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Comparative study for purity assessment of recombinant human growth hormone using mass balance approach and amino acid-based isotope dilution mass spectrometry

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ABSTRACT

Growth hormone plays an unreplaceable role in regulating human growth and promoting protein synthesis, *etc*. For this reason, accurate quantification of its purity has become a focus of attention in the pharmaceutical industry and medical research. Herein, the purity assessment of human growth hormone (hGH) was established on the basis of mass balance approach and amino acid (AA)-based isotope dilution mass spectrometry (IDMS). In the first method, hGH purity was quantified by the determination of all the impurities in a hGH study material. In the second method, hGH purity was assessed by quantifying hydrolyzed AAs, e.g. leucine (Leu), phenylalanine (Phe), and proline (Pro). To accurately quantify these AAs, the corresponding isotope-labeled AAs were used as internal standards using high-performance liquid chromatography- mass spectrometry (HPLC-MS). The results of the two methods for determining the purity of hGH were consistent, with the mass balance method giving a result of (0.352 ± 0.010) g/g and IDMS a result of (0.363 ± 0.028) g/g. The work in this study could give a benefit for protein quantification and provides a reference for the development of hGH certified reference materials.

1. Introduction

Human growth hormone (hGH), which consists of 191 amino acids (AAs), is an important peptide hormone secreted by the somatotrophs located in anterior pituitary gland [1]. hGH plays a key role in human growth and development [2]. It is clinically used in the treatment of short stature in children with growth hormone deficiency, acute necrotizing pancreatitis, burns, sepsis and other diseases [3]. The advent of recombinant technology has enabled the production of hGH through recombinant technology. The extensive range of biological functions exhibited by hGH has led to its extensive utilization in a multitude of applications. In most countries, growth hormone is only legally available as a prescription drug, however, the efficacy and safety of hGH have not been established in clinical trials [4]. Improper use of hGH could cause adverse results, such as acromegaly, diabetes, high blood pressure, liver damage and heart problems [5]. Therefore, the accurate quantification of hGH has important clinical significance and attracted more and more attention from the world.

Accurate and comparable measurements of proteins can support diagnosis, prognosis, monitoring and treatment of diseases. To date, measurement methods for proteins include ultraviolet (UV) absorption method [6], colorimetric methods [7], fluorescence-based assay [8], enzyme-linked immunosorbent assay [9], mass spectrometry [10], *etc*. Among these methods, instrumental methods of analysis are often high accurate and repeatable, but the analytical process is very time-consuming [11]. In addition, biological methods have disadvantages such as antibody specificity differences, matrix disturbances, and false-positive results, making the accuracy of clinical results uncertain [12]. Above all, the analytical results from different methods are usually inconsistent. Therefore, development of comparable protein quantification methods is highly required.

Mass balance method has been proved to be a high precious method for the quantification of main component in high purity material, in which the content of moisture, ash, volatile components, inorganic elements, and other impurities are deducted from 100 %. Then the purity of the substance is determined according to mass fraction of the main components in the sample [13,14]. Mass balance method is considered as a gold standard approach and has been widely applied by the

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European Pharmacopoeia and International Pharmacopoeia for measurement of small molecules [15,16]. In the fields of protein and peptide quantification, a mass balance method was established for angiotensin I, which is a successful application of this method to peptides [17]. As for the quantification of large proteins, the use of mass balance method has also increased in recent years [15,18–20].

Isotope dilution mass spectrometry (IDMS) is an analytical technique based on the modification of the natural isotope composition of elements or compounds after the addition to the sample of an enriched isotope or an isotopically labeled form of the analyte. The isotope composition in the mixture, measured by mass spectrometry, provides the concentration of the analyte in the sample after simple calculations [21]. IDMS has widely used in the quantification of peptides and proteins, which provides the accurate result of purity [22]. In this approach, proteins are characterized in different forms for analysis, including intact protein, peptides or AAs [23]. Generally, signature peptides and AAs are commonly used as target analytes. However, the process of trypsin digestion often renders the selection of the target peptide challenging. Moreover, the accuracy of this approach is contingent upon the correct characterization of the peptide standard [24]. Alternatively, AA-based IDMS method is used to determine the concentration value of a protein and peptide reference material that will be used in the calibration of various protein and peptide analyses, such as disease markers for clinical diagnosis [25]. Feng et al. used AA hydrolysis in combination with IDMS to accurately quantify Aβ protein, a biomarker of Alzheimer's disease, and developed a certified reference material (CRM) [22]. Liu et al. developed an insulin-like growth factor-1 (IGF-1) CRM, which was expected to be used as a primary calibrator for quality control in biopharmaceutical manufacturing and clinical diagnostics, by the use of IDMS method [26].

In the AA-based IDMS method for quantitative determination of protein purity, the isotopically labeled target AAs were added into the sample as the internal standard before the protein sample is hydrolysed [27]. The hydrolyzed AAs are separated by reversed-phase liquid chromatography. After entering MS, target AAs and corresponding internal standards are detected under multiple reaction monitoring (MRM) mode. Complete release of AAs is the key to accurate quantification, so it is necessary to optimize the conditions of protein hydrolysis. During hydrolysis, structural analogues in the protein may release corresponding target AAs, or AAs may have some level of loss, which could lead to inaccurate quantitative results [28]. The addition of isotopically labeled AAs before sample analysis avoids effect by random errors during sample preparation and measurement processes leading to inaccurate quantification result [29].

