Journal of Chromatography A, 1218 (2011) 6596–6602

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Quantification of human growth hormone by amino acid composition analysis using isotope dilution liquid-chromatography tandem mass spectrometry

Ji-Seon Jeong, Hyuk-Min Lim, Sook-Kyung Kim, Hyung-Keun Ku, Kyung-Hwa Oh, Sang-Ryoul Park*

Center for Bioanalysis, Department of Metrology for Quality of Life, Korea Research Institute of Standards and Science, 1 Doryong-dong, Yuseong-gu, Daejeon 305-340, South Korea

ARTICLE INFO

Article history:
Received 8 June 2011
Received in revised form 15 July 2011
Accepted 18 July 2011
Available online 27 July 2011

Keywords:
Amino acid (AA) analysis
HPLC–MS/MS
Isotope dilution mass spectrometry (IDMS)
Acid hydrolysis
Protein quantification
Human growth hormone

ABSTRACT

We describe an accurate method for protein quantification based on conventional acid hydrolysis and an isotope dilution–HPLC–mass spectrometry (ID–HPLC–MS) method. Sample purity was confirmed using capillary zone electrophoresis, HPLC and MS. The analyte protein, human growth hormone (hGH), was effectively hydrolyzed by incubation with 8 M hydrochloric acid at 130 °C for 48 h, where at least 1 μM of hGH was treated to avoid possible degradation of released amino acids during hydrolysis. Using a reversed-phase column, the analytes (isoleucine, phenylalanine, proline and valine) were separated within 5 min using an isocratic eluent comprising 10% acetonitrile containing 0.1% trifluoroacetic acid. The detection limit (signal to noise ratio of 3) of amino acids was 5.5–6.2 fmol per injection. The quantification precision (RSD) of amino acids for intra- and inter-day assays was less than 0.98% and 0.39%, respectively. Comparison with other biochemical and instrumental methods revealed substantially higher accuracy and reproducibility of the ID–HPLC–MS/MS method as expected. The optimized hydrolysis and analytical conditions in our study were suitable for accurate quantification of hGH.

1. Introduction

In the last decades, protein-related business has developed significantly in various areas including the biological and pharmaceutical industries. This development has increased the demand for suitable protein analytical methods since determining the content of a reagent can serve as a quality control standard and may help establish safety guidelines for human use [1,2]. For this reason, national metrology institutes (NMIs) are actively working on the establishment of measurement standards for protein quantification. Our research team has worked on the establishment of a higher order analytical method for accurate protein quantification using human growth hormone as a model protein.

Human growth hormone (hGH) is a peptide secreted by the anterior pituitary gland. hGH contains 191 amino acids, has a molecular weight of 22,124 Da and a p*I* of about 5.1. Its primary use in medicine is to treat child growth disorders and adult growth hormone deficiency [2,3]. hGH has also been used as an anabolic agent by athletes, although its use has been banned in professional sports [4]. Another use of hGH is to increase livestock production in the area of industrial agriculture [5]. Growth hormone is legally available only as a prescription drug in most countries. However, the efficacy and safety of hGH have not been determined in clinical trials, and the breadth of its biological functions is considerable in various applications [6].

Generally, proteins are quantified using biochemical methods such as bioassays, immunoassays, and instrumental methods such as chromatography [7–10]. These methods have been previously used to quantify hGH [2,11–15]. Biological methods are widely used owing to their simplicity and low cost, but are hampered by poor accuracy and reproducibility since their responses vary depending on the type of calibrator used and amino acid residue composition of the protein being analyzed. On the other hand, instrumental analytical methods including chromatographic techniques (HPLC, GC), capillary electrophoresis (CE), and mass spectrometry (MS) are often favored given their higher accuracy and reproducibility. These methods may require time-consuming sample preparation, which leads to higher costs compared with biological methods. Nevertheless, both bioassay and instrumental analyses require calibration using a highly reliable protein standard material to ensure accurate and comparable results. A higher order analytical method needs to be established for the accurate determination of such protein standard materials.

In the establishment of a higher order analytical method for protein quantification, two approaches can be considered. As no analytical method can yet directly quantify whole proteins to the desired level of accuracy, proteins are reduced to analyzable entities, either amino acids (AAs) or peptides, which are then analyzed to deduce the quantity of original protein. In a recent approach concerning the analysis of peptide compositions, peptide residues are released through enzymatic (typically tryptic) hydrolysis whereas

* Corresponding author. Tel.: +82 42 868 5658; fax: +82 42 868 5801.
E-mail address: srpark@kriss.re.kr (S.-R. Park).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.07.053

residual AAs are released typically by acid hydrolysis of peptide-bonds. In contrast to the AA-approach, the peptide-approach is compatible when using a mixture of proteins as the peptides can be traced to their protein origins based on their unique AA sequences [1,16,17]. The peptide-approach applied to hGH in complex human-serum matrices has recently been reported by several research groups [15,18]. With respect to the accurate quantification of a pure protein or peptide as a primary calibrator, however, the AA-approach is more straightforward not only in terms of protein hydrolysis, but also in the final composition analysis (see the following section). In fact, calibrators of the peptide analysis with the peptide-approach have to be determined by the AA-approach. In an effort to arrive at an accurate quantitative standard of hGH, we developed a higher order analytical method based on the AA-approach.

