Chapter 6

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

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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

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-$13C_6$), 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 phosphoethanol-amine (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 microcentrifuge 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 $12,000 \times g$.

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-13C$_6$)	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.

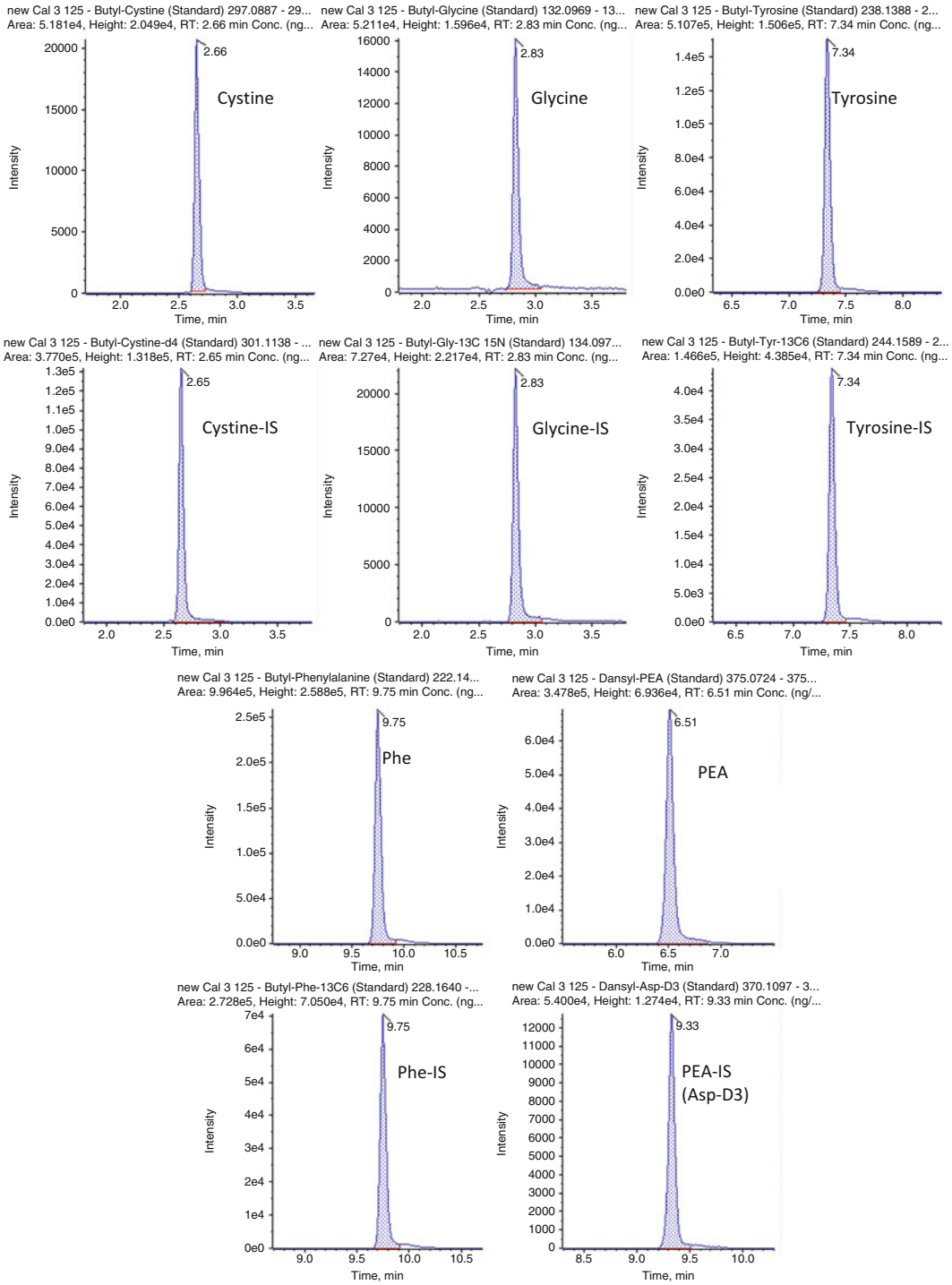2. Drying time is ~5 min. May vary with nitrogen flow rate and type of equipment.

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.

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