In this study, two quantitative methods (e.g. mass balance approach and AA-based IDMS) were established analyzing purity of recombined hGH. All the impurities in a hGH study material were accurately detected and measured for mass balance approach. For AA-based IDMS, hGH purity was assessed by quantifying hydrolyzed AAs.

2. Materials and methods

2.1. Chemicals and instruments

Recombinant human growth hormone (white lyophilized powder, recombinant expression from *Escherichia coli*) was purchased from novasygen biotechnology Co., Ltd (Beijing, China). The CRMs of leucine (Leu, GB(E)100058, 99.1 %), phenylalanine (Phe, GB(E)100061, 99.9 %), and proline (Pro, GB(E)100084, 99 %) were provided by the National Institute of Metrology (Beijing, China). $^{13}C_6$, ^{15}N-leucine (95 %), $^{13}C_5$, ^{15}N-proline (95 %) and $^{13}C_9$, ^{15}N-phenylalanine (95 %) were purchased from Sigma-Aldrich (St. louis, USA). Ultrapure water (18 MΩ/cm) was prepared using a Milli-Q Academic water purification system (MING-CHE 24 V, Millipore, France). Trypsin was purchased from Promega (Beijing, China). Tris (hydroxymethyl) aminomethane (Tris) and hydrochloric acid (HCl), used to prepare 10 mM Tris-HCl buffer solution (pH 7.4), were purchased from Sinopharm chemical reagent Co., Ltd (Shanghai, China). HPLC-grade acetonitrile was purchased from Fisher Scientific Company (Pittsburgh, USA).

The structural identification of hGH was investigated by an Agilent 1290 Infinity LC system coupled with micrOTOF QII mass spectrometer (Bruker, Germany). The concentration of AAs was measured by Agilent 1260 Infinity II HPLC tandem TSQ Quantum Access MAX triple-quadrupole mass spectrometry (Thermo, USA) equipped with a Poroshell 120 EC C18 (3.0 × 150 mm, 2.7 μm, Agilent, USA) analytical column and an electrospray ion source. The UPLC system (Shimadzu, Japan) with LC-20ADXR UV detector and an eclipse XDB-C18 column (150 mm × 4.6 mm, 5.0 μm particle size, Agilent, USA) was used for LC purity assessment of hGH. A Metrohm 852-Titrando Karl Fischer titration was used for moisture determination. Metal ions analysis was performed on Agilent 7800 ICP-MS, and anions analysis was conducted on a Thermo Fischer Scientific DIONEX ICS-1000 ion chromatography.

2.2. Purity assessment via mass balance approach

2.2.1. Mass fraction of structurally related impurities

A UPLC (Shimadzu, Japan) with UV detector LC-20ADXR was used to determine the mass fraction of the structurally related impurities. Three independent samples of hGH were prepared for analysis at a concentration of 1 mg/g. An eclipse XDB-C18 column (150 mm × 4.6 mm, particle size 5.0 μm, Agilent, USA) was used to separate the hGH main components and the structurally related impurities, and gradient elution was performed as listed in Table S1 with eluent A (water with 0.1 % TFA) and eluent B (acetonitrile). The column temperature was maintained at 30 ℃, and the elution flow rate was set at 1.0 mL/min. The injection volume was set at 20 μL and the monitoring wavelength was set at 277 nm.

2.2.2. Moisture content

The content of moisture was carried out on a Metrohm 852-Titrando Karl Fischer titration (Metrohm, Switzerland). The hGH samples were accurately weighed and added into the titration cell and then titrated to the end point with Karl Fischer reagent. The moisture content of the sample was calculated according to the principle of Karl Fischer titration.

2.2.3. Impurity of metal ions

The metal ions were measured using an Agilent 7800 ICP-MS. The instrument parameters were set as follows: pump rate, 100 r/min; sample flush time, 20 s; radio frequency power, 1550 W; nebulizer gas flow rate, 0.8 L/min; auxiliary gas flow rate, 0.8 L/min; cooling gas flow rate, 14 L/min. Prior to entered analysis system, hGH sample was first digested using a Milestone ETHOS-UP microwave digestion system.

2.2.4. Impurity of anions

The determination of anions in the hGH sample was conducted on a Thermo Fischer Scientific DIONEX ICS-1000 ion chromatography system. Separation of anions was carried out on a Dionex IonPac AS11-HC column (4 × 250 mm). Potassium hydroxide solution (25 mM) was used as eluent.

2.3. Enzymatic digestion for protein identification

0.5 mg of hGH was weighed into a centrifuge tube and then 80 μ L of 2 mg/mL trypsin (protein: enzyme = 1:10, n: n) was added. 50 mM Tris-HCl buffer (pH 7.4) was added until the final volume reached 1 mL. Proteolytic digestion was incubated at 37℃ for 4 h. After the reaction was completed, the solution was diluted and filtered through 0.22 μm filters prior to analysis.

All the LC-MS experiments were performed on an Agilent 1290 Infinity LC system coupled with Bruker micrOTOF QII mass spectrometer. LC separation of the peptides was performed using an Agilent Zorbax

300SB-C18 reversed-phase column (4.6 × 250 mm, 5 μm). LC column temperature was maintained at 40 °C. 0.1 % FA aqueous solution and 0.1 % FA acetonitrile solution were used as mobile phase A and mobile phase B, respectively. Flow rate of mobile phase was set at 1 mL/min. A linear gradient was adopted as below: 5 % B in 5 min, 5–60 % B in 50 min, 60–98 % B in 5 min.

