubes (Z-span capability). The pressure pattern during aspiration is monitored (TADM) and compared to a reference pattern to detect clots and notify the user of pipetting errors. TADM can be customized with user defined parameters for optimal clot detection. If a clot is detected—the remaining seven samples are delivered to the wells but the well intended for that sample remains empty. The analyst is notified with a screen message and a printed report listing the sample ID and rack position of the clotted sample. After a plate is completed, the sample racks move off the deck back out to the autoload tray, the clot can be removed and the sample can be placed in a new rack position.

   Initially the robot was programmed to respond to clot detection with a pause after serum aspiration before delivering the other seven samples to the plate. The rack with the clotted sample would move back off the deck to the front autoload tray so the analyst could inspect and remove the clot before returning the sample and tray to the deck. Another attempt would be made by the robot to successfully pipet the problem sample and serum could then be delivered to all eight wells at once (recovery from a clotted sample without wasting a well). However—we found if there was too much delay by the analyst in returning the rack to the deck—the aspirated serum

could drip out of the other seven pipet tips, causing a false low result. The programmed response to a clotted sample was then revised as described.

6. The plate map is automatically transferred by the liquid handler software to DI middleware (using the Instrument Manager Hamilton STAR, STARlet, STARPlus Worklist driver) and reformatted there using the DI Worklist Generator module to match with the AB Sciex Cliquid® sample table. Nominal concentrations for the current lot of calibrators are automatically added to the sample table in DI when it is reformatted. The plate map cannot be displayed in the DI middleware (Instrument Manager); therefore a .txt copy of the file, for easy reference via MS Excel, is written to an archive directory on the liquid handler C drive.
7. One of the replicate barcode labels is applied to an empty 1 mL, conical bottom, polypropylene, 96-well plate—the matching collection (injection) plate for those samples. The other replicate barcode label is applied across the handles of the three racks of 32 samples each (total 96) that were sampled for that plate. The three sample tube racks are clamped together using a custom designed stainless steel handle and covered with parafilm for storage. Samples stored in this manner are simple to transport and resistant to tipping to one side, and can be stacked with a minimal footprint in the refrigerator. Samples needed for repeat testing are easily located by the plate barcode and well ID (mapped to sample rack position and rack ID).
8. We experienced sporadic problems with incomplete shucking of the acetonitrile wide bore tips because of static cling by tips to the 96-channel pipet head. Numerous strategies to reduce static charge on the deck were tested; none worked. Avoiding tip boxes that were individually wrapped in plastic and limiting the number of times the Acetonitrile tips were reused (maximum ten times) reduced the problems with static.
9. The original protocol called for addition of serum and precipitation reagent directly to the wells of the Hybrid SPE® plate, rather than in the sample plate. This protocol worked with frozen/thawed samples and calibrators, but with fresh patient serum we found that a few wells in each Hybrid SPE® plate became clogged with precipitated protein such that filtrate did not flow through to the collection plate. Precipitation in the sample plate followed by off-deck centrifugation and transfer of clear supernatant to the Hybrid SPE® plate prevented clogging, but was too cumbersome for routine production. A 4-min pause after precipitation/mixing in the sample plate,

followed by aspiration of only the upper third of the cloudy precipitate mixture left larger precipitates in the bottom of the sample plate, prevented clogging in the Hybrid SPE® plate, and allowed the entire extraction to be performed by the robot on the ALH deck.

10. Daily LC-MSMS maintenance
    (a) Check for sufficient volume of mobile phases and LC wash solutions.
    (b) Prime all mobile phases, prime autosampler needle wash if not used for >24 h.
    (c) If necessary—empty mobile-phase waste container.
    (d) Check the number of injections/column and change columns if necessary.
    (e) Clean the MSMS curtain plate and orifice with 50:50 methanol:$H_2O$ and an ultraclean sponge swab. Note that the hardware profile must be deactivated before removing the ion source. The curtain plate is handled only with a linen cloth and nitrile gloves to avoid contaminants. Cotton swabs and paper wipes of any kind are avoided to prevent fibers becoming caught in the orifice.
    (f) The LC pressures for both streams and for the loading pump are recorded after the LC has equilibrated and during the same carefully defined time interval of the injection cycle each day. A change in pressure > 300 psi (21 bar) from the previous day is cause for checking for a leak or a source of overpressure.
    (g) The SST is three injections of unextracted standard on each stream. The SST is run and evaluated after 30 min of discard injections to thermo-equilibrate the APCI source. The average of the three unextracted standard injections for each stream are recorded in a spreadsheet and evaluated against a minimum threshold and for downward trends.
11. After several months of use, the plastic tabs of the plate racks used in the rack changers proved to be inadequate to retain both sides of the plate flat on the rack during injection. When the injector pressed down on one side of the plate—the other side of the plate would tilt up. When the injector reached the wells on the tilted edge of the plate—a needle protect error occurred, presumably because when the needle descended to the defined depth before aspirating, it would hit the side or bottom of the well. AB Sciex modified the racks with metal clips that retained the plate more firmly in the rack.

