Fully Traceable Absolute Protein Quantification of Somatropin that Allows Independent Comparison of Somatropin Standards

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BACKGROUND: Measurement traceability in clinical chemistry is required to standardize clinical results irrespective of the measurement procedure and laboratory. The traceability of many protein substances is maintained by reference to the first standard produced, which may no longer exist, with values assigned by consensus. Independent methods that provide traceability to the Système d’Unité International for all relevant properties of a protein standard could remove reliance on the original standard preparations.

METHODS: We developed a method based on the traceable quantification of tryptic peptides released from the protein by isotope dilution mass spectrometry to compare 2 standard preparations of somatropin (recombinant human growth hormone), WHO 98/574 and Ph.Eur.CRS S0947000. Relative quantification using isotope-coded affinity tagging, isobaric tagging for relative and absolute quantification, and standard additions were also performed to validate the digestion method used and to determine whether any modifications were present.

RESULTS: The total somatropin content in both materials was determined and an uncertainty estimation undertaken [WHO 2.19 (0.21) mg/vial, European Pharmacopeia 2.06 (0.21) mg/vial]. Isotope coded affinity tag and standard addition results fully validated the robustness of the digestion method used. In addition, iTRAQ (isobaric tagging for relative and absolute quantification analysis) identified 2 modifications, neither of which impacted the quantification.

CONCLUSIONS: An independent method that does not rely on a preexisting protein standard has been developed and validated for the traceable value-assignment of total somatropin. The methods reported here address the amount of substance (mass fraction) of the standard materials but address neither biological activity nor other characteristics that may be important in assessing suitability for use as a calibrator.

The requirement for biological reference materials and certified reference materials is recognized within the clinical community and by the IFCC. Use of suitable reference materials supports results that are accurate, specific, and most importantly comparable between laboratories. For well-defined measurands comparability is assured according to the principle of traceability, the unbroken chain of comparisons relating an individual patient result back to a national or international standard (1–3). Traceability allows for the appropriate assessment of threshold values used in diagnosis and prognosis of disease states and avoids potential misclassification of disease risk or administration of inappropriate intervention strategies or therapeutics for the patient. It is now a requirement for calibrators of all commercial clinical tests marketed within the European Union to show traceability to higher order standards “where available” (4).

Ideally, the highest order (primary) standard in the traceability chain is defined using a primary method, or if this is not practical a primary ratio method or method of highest achievable metrological order, such as isotope dilution mass spectrometry (IDMS)1 (3). This type of method, when practiced correctly, relates directly to the Système d’Unité International (SI) with small measurement uncertainties. Adopting this approach for cholesterol showed a 4-fold reduction in measurement uncertainty and a saving of approximately $100 million/year (5). However, although requirements for IDMS, such as isotopically labeled materials, can be realized for such well-defined measurands, larger biologicals are not directly amenable to this approach (6, 7).

1 Nonstandard abbreviations: IDMS, isotope dilution mass spectrometry; SI, Système d’Unité International; hGH, human growth hormone; AAA, amino acid analysis; IS, independent standard; EP European Pharmacopoeia; iTRAQ, isobaric tagging for relative and absolute quantification; ICAT, isotope coded affinity tag; EM, exact-matching.
Most biological standards are currently defined according to arbitrarily assigned biological activity units (IU), a best-fit approach to the comparison of quantitative measurements for heterogeneous biologicals for which SI traceability would be difficult to achieve. Often, the exact description of the measurand is not well defined or may incorporate a number of different measurands, owing to inherent protein heterogeneity. It may even consist of a mixture of similar analytes observed to have biological activity. Maintaining continuity of the IU unit over time, however, is still not well defined or may incorporate a number of different measurands, owing to inherent protein heterogeneity.

Various somatropin standards are now available; however, without reference to the first WHO material, the SI values assigned cannot be directly compared. The European Pharmacopeia material was a freeze-dried preparation of recombinant somatropin. This material was a replacement for a previous standard (CRS batch 1) and was prepared in a similar manner to that of the WHO material. The mass of somatropin monomer was determined by size-exclusion chromatography using CRS1 or the second WHO material as a calibrant. Issues arose, however, whereby the different calibrators returned different results (11). Many of the preparations contain excipients, e.g., stabilizers, and thus are not amenable to AAA. Here we report the use of a primary method of comparison for total somatropin that maintained SI-traceability to evaluate two standard preparations, WHO 98/574 and Ph. Eur. CRS S0947000, enabling comparison of the amounts.