2.4. Sample preparation for AAs analysis

The hGH stock solution with a concentration of 1 mg/g was prepared with ultrapure water and stocked in −20 °C refrigerator. Before experiment, the solution was diluted to 0.1 mg/g. All AA CRMs (1 mg/g) and labeled AAs (1 mg/g) solution were separately prepared. A standard AA mixture containing targeted AAs was prepared according to the metrological ratios of AAs in the hGH hydrolyzed solution. An isotopically labeled AA mixture was prepared in the same way. The diluted hGH samples were accurately measured and weighed, and the mixed solution of isotopically labeled AAs was added at the same concentration, so that the ratio of AA content to the hydrolyzed AA content of hGH was about 1:1. High-level and low-level solutions were prepared with the ratio of unlabeled AA and labeled AA content reaching approximately 1 and 0.8, respectively.

2.5. Hydrolysis of the hGH sample

The hGH sample with labeled AAs were weighed and transferred into ampoules so that the mole ratio of the target AA was 1:1. Then, 600 μL of 6 M HCl was added. After the solution was purged in nitrogen atmosphere for 1 min, the ampoule was melting sealed to maintain an oxygen-free environment. The protein solution in ampoule was reacted at 110 °C in an oven. After hydrolysis for 36 h, it was taken out and dried in nitrogen flow. Subsequently, the sample was reconstituted with 0.1 M HCl and filtered through a 0.22 μm filter for LC-MS/MS analysis. The process of sample hydrolysis is shown in Fig. 1.

2.6. LC-MS/MS conditions

5 μL of hydrolysis sample was injected into a Poroshell 120 EC C18 (3.0 × 150 mm, 2.7 μm, Agilent, USA) at a flow rate of 0.3 mL/min and eluted at 35 °C. The mobile phase consisted of solvent A (water with 0.1 % FA) and solvent B (acetonitrile with 0.1 % FA). The elution gradient started at 5 % of solvent B and increased linearly to 15 % in 10 min, followed by getting back to 5 % in 5 min and maintained for 5 min equilibrium. The total running time for each injection was 20 min. A positive ion multiple reaction monitoring mode was adopted. The tube lens, collision energy and ion transitions of Leu, $^{13}C^{15}N$-Leu, Phe, $^{13}C^{15}N$ −Phe, Pro, $^{13}C^{15}N$ −Pro were optimized (seen in Table S2). MS operating parameters were as follows: spray voltage: 3500 V, capillary temperature: 320 °C, vaporizer temperature: 250 °C, sheath gas pressure: 35 arb, ion sweep gas pressure: 0 Arb, aux valve flow: 12 arb.

3. Result and discussion

3.1. Protein identification of hGH

hGH sequence (shown in Fig.S1) was achieved in UniProt database, which was obtained by shearing off the signal peptide of the front 26 AAs by the growth hormone precursor (P01241).

After enzymatic digestion of hGH, peptides can be identified by peptide mass fingerprinting (PMF). Peptide sequence identification was conducted through Expasy (https://web.expasy.org/peptide_mass/), enter the AA sequence of hGH, and then to the following Settings: Charge states: $[M + H]^+$, $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$, Enzyme: trypsin, Missed cleavages: 0, Monoisotopic masses.

The base peak chromatogram (BPC) of the product after tryptic digestion of hGH sample is shown in Fig. 2. The signal of 14 peptide segments was detected in Expasy database. Compared with that of hGH theoretical trypsin, the coverage rate was 66.7 %. The result showed that the sample was hGH. The corresponding results are listed in Table 1, which also provides the corresponding trypsin peptide sequences.

The characteristic peaks of some peptide fragments could not be reflected in the BPC, and the possible reasons are as follows: the peptides with small molecular weight were suppressed by high background interference. The inability of trypsin to reach the sites hidden in the three-dimensional structure of the protein results in incomplete digestion and failure to release the corresponding peptide.

3.2. Result of mass balance approach

Purity assessment of hGH *via* mass balance approach was achieved using the following Eq. (1).

$$w = P \times (1 - A - B - C) \quad (1)$$

where *w* is the hGH purity, and *P* is the peak area ratio of the hGH peak to the sum of hGH peak and other impurities peaks measured by UPLC. *A* is the moisture content, *B* is the metal ions content, *C* is the anions content.

In this study, above impurities were quantified separately and the results are listed in Table 2.

Complete separation of hGH and structurally related impurities is the basis for accurate quantification using LC peak area normalization. After 20 min gradient elution, a baseline separation of hGH and impurities was achieved (shown in Fig. 3). The result of LC purity was calculated to be 93.69 %. Three independent hGH samples was analyzed under Karl Fischer titration using volumetric method. The result indicated that moisture content in hGH sample was 2.98 %. Screening for metal ion impurities was performed using ICP-MS and 19.91 % of impurities was found in the hGH study material, metal ions with contents greater than