## Acknowledgements

The author would like to thank the major contributors to this work: At TMPG Kaiser Regional Laboratories: Julia Drees, Ph.D., Scientific Director; Antonio DaSilva—Applications Development Manager, Jonathan Agustin—Automation Manager. At AB Sciex: Larry McAndrew, Christopher Borton, Ph.D., John Gibbons, Ph.D., Shamim Haider, Ph.D., Hua-fen Liu, Ph.D., Alexandre Wang, Ph.D. At Data Innovations: Don Jarvis, Patrick Kelly. At Hamilton Co.: Bret Martin. At Perkin-Elmer: Lynn Jordan, Benjamin Mendoza.

### References

1. Farrell C-J, Martin S, McWhinney B et al (2012) State-of-the-art vitamin d assays: a comparison of automated immunoassays with liquid chromatography–tandem mass spectrometry methods. Clin Chem 58:531–542
2. Grant RP (2011) High throughput automated LC-MS/MS analysis of endogenous small molecule biomarkers. Clin Lab Med 31: 429–441
3. Ismaiela OA, Zhang T, Jenkins RG et al (2010) Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry. J Chromatogr B 878:3303–3316
4. Grebe SKG, Singh RJ (2011) LC-MS/MS in the clinical laboratory—where to from here? Clin Biochem Rev 32:5–31
5. Online product guide for middleware (2014) College of American Pathologists. http://www.captdoayonline.com/productguides/software-systems/middleware-systems-June-2014. Accessed 28 Feb 2015
6. Stangegaard M, Hansen AJ, Frøslev TG et al (2011) A simple method for validation and verification of pipettes mounted on automated liquid handlers. J Lab Autom 16:381–386
7. CLSI (2014) Liquid chromatography-mass spectrometry methods; approved guideline. CLIS document C62-A. Clinical and Laboratory Standards Institute, Wayne, PA
8. Couchman L, Benton CM, Caietan FM et al (2012) Variability in the analysis of 25-hydroxyvitamin D by liquid chromatography–tandem mass spectrometry: the devil is in the details. Clin Chim Acta 413:1239–1243

# Chapter 33

# Quantitation of 25-OH-Vitamin-$D_2$ and 25-OH-Vitamin-$D_3$ in Urine Using LC-MS/MS

**Dean C. Carlow, Ryan C. Schofield, and Michelle Denburg**

## Abstract

Patients with significant proteinuria represent a unique population with respect to vitamin D status due to the urinary losses of vitamin D-binding protein (DBP) to which >99 % of circulating 25-hydroxy vitamin D (25(OH)D) is bound. Low serum concentrations of 25(OH)D have been found in children and adults with nephrotic syndrome (NS). However, previously described assays developed to quantify the magnitude of urinary loss are technically challenging. This chapter describes a simple and sensitive method to quantify 25(OH)$D_2$ and 25(OH)$D_3$ in urine specimens in a single analytical LC-MS/MS analysis. This assay is more sensitive than previously described radioimmunoassays and offers the ability to quantitate both forms of 25-hydroxy vitamin D. The assay involves no chemical derivitization, has a linear measurement range of 20–1500 pg/mL and displays imprecision (CVs) below 7 % at various concentrations across the analytical measurement range.

**Key words** 25-Hydroxy vitamin $D_2$, 25-Hydroxy vitamin $D_3$, Mass spectrometry, Liquid chromatography, Nephrotic syndrome, Urine

## 1 Introduction

Vitamin D deficiency is common worldwide, and its adverse effects extend beyond bone and mineral metabolism. Numerous studies have demonstrated associations with mortality, cardiovascular disease, insulin resistance, diabetes, autoimmunity, infection, and inflammation [1]. The kidney plays a major role in vitamin D homeostasis, and vitamin D deficiency in turn may contribute to podocyte injury [2, 3]. Vitamin D metabolites circulate highly bound, either to vitamin D-binding protein (DBP, 85–88 %) or albumin, with <1 % in their free forms [4]. In the kidney, vitamin D-DBP complexes are freely filtered by the glomerulus and reabsorbed via megalin/cubilin-mediated endocytosis in the proximal tubule. The conversion of 25-hydroxy vitamin D (25(OH)D) to 1,25-dihydroxy vitamin D depends on this endocytic pathway [5], and animal studies have shown that blocking it has considerably

Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8_33, 

greater adverse effects on mineral metabolism and bone health than DBP deficiency [6, 7].