Materials and Methods

We obtained the natural amino acids L-alanine, L-arginine, L-isoleucine, L-leucine, L-lysine, and L-phenylalanine, all purities >99%, from Fluka. Labeled amino acids were from Cambridge Isotopes Laboratory: L-alanine (3-13C, 99%), L-arginine:HCl (15N2, 98%), L-isoleucine (13C6, 98%), L-leucine (13C6, 98%), L-lysine:2HCl (15N2, 98%), L-phenylalanine (3-13C, 99%).

The peptides T2 (sequence LFDNAMLR), T2* ([U-13C6, 15N]LFDNAMLR), T13 (TGQIFK), and T13* (TGQ[13C9,15N]FK), all stated purities >95%, were custom synthesized (Cambridge Research Biochemicals). Recombinant hGH (22-kDa isoform, Fig. 1), used as the independent standard (IS), was purchased from CytoShop; 10 mg of the IS material was further purified at the Physikalisch-Technische Bundesanstalt (PTB, Braunschweig, Germany) and analyzed by AAA and IDMS as described by Arsen et al. (12). Briefly, the purified standard solution was assigned a mass fraction...
of somatropin via AAA using IDMS principles and peptide IDMS. The mean (SD) reported concentration was 30.8 (0.7) nmol/g (0.681 (0.016) mg/g).

Reference somatropin standards were WHO 98/574 (National Institute for Biological Standards and Control) and European Pharmacopeia (EP) CRS S0947000 (LGC Standards).

Other materials were: ultra pure water (18 mol/L cm⁻¹), acetic acid, formic acid (Fisher Scientific), sequencing-grade trypsin (Roche), trifluoroacetic acid (Fluka), hydrochloric acid (37 wt. % in water, 99.999%, SigmaAldrich), acetonitrile (Optigrade HPLC Special Grade, LGC Standards). The digestion reagents triethylammonium bicarbonate pH 8.5, tris-acid (Fluka), hydrochloric acid (37 wt. % in water, and 92.0% (2.3%), leucine 99.6%

Sulfate were acquired from Applied Biosystems, as were (2-carboxyethyl)phosphine, and methylmethanethiosulfate were acquired from Applied Biosystems, as were the isobaric tagging for relative and absolute quantification.

Amino acid standard solutions were prepared in 100 mmol/L HCl and stored at 4 °C. Natural amino acid solution concentrations were compared against NIST standard reference material 2389 using exact-matching (EM)-IDMS, as described by Burkitt et al. (13). Amino acid purities were: alanine 99.0% + 1.0%/−2.3%, arginine 97.8% + 2.2%/−2.6%, isoleucine 92.0% (2.3%), leucine 99.6% +0.4%/−3.0%, lysine 98.8% +1.2%/−2.5%, phenylalanine 95.3% (2.5%).

Peptide standard solutions were prepared in water and stored at −20 °C. Peptides were quantified by un-derivatized AAA as described by Burkitt et al. (13). The measured concentrations were: T2 59.0 (3.7) nmol/g, T13 69.0 (2.2) nmol/g.

Protein standards were prepared in 50:50 (vol:vol) water:acetonitrile and stored at −20 °C. The certified value for the WHO material was 1.95 mg total somatropin and related proteins per vial (10). The gravimetric preparation showed an expected value of 0.98 (0.05) mg/g. The EP certified value was 1.69 mg somatropin monomer per vial (11). The value expected from gravimetric preparation, corrected using the consensus value for monomer content of 0.9836 (14), was 0.86 (0.05) mg/g.

For EM-IDMS, 3 pairs of blends were prepared for each standard (IS, EP, WHO), with the sample blend containing 10 µg of protein standard and labeled peptides, and the calibration blend containing natural and labeled peptides. Mass fractions of labeled peptides were equivalent in both blends and added such that equal instrument response for the natural and label signals was observed. For iTRAQ analysis, 3 samples containing 10 µg of the protein standards at 1:1:1 ratios were prepared. For standard addition experiments, samples contained 10 µg of the WHO/EP protein standard and equivalent molar amounts of the labeled peptides with 0, 5, 10, or 15 µg of IS somatropin added to each. For ICAT 4 samples, containing 10 µg of protein, 2 of IS, and 1 each of the WHO and EP materials, were prepared and repeat digests performed.

Digestion of samples analyzed by EM-IDMS, iTRAQ, and standard addition experiments was performed as described by Quaglia et al. (14) using 3 trypsin additions. Digestion of ICAT samples was performed according to the Applied Biosystems protocol with a single trypsin addition.