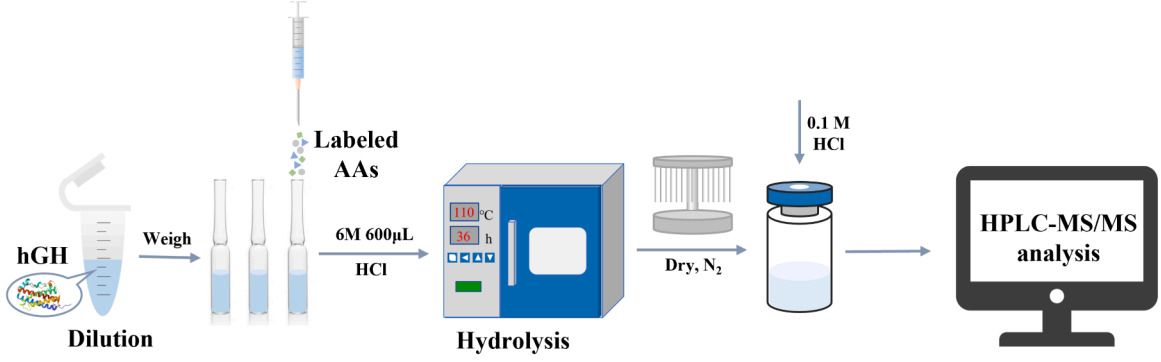

Fig. 1. Process of hGH sample hydrolysis.

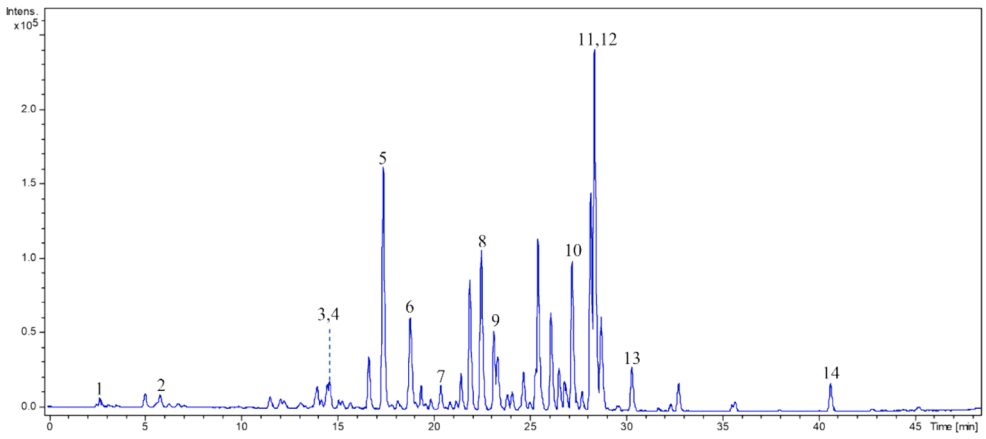

Fig. 2. Base peak chromatogram of trypsin digestion of human growth hormone by TOF-MS.

Table 1
hGH digestion products.

Peak no.	Sequence	Position	$[M + H]^+$ *m/z*	$[M + 2H]^{2+}$ *m/z*	$[M + 3H]^{3+}$ *m/z*
	YSFLQNPQTSLCFSESIPTPSNR	42–64	2616.2399	1308.6236	872.7515
11	LHQLAFDTYQEFEEAYIPK	20–38	2342.1339	1171.5706	781.3828
13	SVFANSLVYGASDSNVYDLLK	95–115	2262.1288	1131.5680	754.7144
14	ISLLLIQSWLEPVQFLR	78–94	2055.2001	1028.1037	685.7382
5	FDTNSHNDDALLK	146–158	1489.6917	745.3495	497.2354
12	DLEEGIQTLMGR	116–127	1361.6729	681.3401	454.5625
10	NYGLLYCFR	159–167	1148.5557	574.7815	383.5234
8	LFDNAMLR	9–16	979.5029	490.2551	327.1725
9	FPTIPLSR	1–8	930.5407	465.7740	310.8517
7	SNLELLR	71–77	844.4887	422.7480	282.1677
	SVEGSCGF	184–191	785.3134	393.1603	262.4426
2	LEDGSPR	128–134	773.3788	387.1930	258.4644
6	VETFLR	173–178	764.4301	382.7187	255.4815
1	EETQQK	65–70	762.3628	381.6850	254.7924
3	TGQIFK	135–140	693.3930	347.2001	231.8025
	QTYSK	141–145	626.3144	313.6608	209.4430
	IVQCR	179–183	618.3392	309.6732	206.7846
4	DMDK	169–172	508.2072	254.6072	170.0739
	EQK	39–41	404.2140	202.6106	135.4095
	AHR	17–19	383.2150	192.1111	128.4098
	K	168–168	147.1128	74.0600	49.7091

Table 2
The results of hGH impurity for the mass balance approach.

Subject	Method	Result (%)
LC purity	UV-UPLC	93.69
Moisture content	Karl Fischer titration	2.98
Metal ions	ICP-MS	19.91
Cl^-	Ion chromatography	12.00
PO_4^{3-}	Ion chromatography	27.55

or equal to 0.1 % were counted. Similarly, Cl^- and PO_4^{3-} were detected to be 12.00 % and 27.55 % by using ion chromatography. As a result, the purity of hGH was calculated to be 35.19 % according to Eq. (1).