Patients with nephrotic syndrome (NS) present unique challenges to the assessment of vitamin D status. NS is the most common chronic kidney disease in childhood, and glomerular diseases associated with NS and nephrotic-range proteinuria are leading causes of end-stage kidney disease in children and young adults. Many small studies have documented very low 25(OH)D concentrations in patients with NS [8–16], attributed to the urinary loss of DBP and albumin, however the pathophysiology is poorly understood. Most of these studies reported low serum DBP concentrations in NS [8, 11, 12]. Studies that directly quantify urinary loss of 25(OH)D, rather than DBP, in NS are very limited and involved the use of radioactive tracers [11, 14].

The ability to reliably quantify urinary loss of 25(OH)D is fundamental to understanding aberrant vitamin D homeostasis in the setting of proteinuria. Previously reported methods employed for quantifying urinary vitamin D metabolites have involved laborious procedures not amenable to high-throughput analysis [14, 17]. This chapter describes a simple and sensitive method to quantify $25(OH)D_2$ and $25(OH)D_3$ in urine specimens in a single analytical LC-MS/MS run. This assay is more sensitive than previously described radioimmunoassays and offers the ability to quantitate both forms of 25-hydroxy vitamin D with a higher throughput.

## 2 Materials

### 2.1 Samples

Urine samples are acceptable for this procedure. Samples are stable 10 days when refrigerated or 4 months when frozen at −20 °C.

### 2.2 Solvents and Reagents

1. Mobile phase A (2.5 mM ammonium formate in water containing 1 % methanol): Add 980 mL of water to a 1 L graduated cylinder. To the cylinder add 10 mL methanol and 10 mL of 250 mM ammonium formate. Decant into a 1 L HPLC solvent bottle, cap, and invert ten times. Degas for 5 min by sonication. The mobile phase is stable at room temperature, 18–24 °C, for 1 month.
2. Mobile phase B (2.5 mM ammonium formate in methanol): Add 990 mL of methanol to a 1 L graduated cylinder. To the cylinder add 10 mL of 250 mM ammonium formate. Decant into a 1 L HPLC solvent bottle, cap, and invert ten times. Degas for 5 min by sonication. The mobile phase is stable at room temperature, 18–24 °C, for 1 month.
3. Wash Solvent (water:2-propanol, 7:3 v/v): In a 500 mL graduated cylinder, add 350 mL of water and 150 mL of 2-propanol and transfer into a HPLC wash bottle. Degas the solution for

5 min by sonication. The solution is stable at room temperature, 18–24 °C, for 1 month.

4. Needle wash solvent (methanol/2-propanol/water, 7:2:1 v/v): In a 500 mL graduated cylinder, add 350 mL of methanol, 100 mL of 2-propanol, and 50 mL of water and transfer into an HPLC wash bottle. Degas the solution for 5 min by sonication. The solution is stable at room temperature, 18–24 °C, for 1 month.
5. Sample elution solvent (hexanes/ethyl acetate, 8:2 v/v): In a 500 mL graduated cylinder, add 400 mL of hexanes and 100 mL of 2-propanol and transfer into a HPLC wash bottle. Degas the solution for 5 min by sonication. The solution is stable at room temperature, 18–24 °C, for 1 month.
6. Reconstitution solvent (mobile phase A/mobile phase B, 6:4 v/v): Using a 500 mL graduated cylinder add 300 mL mobile phase A and 200 mL mobile phase B. Decant into a 1 L HPLC solvent bottle, cap, and invert ten times. Degas for 5 min by sonication. The solution is stable at room temperature, 18–24 °C, for 1 month.

### 2.3 Internal Standards and Standards

1. Primary standards: 25-hydroxy vitamin $D_2$ and 25-hydroxy vitamin $D_3$ (Sigma-Aldrich).
2. Primary internal standards (I.S.): 25-Hydroxy vitamin $D_2$ (26, 26, 26, 27, 27, 27-$d_6$) and 25-hydroxy vitamin $D_3$ (26, 26, 26, 27, 27, 27-$d_6$) (Medical Isotopes, Inc.).
3. Internal standard solutions:
   (a) Internal standards stock solution (10 μg/mL): Weigh 1 mg each of 25(OH)$D_2$-$d_6$ and 25(OH)$D_3$-$d_6$ and transfer into a 100 mL volumetric flask. Bring to volume with methanol and mix well. The solution is stable for 1 year when stored at −80 °C.
   (b) Internal standards working solution (500 pg/mL): Add approximately 75 mL of methanol into a 100 mL volumetric flask. Add 5 μL of the internal standard solution (10 μg/mL) to the same volumetric flask. Bring to volume with methanol and mix well. The Internal Standard Working Solution is stable for 1 year when stored at −80 °C.
4. Standard stock solutions:
   (a) 25-Hydroxy vitamin $D_2$ (approximately 20 μg/mL): Weigh 1 mg of 25(OH)$D_2$ and transfer into a 50 mL volumetric flask. Bring to volume with methanol and mix well. Determine the precise concentration by measuring the absorbance using a UV-Vis spectrophotometer set at a wavelength of 264 nm. The solution is stable for 1 year when stored at −80 °C (*see* **Note 1**).