**PROTEIN QUANTIFICATION**

For EM-IDMS and standard addition analysis, aliquots of digested protein samples (10 µL) were diluted 1:10 in 0.1% formic acid before liquid chromatography–tandem mass spectrometry analysis on a Waters Ultima triple-quadrupole instrument coupled with a Waters Alliance 2965 HPLC system using a Phenomenex Luna HST C18 (4) 2.5-µm column, 2.0 × 100 mm at 30 °C. Mobile phases were: (A) water:0.1% formic acid (vol:vol) and (B) acetonitrile:0.1% formic acid (vol:vol). Linear gradient steps of 0–0.2 min 95% A, 0.2–7 min 95%–55% A and 7–7.5 min 55%–95% A were performed at a flow rate of 300 µL/min. The [M+2H]²⁺ ions for natural and labeled peptides were monitored using single-reaction monitoring: T2 m/z 490.3 > 233.1, T2* m/z 493.8 > 240.2, T13 m/z 347.2 > 294.1, T13* m/z 352.2 > 304.1. A collision energy of 18 V and dwell time of 0.1 s were used for all transitions.

iTRAQ labeling was performed gravimetrically, following the Applied Biosystems protocol (15). IS samples were labeled with light (¹²C₉) and WHO/EP materials with heavy (¹³C₉) ICAT reagent. Dried samples were dissolved in 100 µL water before LC-MS analysis on a triple quadrupole instrument (details above). A Waters Atlantis C18 5-µm column (2.1 × 150 mm) was used for peptide separation. Mobile phases were: (A) water:0.1% formic acid (vol:vol) and (B) acetonitrile:0.1% formic acid (vol:vol) with linear gradient steps of 0–45 min 95%–5% A, 45–50 min 5%–95% A, flow rate 200 µL/min. Selected ion recording for the 3 observed ICAT-labeled Cys-containing peptide pairs was performed: T16 m/z 688.5/693.0, T6-T7 (missed cleavage) m/z 898.0/900.2, T15-T16 (missed cleavage) m/z 949.1/952.2. Dwell times of 0.1 s and cone voltages of 20 V were applied.

iTRAQ sample preparation was performed as described by Quaglia et al. (14) using 3 protein samples (IS, EP, and WHO). After derivatization, vials were gravimetrically pooled in a 1:1:1 ratio of 114-labeled IS, 115-labeled WHO, and 116-labeled EP, and analyzed.
by liquid chromatography–tandem mass spectrometry analysis on a quadrupole time-of-flight instrument.

**EM-IDMS QUANTIFICATION**
Samples were injected 5 times each, bracketed by the respective calibration blend, and the results determined using the EM-IDMS equation as described by Quaglia et al. (14).

**STANDARD ADDITION QUANTIFICATION**
Standard addition calibration curves for each sample were determined by plotting the mean ratio of the natural- to labeled-peptide response (3 injections) against the known amount of standard protein added for each peptide. For all samples the $R^2$ was $>0.99$. Molar concentrations of the 2 peptides were calculated from these plots, and the results were combined to give the final protein concentration.

**ICAT QUANTIFICATION**
Three Cys-containing peptides were detected, and the measured protein concentration was determined from the ratio of light to heavy peptide present. The final protein concentration was a mean of the results of the 3 ICAT labeled peptide pairs.

**iTRAQ QUANTIFICATION**
Results were processed using ProQuant and confirmed with Mascot, for which the ratio of 115 (WHO) or 116 (EP) against 114 (IS) was used to determine protein concentration. The final results were a mean of 3 separate mixes.

**UNCERTAINTY CALCULATIONS**
Standard and combined uncertainties were estimated as described in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue11.

**Results and Discussion**
Quantification of the IS, WHO, and EP somatropin materials was performed using the 4 independent techniques of EM-IDMS, standard addition, ICAT, and iTRAQ. Results of the analyses are shown in Table 1.

**COMBINED RESULTS**
Results of the independent methods EM-IDMS, standard addition, and iTRAQ were combined to give a final value for the standards. The determined mass fractions were: EP 1.03 (0.05) mg/g and WHO 1.09 (0.05) mg/g. With use of the gravimetric preparation of the solutions, these values gave amounts of total protein per vial containing the somatropin-specific peptides chosen of 2.06 (0.21) mg/vial for EP and 2.19 (0.21) mg/vial for WHO (Fig. 2).