3.3. Optimization of hydrolysis conditions

The accuracy of AA-based IDMS quantitative results highly depends on the completeness of protein hydrolysis into individual AAs. The most critical step in protein quantification is chemical hydrolysis. HCl was selected as a hydrolysis reagent in this work. In this study, Leu, Phe, Pro were selected for protein quantification because they present in hGH at high level and acid-stable. In order to maximize the degree of hydrolysis, the reaction conditions, including the concentration and volume of

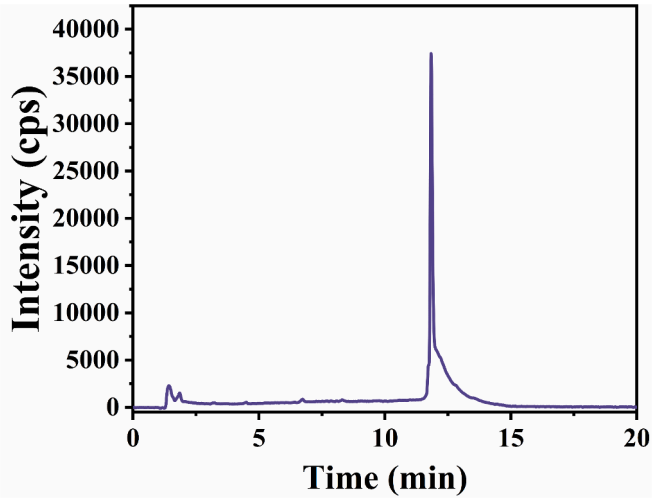

Fig. 3. Chromatography of hGH detected by UPLC.

hydrochloric acid, the reaction temperature and time were carefully optimized. The ratio of AAs peak area to hGH sample amount under different hydrolysis conditions reflects the extent of hydrolysis.

Consequently, the optimal hydrolysis conditions for hGH were 36 h at 110 °C in an oven with the addition of HCl (6 M, 600 μL) (seen in Fig. S2).

3.4. Result of AA-based IDMS method

In the AA-based IDMS method, Leu, Phe, and Pro were selected as the targeted AAs, and corresponding isotope labeled AAs (e.g. $^{13}C^{15}N$-Leu, $^{13}C^{15}N$ −Phe, and $^{13}C^{15}N$ −Pro) were used as internal standards to minimize influences from drifts and fluctuations in LC-MS/MS analysis. The internal standard AAs undergoing the same treatment as the samples were used as calibrants. All hydrolysis reactions were carried out under optimal conditions. After 10 min gradient elution, the three AAs were completely separated in a hydrolyzed hGH sample (Fig. 4).

After the peak area ratios of specific AAs was obtained, the concentration of each AA was calculated according to Eq. (2). Following this, the concentration of the hGH samples was calculated according to the stoichiometric presence of each AA in hGH by Eq. (3).

$$c_a = \frac{P_1 \times P_H \times m_{labeled} \times [R_s \times (I_1 - I_2) - (I_1 \times R_2 - I_2 \times R_1)]}{M \times (R_1 - R_2)} \quad (2)$$

$$c_{hGH} = P_2 \times \frac{M_{hGH} c_a}{n M_{AA}} \quad (3)$$

where P_1 is the purity of AA CRM; P_H is the hydrolysis efficiency of hGH (The experiments defaulted to hGH reaching complete hydrolysis under optimized hydrolysis conditions); $m_{labeled}$ is the mass of labeled AAs; R_s is the peak area ratio of unlabeled and labeled AAs in hGH sample; I_1 and I_2 are the mass ratio of unlabeled and labeled AAs in high-level and low-level solutions, respectively; R_1 and R_2 are the peak area ratio of unlabeled and labeled AAs in high-level and low-level solutions, respectively; M is the mass of hGH; P_2 is purity of hGH assessed by LC; M_{hGH} is molar mass of hGH; n is the number of target AAs in a hGH molecule; M_{AA} is molar mass of target AAs.

As shown in Fig. 5, the hydrolysis efficiencies of the different AAs were generally different, but the hGH concentrations calculated from Leu, Phe, and Pro with the stoichiometric presence of those AAs in the protein were consistent. Three hydrolyzed hGH samples were prepared and measured separately. The hGH quantitative concentrations and standard deviations (SD) calculated from the three AAs (Leu, Phe, Pro)

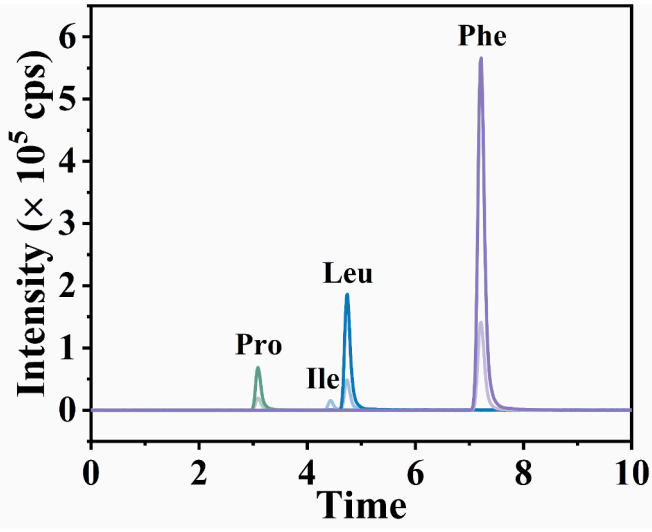

Fig. 4. Typical overlayed extracted chromatogram of the unlabeled and labeled AAs in a hydrolyzed hGH sample.