(b) 25-Hydroxy vitamin $D_3$ (approximately 20 μg/mL): Weigh 1 mg of 25(OH)$D_3$ and transfer into a 50 mL volumetric flask. Bring to volume with methanol and mix well. Determine the precise concentration by measuring the absorbance using a UV-Vis spectrophotometer set a wavelength of 264 nm. The solution is stable for 1 year when stored at −80 °C (*see* **Note 1**).

5. Working stock solution:
   (a) Working standard (approximately 200 ng/mL 25(OH)$D_2$ and 25(OH)$D_3$).
   (b) Add 1 mL of both 25(OH)$D_2$ and 25(OH)$D_3$ from their respective standard stock solutions into a 100 mL volumetric flask. Bring to volume with methanol and mix well. The solution is stable for 1 year when stored at −80 °C.

### 2.4 Calibrators and Controls

1. Calibrators: six calibrators are used when generating a standard calibration curve with the following concentrations: 20, 100, 250, 1000, and 1500 pg/mL (*see* **Note 2**). All calibrators are prepared by diluting working stock solution into pooled vitamin D-free urine and will be prepared by following Table 1. The calibrators are stable for 4 months when stored at −20 °C.
2. Check the new lot of calibrators by verifying five unknown patient specimen with the current lot of calibrators. The agreement between the two calculated concentrations must be within 15 %.
3. Controls: Prepare high and normal control materials by pooling previously analyzed samples. The high-quality control material may be prepared by the addition of 25(OH)$D_2$ and

**Table 1**
**Calibrator preparation**

| Calibrator | Vol. of 200 ng/mL stock added (μL) | Vol. of MeOH added (μL) | Urine added (μL) | Total vol. (mL) | Approx. final conc. (pg/mL)[a] |
|---|---|---|---|---|---|
| 1 | 0 | 750 | 99,250 | 100 | 0 |
| 2 | 10 | 740 | 99,250 | 100 | 20 |
| 3 | 125 | 625 | 99,250 | 100 | 250 |
| 4 | 250 | 500 | 99,250 | 100 | 500 |
| 5 | 500 | 250 | 99,250 | 100 | 1000 |
| 6 | 750 | 0 | 99,250 | 100 | 1500 |

[a]Values are approximate and based on gravimetric determination; actual values are determined at 264 nm as described in the text

$25(OH)D_3$ to pooled normal urine. The controls are stable for 4 months at −20 °C.

4. Establish a range for the new lot of controls by collecting data points over 20 consecutive runs and establish the mean and standard deviation.

### 2.5 Analytical Equipment and Supplies

1. Prominence LC-20 (Shimadzu) coupled to an Applied Biosystems API 5000 triple quadrupole mass spectrometer (AB Sciex).
2. HPLC Column: Gemini $C_{18}$, 50 × 2.0 mm i.d., 3 μm particle (Phenomenex).
3. Centrifuge tubes and National Scientific 2 mL amber glass vials with inserts and pre-slit caps or equivalent.
4. TurboVap LV evaporator (Biotage).
5. CEREX 48 positive pressure manifold (SPEware).
6. Trace-N SPE cartridge, 3CC (SPEware).

### 2.6 Instrument Operating Conditions

1. High-performance liquid chromatography (HPLC): A Schimadzu Prominence LC-20 system consisted of an autosampler, column oven, and two micro pumps. Chromatographic separations of $25(OH)D_2$ and $25(OH)D_3$ as well as the internal standard were achieved using a 50 × 2.0 mm i.d., 3 μm particle size Gemini $C_{18}$ column maintained at 45 °C. mobile phase A consisted of 1 % methanol in water and 2.5 mM ammonium formate, and mobile phase B consisted of methanol and 2.5 mM ammonium formate. The HPLC method is described in Table 2. The injection volume is 50 μL (*see* **Note 4**), with a syringe wash volume of 250 μL using the syringe wash solvent. The autosampler performs one wash pre-injection and three washes post-injection.

**Table 2**

**HPLC method**

| Step | Total time (Min) | Flow rate (μL/min) | %A | %B |
|---|---|---|---|---|
| 1 | 1.0 | 250 | 25 | 75 |
| 2 | 6.0 | 250 | 5 | 95 |
| 3 | 6.1 | 300 | 5 | 95 |
| 4 | 6.5 | 300 | 5 | 95 |
| 5 | 6.6 | 300 | 25 | 75 |
| 6 | 7.5 | 300 | 25 | 75 |

2. Tandem mass spectrometry: Mass spectrometric detection was performed using an Applied Biosystems API 5000 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in a positive ion mode. Multiple reaction monitoring (MRM) was selected for detection of 25(OH)$D_2$ and 25(OH)$D_3$ and their respective internal standards with a dwell time of 250 ms (Fig. 1). As shown in Table 3, two mass transitions were monitored for each analyte. The tune parameters used for data acquisition were: source temperature of 300 °C; collision activation dissociation (CAD) gas value of 3; curtain gas of 20 psi; nebulizer gas of 35 psi;

