Chapter 15

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

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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

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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.

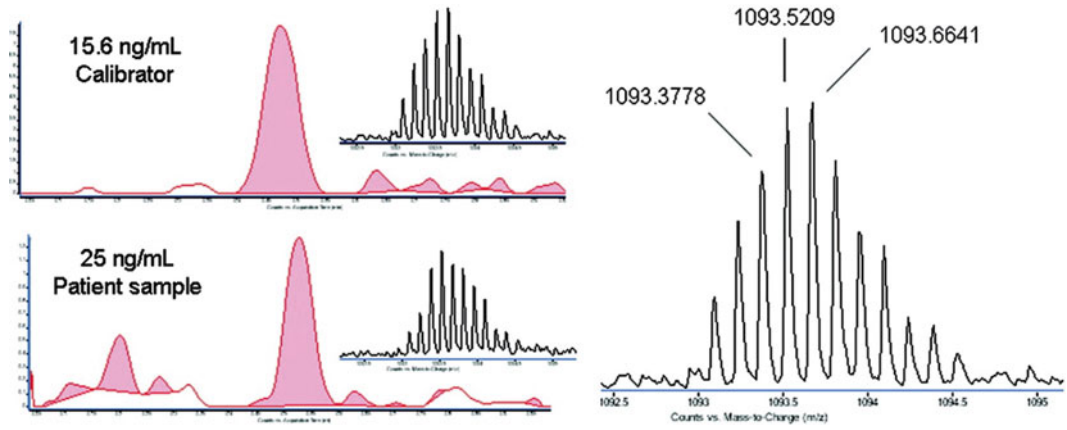

Fig. 1 Example chromatograms and averaged spectra for IGF-1 in calibrators (15.6 ng/mL) and patient samples (25 ng/mL). The isotopic resolution of the most abundant charge state (7+) for IGF-1 is shown with the quantifier (*m/z* 1093.5209) and two qualifier ions (*m/z* 1093.3778 and 1093.6641) indicated. 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.

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