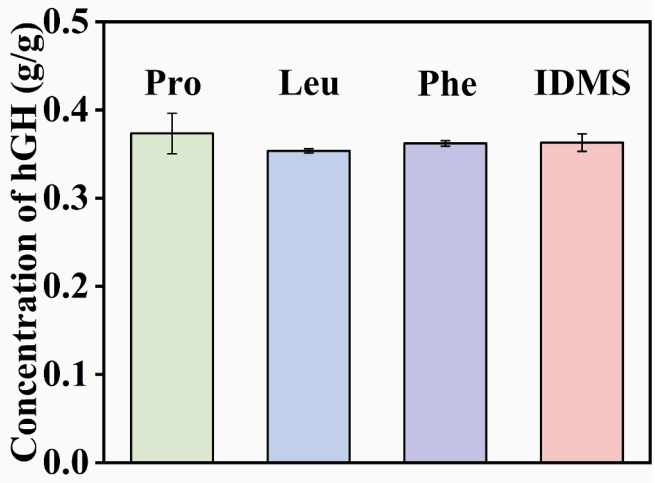

Fig. 5. Hgh purity results by aa-based idms.

were calculated to be (0.373 ± 0.023) g/g, (0.354 ± 0.002) g/g, (0.362 ± 0.003) g/g, respectively.

It is worth noting that despite the optimal hydrolysis conditions were applied and internal standards were used to minimize potential errors caused by instrument and measuring method, the quantitative results of the various AAs still may be somewhat biased. There are some reasons lead to inconsistent results. Primarily, different AAs and their positions in the AA sequence of hGH result in different releasing times and efficiencies during hydrolysis. In this case, even under optimal hydrolysis condition, incomplete hydrolysis may lead to low quantitative results. In contrast, due to the presence of peptide and protein impurities, target AAs released from them may overestimate quantitative results.

Table 3 depicts the results of the inter- and intra- day assays, which were used to assess the precision, and reproducibility of the sample analysis. As can be seen in Table 3, the mean inter-day and intra-day RSDs of hGH purity determined by AA-based IDMS analysis were within 1.98 %, which indicates that the hydrolysis method has good reproducibility.

3.5. Comparison of the results from different methods and uncertainty assessment

In this study, two independent quantification strategies, e.g. mass balance method and AA-based IDMS were used to assess the purity of hGH. Fig. 6 shows the hGH purity results characterized by mass balance method and AA-based IDMS with Leu, Phe and Pro as target analytes. The result from mass balance method (0.352 g/g) is in good agreement with the result from AA-based IDMS mean (0.363 g/g). Despite the fact that possible peptide or protein impurities were eliminated, the result of the IDMS method is still slightly higher than that of the mass balance method, which might be due to the presence of a small amount of free AAs in the samples.

Uncertainties of mass balance approach and AA-based IDMS were determined according to JJF 1343–2022. Uncertainties comes from

Table 3
Intra-day and inter-day precisions for hGH purity assessment using AA-IDMS (g/g).

	Intra-day assays (n = 6)		Inter-day assays (n = 6)	
	Concentration (mean + SD)	RSD (%)	Concentration (mean + SD)	RSD (%)
Pro	0.377 + 0.007	1.89	0.376 + 0.005	1.35
Leu	0.350 + 0.007	1.98	0.346 + 0.003	0.76
Phe	0.355 + 0.005	1.43	0.356 + 0.004	0.99

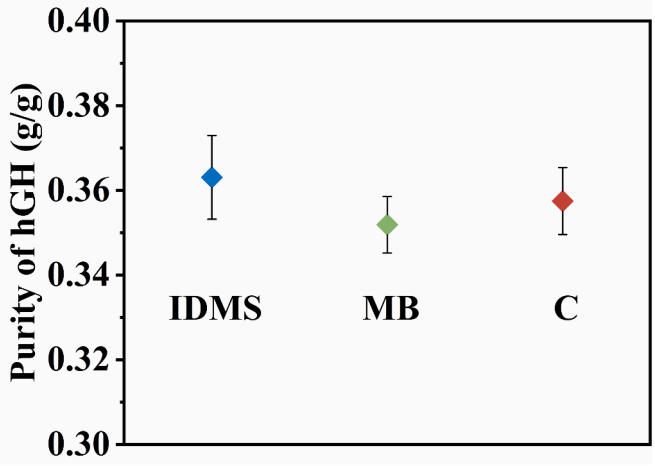

Fig. 6. Hgh purity results by mass balance approach and aa-based idms. c represents the mean value of hgh mass fraction from these two independent methods.

various sources.

In the mass balance approach, uncertainties of measurement of LC purity and moisture content mainly came from the measurement repeatability, which were obtained by Eq. S1. The results were calculated to be 0.005 g/g and 0.004 g/g, respectively. As for uncertainty of metal ions and anions assessment, we refer to previously reported literature and obtain final uncertainties results of 1.99×10^{-7} [30] and 4.4×10^{-8} [31]. Finally, the uncertainty of mass balance approach was calculated to be 0.005 g/g *via* Eq. S2, thus the expanded uncertainty was 0.010 g/g according to Eq. S3.

The main sources of uncertainty in the quantification of hGH purity by AA-based IDMS are the reproducibility of the method, the efficiency of hydrolysis, reagent weighing, and certified values for AAs. The contents of Leu, Phe and Pro in hGH were measured by AA-based IDMS, and finally hGH content was obtained according to the number of AAs in a hGH molecule. The average of the three AAs quantitative hGH results, so the uncertainties of the three AAs on the quantitative results of hGH need to be calculated, respectively, which include the standard deviation of the mean of Leu, Phe and Pro, uncertainty introduced by hydrolysis efficiency is already included in the differences in the quantitative results for different AAs [32], uncertainty introduced by the balance weighing was less than 0.1 % and can be neglected, and uncertainty contribution of AA-CRMs was calculated based on the certificate of reference material according to the type B evaluation of measurement uncertainty. The uncertainty evaluation of the purity assessment for hGH using AA-based IDMS is listed in Table 4 and Eqs. S4–S7.