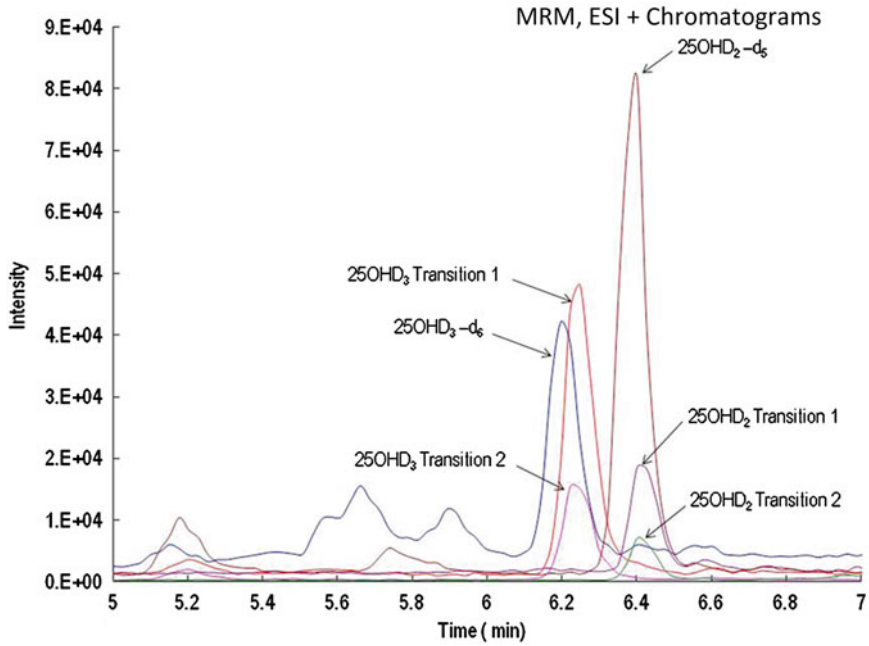



**Fig. 1** LC-ESI-MS/MS ion chromatograms of 25(OH)$D_2$, 25(OH)$D_3$, 25(OH)$D_2$-$d_6$, and 25(OH)$D_3$-$d_6$ primary and secondary product ions from a patient sample

**Table 3**

**Analyte precursor and product ions (*m/z*)**

| Analyte | Precursor ion | 1° product ion | 2° product ion | CE (V) |
|---|---|---|---|---|
| 25(OH)$D_2$ | 413.2 | 395.2 | 355.2 | 17 |
| 25(OH)$D_3$ | 401.2 | 383.2 | 365.2 | 17 |
| 25(OH)$D_2$-$d_6$ | 419.3 | 401.2 | N/A | 17 |
| 25(OH)$D_3$-$d_6$ | 407.3 | 389.3 | N/A | 17 |

Optimized *m/z* may change based on instrument and tuning parameters

heating gas of 30 psi; and a spray voltage of 4800 V. The DP, EP, CXP, CEM, and DF were 52, 13, 24, 2200, and 100 V, respectively. Nitrogen (99.995 % purity) was used as the desolvation and collision gas. The MRM acquisition method was run in unit resolution (0.7 amu) in both Q1 and Q3.

## 3 Methods

### 3.1 Stepwise Procedure

1. Run a system suitability to confirm the system performance (*see* **Note 3**).
2. Aliquot 5 mL of samples (calibrators, controls, or patient specimen) into the corresponding labeled centrifuge tubes.
3. Add 50 μL of the internal standard solution (500 pg/mL 25-hydroxy vitamin $D_2$-$d_6$ and 25-hydroxy vitamin $D_3$-$d_6$ in methanol).
4. Add 5 mL of methanol and vortex mix for 1 min.
5. Centrifuge samples for 10 min at 2500 × *g*.
6. Condition the Trace-N SPE cartridge with 0.5 mL of methanol and 0.5 mL of water. Slowly load the sample supernatant onto the cartridge.
7. Wash cartridges with 1 mL of the wash solvent and then dry the cartridge in the CEREX 48 with nitrogen set at a maximum flow for 5 min.
8. Elute samples with 1 mL of elution solvent.
9. Evaporate the samples to dryness in the TurboVap under nitrogen at 40 °C for 6 min.
10. Reconstitute the samples with a 100 μL of the reconstitution solvent and vortex mix thoroughly.

### 3.2 Data Analysis

1. The data are analyzed using Analyst 1.4.1 software (AB Sciex).
2. Standard curves are based on a linear regression for all analytes. Weighted linear regression models with weights inversely proportional to the *X* values were used. The analysis compared I.S. peak area to sample peak area (*y*-axis) versus analyte concentration (*x*-axis) using the quantifying ions indicated in Table 3.
3. Acceptability of each run is confirmed if the calculated control concentrations fall within two standard deviations of the target mean values. Target values are established as the mean of 20 runs. If any of the controls are greater than 3 standard deviations the run cannot proceed and troubleshooting procedures must commence.
4. Typical coefficients of correlation of the standard curve are >0.95 (*see* **Note 5**).