Amino acid (AA) compositional analysis using various methods is a classical protein analysis technique [19–21]. It is indispensible for the quantification of peptides and proteins as it directly links such quantification to the quantification of amino acids which can be performed in an accurate and precise manner. Prior to analysis, the protein has to be completely hydrolyzed to yield free AAs [19]. Hydrolysis is an important step, and its proper execution is a prerequisite for a successful analysis. Although many hydrolysis protocols and applications exist, standardization of hydrolysis conditions is difficult to achieve since the range of sizes and specificity differs for each protein. Furthermore, the specificity of different AAs varies [22,23]. For example, differences in the ease of peptide-bond cleavage and acid stability have significant effects on estimating protein quantity through AA composition analysis. Disagreement between the end-point of hydrolysis and the start-point of degradation may lead to estimation errors of protein content according to the quantified residues. To overcome this error, comparative studies have been performed [21–27]. Conventional acidic hydrolysis involves incubation with 6 M HCl for 24 h at 110 °C as a standard procedure. Based on these standard acidic hydrolysis conditions, the type and concentration of acid, in addition to the time and temperature of hydrolysis, were examined. Some studies have proposed the use of correction factors [22,25–28]. Correction factors were calculated for AA residues that were not maximally hydrolyzed under standard conditions. Of all the various methods attempted for improving the accuracy of hydrolysis techniques, none are perfectly suitable for all cases. As AA composition analysis is the primary approach of establishing the measurement standards for protein quantification, further studies are needed to minimize such errors in protein hydrolysis.

There are several methods available for quantifying AAs. Numerous chromatographic methods have generally been used for AA analyses [20,21,24,29–31]. An analytical method requires not only high accuracy, precision and sensitivity, but also a reliable standard. The isotope dilution (ID) method involves spiking the isotope standard of an element or molecular compound labeled with an isotope for use as an internal standard. The ID–MS technique has been used for a wide range of analytical chemistry applications to provide high accuracy and precision, and the effectiveness of this method has been verified [1,16–18,21,29,30].

ID–LC tandem mass spectrometry (MS/MS) was used in this study. Analytical interference from matrix or unknown factors would be minimized by LC separation, and subsequent analyte identification improved by the use of MS/MS. Analytical precision, especially involving possible poor reproducibility of MS ionization, would be overcome by using an isotopically labeled internal standard. Advanced MS instrumentation would yield high sensitivity.

In this study, we report on the optimization of hydrolysis conditions and an ID–LC–MS/MS method for quantifying hGH. The purity of hGH was first assessed using CE and HPLC (according to the European Pharmacopoeia [32,33]) and as well as using MALDI–TOF–MS [34]. The hydrolysis conditions were optimized using purified hGH. Conditions for separating four AAs (isoleucine, phenylalanine, proline and valine) were validated. hGH quantification was finally performed under the optimized hydrolysis and separation conditions. The performance of the suggested analytical procedure was compared with the results of common biochemical or instrumental methods.

2. Materials and methods

2.1. Materials

The recombinant 22-kDa hGH in solution was purchased from LG Life Science (Daejeon, South Korea). The content of hGH (6.7 mg/mL) was determined by the manufacturer using size-exclusion HPLC and the composition of the sample solution included 2.3% mannitol, 0.5% glycine and 0.15% Na_2HPO_4. The hGH solution was stored at −70 °C before use. The AA mixture used for calibration was a certified reference material comprising AAs in 0.1 M HCl from the National Institute of Standard and Technology (SRM2389a; NIST, Gaithersburg, MD, USA) [35]. The AA mixture was stored at 4 °C before use. The following unlabeled AAs (>99% purity) were purchased from Sigma (St. Louis, MO, USA): L-valine (Val), L-isoleucine (Ile), L-proline (Pro), L-phenylalanine (Phe) and L-leucine (Leu). The following labeled AAs were obtained from Cambridge Isotopes Laboratory (Andover, MA, USA): L-valine (U-$^{13}C_5$, 98%; ^{15}N, 98%; Val*), L-isoleucine (U-$^{13}C_6$, 98%; ^{15}N, 98%; Ile*), L-proline (U-$^{13}C_5$, 98%; ^{15}N, 98%; Pro*) and L-phenylalanine (RING-$^{13}C_6$, 99%; Phe*).

Hydrochloric acid (HCl) and trifluoroacetic acid (TFA) were purchased from Junsei (Tokyo, Japan), and acetonitrile (ACN) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Other reagents and solvents used were all of analytical grade. Water used to prepare the standard solutions, sample solutions, and mobile phase was purified using a Millipore Alpha-Q water purification system (Millipore, Billerica, MA, USA), and solvents were filtered through a membrane filter (pore size 0.2 μm). Prior to analysis, all samples were passed through disposable centrifugal filter devices (Ultrafree-MC, PVDF membrane filters; pore size 0.22 μm; Millipore).

2.2. Sample preparation

The hGH sample was diluted gravimetrically with 0.1 M HCl to a final concentration of about 5 μM. According to the "Vocabulary in Metrology (VIM)" [36], chemical quantity is described in the term of "amount-of-substance concentration". While acknowledging the new terminology, 'concentration' is used in this work for convenience. All stock solutions of unlabeled and labeled AAs were prepared gravimetrically in 0.1 M HCl. A working internal standard solution was prepared gravimetrically from labeled stocks and concentrations were adjusted to hydrolyzed AA concentrations by multiplying the AA residue number (stoichiometric value) by the hGH concentration. The mass fraction of labeled AAs was equal to that of the unlabeled AAs in the hydrolyzed sample and standard AA solution. The solutions were stored at 4 °C before use.

Two hundred microliters of diluted hGH and internal standard were placed gravimetrically into a glass tube (15 mL, PYREX screw cap culture tubes with Teflon-lined phenolic caps, cat # 9826-16x; Corning, NY, USA) and mixed well. HCl (1.6 mL) was added to yield a final concentration of 8 M. The tubes were sealed, the solution was mixed, and hGH was hydrolyzed at 130 °C for 48 h. Following hydrolysis, the tubes were cooled at room temperature with gentle rotation on a shaker. The acid was evaporated under nitrogen gas and the residue was dissolved in 400 μL of 0.1 M HCl,