In summary, the hGH purity determined by mass balance approach was (0.352 ± 0.010) g/g, while the result obtained by IDMS was (0.363 ± 0.028) g/g which well covers all three results for the determination of Leu Phe and Pro. Therefore, the mean value of hGH purity in the study material was calculated to be 0.358 g/g. According to JJF 1343–2022, Eq. S8 was applied to assess the uncertainty of the value assignment and the result of u_c was estimated to be 0.008 g/g, expanded uncertainty was 0.016 g/g. Eventually, the purity of hGH in the study material was (0.358 ± 0.016) g/g.

4. Conclusion

In this study, two methods for hGH purity assessment were established. Mass balance approach and AA-based IDMS were adopted in the purity assessment process and the results were in agreement with each other and with a small uncertainty. The mean value of hGH purity was calculated to be (0.358 ± 0.016) g/g. The hGH quantification method established in this study provides valuable information for development of other protein quantification methods, and lay the foundation for the research of accurate quantification methods for peptide and protein contents for disease diagnostic markers, the development of hGH standard substances, and quality control of protein medicine.

Table 4
Uncertainty evaluation of the purity assessment for hGH using AA-based IDMS.

Source of uncertainty	Pro (%)	Leu (%)	Phe (%)
Method repeatability (u_r)	3.59	0.41	0.51
AA-CRM (u_{CRM})	0.76	0.76	0.75
Uncertainty value ($u_c(AA)$)	3.77	1.32	1.35
Combined standard uncertainty value ($u_c(IDMS)$)	1.41		
Expanded uncertainty (U) ($k = 2$)	2.28		

CRediT authorship contribution statement

Jiahui Li: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Jingkang Li:** Validation, Data curation. **Haihong He:** Writing – review & editing, Visualization. **Ming Li:** Writing – review & editing, Supervision, Resources, Conceptualization. **Pinyi Ma:** Writing – review & editing, Visualization. **Daqian Song:** Writing – review & editing, Visualization. **Qiang Fei:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2024.111144.

References

[1] D.C. Perez-Ibave, I.P. Rodriguez-Sanchez, L. Garza-Rodriguez Mde, H.A. Barrera-Saldana, Extrapituitary growth hormone synthesis in humans, Growth Horm IGF Res 24 (2014) 47–53.
[2] S. Wu, W. Yang, F. De Luca, Insulin-like growth factor-independent effects of growth hormone on growth plate chondrogenesis and longitudinal bone growth, Endocrinology 156 (2015) 2541–2551.
[3] M. Dattani, M. Preece, Growth hormone deficiency and related disorders: insights into causation, diagnosis, and treatment, Lancet 363 (2004) 1977–1987.
[4] P.J. Trainer, J. Barth, C. Sturgeon, G. Wieringaon, Consensus statement on the standardisation of GH assays, Eur J Endocrinol 155 (2006) 1–2.
[5] S.A. Divall, S. Radovick, Growth hormone and treatment controversy long term safety of rGH, Curr Pediatr Rep 1 (2013) 128–132.
[6] K. Reinmuth-Selzle, T. Tchipilov, A.T. Backes, G. Tscheuschner, K. Tang, K. Ziegler, K. Lucas, U. Poschl, J. Frohlich-Nowoisky, M.G. Weller, Determination of the protein content of complex samples by aromatic amino acid analysis, liquid chromatography-UV absorbance, and colorimetry, Anal Bioanal Chem 414 (2022) 4457–4470.
[7] A.F.S. Silva, F.R.P. Rocha, A novel approach to detect milk adulteration based on the determination of protein content by smartphone-based digital image colorimetry, Food Control 115 (2020) 107299.
[8] F. Mahzabeen, O. Vermesh, J. Levi, M. Tan, I.S. Alam, C.T. Chan, S.S. Gambhir, J. S. Harris, Real-time point-of-care total protein measurement with a miniaturized