## 4 Notes

1. To determine the concentrations of the stock solutions by UV light at 264 nm the molar extinction coefficient of 18,300 $M^{-1}$ $cm^{-1}$ is used. The final concentrations are assigned based on the UV reading.
2. A new standard curve should be generated with each analytical run to ensure method performance.
3. A system suitability should be performed each day the method is run. The suitability includes running a test mix with all analytes to ensure proper retention time, integration, and sensitivity.
4. Retention times are instrument specific and can vary due to column use and PEEK tubing length.
5. The imprecision (CVs) measured at various concentrations across the analytical measurement range (AMR) were less than 7 % for all analytes. The calibration curves were linear over the AMR with correlation coefficients $r \geq 0.95$. The AMR for 25(OH)$D_2$ and 25(OH)$D_3$ was 20–1500 pg/mL. The LOQ for both analytes was determined to be 20 pg/mL. Recovery studies yielded recoveries of 81–89 % at various concentrations across the AMR for both analytes [18]. A cross-sectional study of 20 NS patients and 30 healthy control patients (3–40 years) showed that 25(OH)D was undetectable (<20 pg/mL) in all healthy controls and the mean 25(OH)D for NS patients was 197 pg/mL, with a range from 64 to 685 pg/mL [19].

### References


1. Grober U, Spitz J, Reichrath J, Kisters K, Holick MF (2013) Vitamin D: update 2013: from rickets prophylaxis to general preventive healthcare. Dermatoendocrinol 5:331–347
2. Alborzi P, Patel NA, Peterson C, Bills JE, Bekele DM, Bunaye Z, Light RP, Agarwal R (2008) Paricalcitol reduces albuminuria and inflammation in chronic kidney disease: a randomized double-blind pilot trial. Hypertension 52:249–255
3. Kuhlmann A, Haas CS, Gross ML, Reulbach U, Holzinger M, Schwarz U, Ritz E, Amann K (2004) 1,25-Dihydroxyvitamin D3 decreases podocyte loss and podocyte hypertrophy in the subtotally nephrectomized rat. Am J Physiol Renal Physiol 286:F526–F533
4. White P, Cooke N (2000) The multifunctional properties and characteristics of vitamin D-binding protein. Trends Endocrinol Metab 11:320–327
5. Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI, Willnow TE (1999) An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell 96:507–515
6. Leheste JR, Melsen F, Wellner M, Jansen P, Schlichting U, Renner-Muller I, Andreassen TT, Wolf E, Bachmann S, Nykjaer A, Willnow TE (2003) Hypocalcemia and osteopathy in mice with kidney-specific megalin gene defect. FASEB J 17:247–249
7. Safadi FF, Thornton P, Magiera H, Hollis BW, Gentile M, Haddad JG, Liebhaber SA, Cooke NE (1999) Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. J Clin Invest 103:239–251
8. Koenig KG, Lindberg JS, Zerwekh JE, Padalino PK, Cushner HM, Copley JB (1992) Free and total 1,25-dihydroxyvitamin D levels in subjects with renal disease. Kidney Int 41:161–165
9. Auwerx J, De Keyser L, Bouillon R, De Moor P (1986) Decreased free

1,25-dihydroxycholecalciferol index in patients with the nephrotic syndrome. Nephron 42:231–235

10. Goldstein DA, Haldimann B, Sherman D, Norman AW, Massry SG (1981) Vitamin D metabolites and calcium metabolism in patients with nephrotic syndrome and normal renal function. J Clin Endocrinol Metab 52:116–121
11. Barragry JM, France MW, Carter ND, Auton JA, Beer M, Boucher BJ, Cohen RD (1977) Vitamin-D metabolism in nephrotic syndrome. Lancet 2:629–632
12. Grymonprez A, Proesmans W, Van Dyck M, Jans I, Goos G, Bouillon R (1995) Vitamin D metabolites in childhood nephrotic syndrome. Pediatr Nephrol 9:278–281
13. Freundlich M, Bourgoignie JJ, Zilleruelo G, Abitbol C, Canterbury JM, Strauss J (1986) Calcium and vitamin D metabolism in children with nephrotic syndrome. J Pediatr 108:383–387
14. Sato KA, Gray RW, Lemann J Jr (1982) Urinary excretion of 25-hydroxyvitamin D in health and the nephrotic syndrome. J Lab Clin Med 99:325–330
15. Tessitore N, Bonucci E, D'Angelo A, Lund B, Corgnati A, Valvo E, Lupo A, Loschiavo C, Fabris A et al (1984) Bone histology and calcium metabolism in patients with nephrotic syndrome and normal or reduced renal function. Nephron 37:153–159
16. Mittal SK, Dash SC, Tiwari SC, Agarwal SK, Saxena S, Fishbane S (1999) Bone histology in patients with nephrotic syndrome and normal renal function. Kidney Int 55:1912–1919
17. Thierry-Palmer M, Henderson VM, Hammali RE, Cephas S, Palacios C, Martin BR, Weaver CM (2008) Black and white female adolescents lose vitamin D metabolites into urine. Am J Med Sci 335:278–283
18. Carlow DC, Zhang Y, Leonard MB, Denburg M (2013) Analysis of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in urine using liquid chromatography with tandem mass spectrometry. Arch Pathol Lab Med 137:1519
19. Denburg M, deBoer IH, Carlow D, York A, Chun R, Hewison M, Leonard MB (2012) New insights into vitamin D-related mineral metabolism in nephrotic syndrome. J Am Soc Nephrol 23:316A