optoelectronic biosensor and fast fluorescence-based assay, Biosens Bioelectron 180 (2021) 112823.
[9] E.Y. Kwon, H.A. Abusharkh, X. Ruan, D. Du, E. Hammond-Pereira, B.J. Van Wie, Pd@Pt nanoparticle-linked immunosorbent assay for quantification of Collagen type II, Anal Chim Acta 1266 (2023) 341265.
[10] Z. Ji, J. Zhang, C. Deng, Z. Hu, Q. Du, T. Guo, J. Wang, R. Fan, R. Han, Y. Yang, Identification of mare milk adulteration with cow milk by liquid chromatography-high resolution mass spectrometry based on proteomics and metabolomics approaches, Food Chem 405 (2023) 134901.
[11] J.S. Jeong, H.M. Lim, S.K. Kim, H.K. Ku, K.H. Oh, S.R. Park, Quantification of human growth hormone by amino acid composition analysis using isotope dilution liquid-chromatography tandem mass spectrometry, J Chromatogr A 1218 (2011) 6596–6602.
[12] V.C. Albright 3rd, R.L. Hellmich, J.R. Coats, A review of cry protein detection with enzyme-linked immunosorbent assays, J Agric Food Chem 64 (2016) 2175–2189.
[13] N. Stoppacher, R.D. Josephs, A. Daireaux, T. Choteau, S. Westwood, R.I. Wielgosz, Accurate quantification of impurities in pure peptide material - angiotensin I: comparison of calibration requirements and method performance characteristics of liquid chromatography coupled to hybrid tandem mass spectrometry and linear ion trap high-resolution mass spectrometry, Rapid Commun Mass Spectrom 29 (2015) 1651–1660.
[14] S. Westwood, T. Choteau, A. Daireaux, R.D. Josephs, R.I. Wielgosz, Mass balance method for the SI value assignment of the purity of organic compounds, Anal Chem 85 (2013) 3118–3126.
[15] X. Wang, F. Zhang, H. Li, P. Xiao, F. Su, B. Xu, W. Sun, D. Song, Purity determination of synthetic glucagon using a mass balance approach, Sci Rep 10 (2020) 4423.
[16] J. Zhou, F. Li, M. Wang, C. Yan, M. Yang, T. Wang, L. Zhang, Preparation of clorprenaline certified reference material: purity determination and uncertainty evaluation, Microchem. J. 179 (2022) 107502.
[17] R.D. Josephs, N. Stoppacher, A. Daireaux, T. Choteau, K.A. Lippa, K.W. Phinney, S. Westwood, R.I. Wielgosz, State-of-the-art and trends for the SI traceable value assignment of the purity of peptides using the model compound angiotensin I, TRAC-Trend Anal Chem 101 (2018) 108–119.
[18] S. Wang, P. Wu, M. Li, T. Huang, N. Shi, L. Feng, H. Li, Mass balance method for SI-traceable purity assignment of synthetic oxytocin, J Pharm Biomed Anal 207 (2022) 114401.
[19] L. Wu, A. Takatsu, S.R. Park, B. Yang, H. Yang, T. Kinumi, J. Wang, J. Bi, Y. Wang, Development and co-validation of porcine insulin certified reference material by high-performance liquid chromatography-isotope dilution mass spectrometry, Anal Bioanal Chem 407 (2015) 3125–3135.
[20] L. Wu, B. Yang, J. Bi, J. Wang, Development of bovine serum albumin certified reference material, Anal Bioanal Chem 400 (2011) 3443–3449.
[21] P. Worsfold, C. Poole, A. Townshend, M. Miró, Encyclopedia of Analytical Science, 2019.
[22] L. Feng, Z. Huo, J. Xiong, H. Li, Certification of amyloid-beta (abeta) certified reference materials by amino acid-based isotope dilution high-performance liquid chromatography mass spectrometry and sulfur-based high-performance liquid chromatography isotope dilution inductively coupled plasma mass spectrometry, Anal Chem 92 (2020) 13229–13237.
[23] T. Huang, X. Zhang, D. Song, L. Wu, X. Li, Y. Yang, C. Quan, H. Li, The quantification of human chorionic gonadotropin by two isotope dilution mass spectrometry methods, Anal, Methods 6 (2014) 8690–8697.
[24] T. Slechtova, M. Gilar, K. Kalikova, S.M. Moore, J.W. Jorgenson, E. Tesarova, Performance comparison of three trypsin columns used in liquid chromatography, J Chromatogr A 1490 (2017) 126–132.
[25] J. Liu, W. Zhu, H. Sun, D. Song, P. Xiao, B. Xu, H. Li, Development of a primary reference material of natural C-reactive protein: verification of its natural pentameric structure and certification by two isotope dilution mass spectrometry, Anal Methods 13 (2021) 626–635.
[26] Z. Liu, X. Zhao, Y. Liu, L. Shi, L. Wu, H. Yuan, Y. Jin, Development of an insulin-like growth factor-1 certified reference material by SI-traceable isotope-dilution mass spectrometry, Talanta 273 (2024) 125812.
[27] M. Kato, T. Kinumi, M. Yoshioka, M. Goto, S. Fujii, A. Takatsu, Development of C-reactive protein certified reference material NMIJ CRM 6201-b: optimization of a hydrolysis process to improve the accuracy of amino acid analysis, Anal Bioanal Chem 407 (2015) 3137–3146.
[28] J. Yan, M. Wang, M. Yang, J. Zhou, X. Xue, T. Wang, Study of SI-traceable purity assessment of bovine lactoferrin using mass balance approach and amino acid-based isotope dilution liquid chromatography-mass spectrometry, Food Chem 385 (2022) 132674.
[29] M. Zhu, J. Li, Z. Chu, L. Li, B. Meng, Y. Zhao, X. Gong, Z. Qu, W. Mi, Y. Jiang, L. Wu, X. Dai, X. Fang, R. Zhai, Development of cancer biomarker heat shock protein 90alpha certified reference material using two different isotope dilution mass spectrometry techniques, Anal Bioanal Chem 416 (2024) 913–923.
[30] X. He, Uncertainty evaluation of determination of tin in soil by microwave digestion-inductively coupled plasma mass spectrometry, Shandong Chem. Ind. 51 (2022), 136–138+142.
[31] Y. Zhou, L. Liu, Y. Lin, L. Li, W. Zhang, Uncertainty evaluation for determination of chloride content in drinking water by Ion Chromatography, Stud. Trace Elements Health 30 (2013) 52–53.
[32] C. Pritchard, F.A. Torma, C. Hopley, M. Quaglia, G. O'Connor, Investigating microwave hydrolysis for the traceable quantification of peptide standards using gas chromatography-mass spectrometry, Anal Biochem 412 (2011) 40–46.