# Index


## A

Adenine ..... 225–230, 232, 233
Adenosine ..... 225–233
Albumin ..... 31–36, 59, 62, 63, 99–101, 121, 133, 186, 198, 218–220, 243, 289, 319, 320
Aldosterone ..... 5, 37–40, 42, 44, 45, 242
Amyloidogenic protein ..... 55, 56
Amyloidosis ..... 55, 56
Angiotensin I ..... 37, 241
Arginine ..... 21–26, 28–30, 244
Aspart ..... 121–123, 128
Asymmetric dimethylarginine ..... 21, 24
Automated liquid handling ..... 300

## B

Beta-pleated sheets ..... 55
Butylation ..... 12, 48, 50–52

## C

Cerebrospinal fluid ..... 48, 110, 173, 232
Clinic biomarker ..... 197
Cortisol ..... 5, 38, 45, 71–78, 271
C-peptide ..... 120
Cushing's syndrome ..... 71, 72
Cystine ..... 47, 49–52

## D

Dansylation ..... 51, 52
Deconvolution ..... 159, 165, 169, 170
Dehydroepiandrosterone ..... 5, 272
11-Deoxycortisol ..... 272, 275
Derivatization ..... 3, 38, 48, 88, 100, 104–105, 110, 181, 183, 191, 272, 280, 284
Dihydropyrimidinuria ..... 236
Dihydrothymine ..... 236–240
Dihydrouracil ..... 236–240
Dihydroxyacetone phosphate ..... 81, 82
1,25-Dihydroxy vitamin D ..... 5, 319
Dimethylarginine ..... 21, 23, 24
Dried blood spot ..... 262, 280

## E

Equilibrium dialysis ..... 100
Estradiol ..... 5, 87–90, 93–95, 99–102, 106
Estrogen ..... 87, 88, 95, 99, 101
Estrone ..... 5, 87–91, 93–95

## F

Fatty acid oxidation defects ..... 11
Folate deficiency ..... 173
Free estradiol ..... 99, 100, 105–107

## G

γ-aminobutyric acid (GABA) ..... 109–118
Gas chromatography ..... 11, 38, 158, 181, 236, 262
Glargine ..... 122, 123, 128
Glycine ..... 47, 48, 51, 52, 186, 225
Guanine ..... 225, 226

## H

High performance liquid chromatography (HPLC) ..... 4, 8, 14, 15, 22, 24, 30, 32–34, 39, 43, 45, 48, 50, 53, 57–59, 62–64, 72–75, 77, 82–85, 88, 89, 91, 92, 102, 103, 110, 116, 117, 123, 125–127, 134, 142, 143, 162, 163, 174, 176–179, 202, 203, 212, 213, 218–221, 227, 236, 237, 247, 248, 254, 256–260, 263, 266, 272, 273, 275, 293, 320, 321, 323
High resolution mass spectrometry ..... 121, 131–136, 268
High-throughput ..... 243, 320
HPLC-TOF-MS ..... 47–53, 81–85
Humalog ..... 120, 122
Humulin ..... 120, 128
17-Hydroxyprogesterone ..... 5, 272
25-Hydroxy vitamin D ..... 5, 299–301, 319, 320
25-Hydroxy vitamin $D_2$ ..... 301, 312, 321
25-Hydroxy vitamin $D_3$ ..... 301, 312, 321, 322
Hypertension ..... 22, 37, 242, 244, 245
Hypoglycemia ..... 11, 119, 120
Hypokalemia ..... 37, 242
Hypoxanthine ..... 225–228, 230–233


Uttam Garg (ed.), *Clinical Applications of Mass Spectrometry in Biomolecular Analysis: Methods and Protocols*, Methods in Molecular Biology, vol. 1378, DOI 10.1007/978-1-4939-3182-8, © Springer Science+Business Media New York 2016

## I

Immunocapture ..... 210
Inborn error of metabolism ..... 3, 4
Inherited metabolic disorders ..... 1, 254
Inosine ..... 225, 227, 228, 230–233
Insulin ..... 5, 119–129, 131, 132, 319
Insulin-like growth factor-1 ..... 131–136
Ion pair reagent ..... 85
Isomers ..... 82, 158, 166–168, 170, 280, 286, 287

## L

Levemir ..... 120, 122
Liquid chromatography ..... 11, 22, 32, 61, 72, 116, 117, 120, 121, 131–136, 140–144, 146, 174, 177, 179, 197, 210, 227, 243, 257, 258, 262, 293
Liquid chromatography tandem mass spectrometry (LC-MS/MS) ..... 3, 4, 22, 26, 27, 29, 32, 34, 38, 42, 57, 64, 72, 76, 93, 100, 120, 121, 126, 128, 140, 144, 158, 164–165, 203, 210, 243, 247, 250, 259, 280, 290, 296, 300, 306–308, 313, 315, 320
Lispro ..... 121–123, 128, 129

## M

Mass spectrometry ..... 1–8, 14, 15, 17, 22, 32, 38, 48, 56, 77, 82, 84, 88, 92, 105–106, 132, 135, 158, 182, 197, 208, 230, 237, 250, 262, 264, 267–269, 280, 300, 311–312, 323, 324
MCAD
Medium chain acyl-CoA dehydrogenase deficiency ..... 6
Metanephrine ..... 139–146
Methylation ..... 21, 59, 253
Methylmalonic acid ..... 6, 158, 161
5-Methyltetrahydrofolate ..... 173
Microalbumin
Microalbuminuria ..... 32
Mineralocorticoid ..... 242, 271, 272
Monophasic lipid extraction ..... 262
Multiple reaction monitoring ..... 42, 88, 210, 248, 324
Multiplexing ..... 34, 300, 303, 313

## N

Nephrotic syndrome ..... 320
Newborn screening ..... 1, 4, 6, 218, 226, 272, 280
Normetanephrine ..... 140–145
Novorapid ..... 120, 122

## O

Orbitrap mass spectrometry ..... 56
Organic acidemia ..... 6, 11
Organic acidurias ..... 6, 11

## P

Pancreatic polypeptide ..... 5, 197
Parathyroid hormone (PTH) quantitation ..... 209
Peptide hormone ..... 132, 209
Phenylalanine ..... 47, 51, 52, 217–219, 222
Phenylalanine hydroxylase ..... 217
Phenylketonuria (PKU) ..... 6, 47, 217, 218
Pheochromocytoma ..... 139, 147
Phosphoethanolamine ..... 47–49, 51, 52
Plasma ..... 11, 12, 21, 22, 26, 29–31, 37, 38, 48, 62, 63, 71, 82, 88, 100, 121, 139–142, 144, 158, 159, 161–163, 167, 169, 173, 197–199, 202, 210, 212, 218, 236, 242, 243, 245, 250, 254–256, 259, 261
Plasma renin activity ..... 38, 242
Primary aldosteronism ..... 37
Production ion ..... 82, 85, 117, 160, 170, 206, 324
Proteins ..... 1, 3, 4, 6, 7, 31, 38, 59, 62, 66, 99, 102, 127, 132, 200, 202, 253, 264, 266, 300, 303, 305–306, 312, 316
Purine ..... 7, 225–227, 230–233, 235, 238, 239
Pyrimidine ..... 7, 226, 235–239
Pyrimidinuria ..... 236

## R

Red blood cells ..... 227
Reproductive hormones ..... 87, 88

## S

S-adenosylhomocysteine ..... 7, 253, 254
S-adenosylmethionine ..... 7, 253, 254
Saliva ..... 72, 74
Seizures ..... 48, 110, 226
Sex hormone binding globulin (SHBG) ..... 99, 102
Sphingolipids ..... 261–263, 268
Succinic acid ..... 109, 161, 162
Succinylacetone ..... 7, 186, 280–282, 286–288
Succinyladenosine ..... 227–233
Symmetric dimethylarginine ..... 21

## T

Tandem mass spectrometry ..... 6, 22, 32–36, 72, 120, 140–144, 146, 174, 210, 227, 236, 238, 243, 294, 324
Testosterone ..... 5, 96, 272, 273, 275
Tetrahydrobiopterin ..... 217
Thymidine ..... 235–240
Thymine ..... 186, 236–240
Tributylamine ..... 82
Trimethylsilyl derivatives (TMS) ..... 103, 182, 188, 192–194, 280, 284, 286

Tyrosine ..... 47, 48, 51, 52, 217–219, 222, 279
Tyrosinemia I ..... 279, 286, 287

## U

Ultra performance liquid chromatography (UPLC) ..... 49, 83, 227, 229, 231
Uracil ..... 186, 236–240
Ureidopropionic aciduria ..... 6
Urine organic acids ..... 4, 218, 284, 286

## X

Xanthine ..... 225, 227–233