Naturally occurring growth hormone shows inherent sample heterogeneity, and a number of these forms have been characterized. There are 2 main variants, the more abundant 22-kDa and a shorter 20-kDa variant, with degradation fragments also reported (16) (17). This observed sample heterogeneity in plasma is one reason behind the discrepancies in hGH measurement results observed between commercially available assays (16). Monomeric 22-kDa hGH (Fig. 1) has been chosen as a basis for hGH measurements, but because each assay uses different epitopes, there is little guarantee as to which other forms are being recognized and to what extent (17). It is understood that to improve hGH measurements, assay calibration and assay specificity must be considered; (16). Assay specificity has not been addressed here, but improvements in value-

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**Table 1. Results obtained by EM-IDMS, standard addition, ICAT, and iTRAQ analysis of the independent, WHO and EP somatropin standards.**

<table>
<thead>
<tr>
<th></th>
<th>Certified value</th>
<th>EM-IDMS</th>
<th>Standard addition</th>
<th>ICAT</th>
<th>iTRAQ</th>
<th>Combined value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IS</strong></td>
<td>0.68 (0.02)</td>
<td>0.69 (0.10)</td>
<td>—</td>
<td>0.62 (0.11)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>14.1%</td>
<td>17.4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WHO</strong></td>
<td>0.98 (0.05)</td>
<td>1.05 (0.17)</td>
<td>1.11 (0.37)</td>
<td>0.78 (0.17)</td>
<td>1.11 (0.13)</td>
<td>1.09 (0.05)</td>
</tr>
<tr>
<td></td>
<td>15.8%</td>
<td>33.4%</td>
<td>22.2%</td>
<td>11.7%</td>
<td>9.6%</td>
<td></td>
</tr>
<tr>
<td><strong>EP</strong></td>
<td>0.86 (0.05)</td>
<td>1.05 (0.17)</td>
<td>0.99 (0.20)</td>
<td>1.02 (0.23)</td>
<td>1.05 (0.18)</td>
<td>1.03 (0.05)</td>
</tr>
<tr>
<td></td>
<td>15.8%</td>
<td>20.7%</td>
<td>22.8%</td>
<td>16.8%</td>
<td>10.3%</td>
<td></td>
</tr>
</tbody>
</table>

* Certified values and the combined result incorporating EM-IDMS, standard addition and iTRAQ results also shown. Uncertainties represent the combined uncertainties quoted at 95% confidence ($k = 2$). Percentage uncertainties are shown below each result.
assigning the higher-order standards required for assay calibration have been described.

The method developed for hGH quantification (12, 13) made use of tryptic peptides common to most variants of somatropin. These were quantified using EM-IDMS after total release of the peptides of interest on protein digestion. The peptides were purified and purity values confirmed by acid hydrolysis and IDMS of the released amino acids. A direct traceability chain was established from the determined mass fraction of the released peptides to the mass fractions of the primary amino acid solution prepared for peptide characterization. Discrepancies in protein digestion between the samples, possibly due to different material preparations, were investigated by standard additions by using a separate well-characterized somatropin preparation, as well as a labeling technique enabling digestion under identical conditions (ICAT). Finally, a comparison of relative amounts of different variants was achieved by using iTRAQ, in which all peptides are labeled. The results obtained using different techniques were consistent within the reported measurement uncertainties, suggesting that a direct comparison in terms of mass fraction between the 2 preparations is feasible without the use of a protein standard.

The EM-IDMS method enables SI traceability for the value-assignment of protein standards via the specific peptides chosen. Provided the criteria of complete release of the peptides of interest and stability of labeled and natural peptides during tryptic digestion are satisfied, this method has been shown to provide accurate and precise results for protein quantification (12–14).

The EM-IDMS result for the WHO material was consistent with the certified value within the measurement uncertainties. For the EP material, however, a 23% difference from the certified value was observed, which was not accounted for within the measurement uncertainties (Table 1).

Daas et al. reported a “statistically significant discrepancy” in the results for the EP material (somatropin CRS2) compared to 2 recognized standard preparations (WHO 98/574, EP somatropin CRS1) (11). To assess possible inconsistencies between the IU assigned to different standard preparations, quantification of the current somatropin standards by the WHO/EP was performed relative to earlier standards. During performance of this quantification, the consensus mean may have drifted from the true value and may no longer be equivalent. To retain measurement continuity for the end user, a consensus value was chosen that reflects neither comparison directly and will perpetuate any drift from the true value. This demonstrates the need for standards to be value-assigned such that they are independently traceable for all relevant influence quantities, ideally to the SI, to ensure true comparability of clinical measurements over time (10, 11).

To infer protein concentration from peptide concentration, the peptides of interest must be fully released during digestion. By monitoring multiple peptides, which give equimolar results, the completeness of digestion can be inferred. Release of peptides T2/T13 for the IS material under digestion conditions used for EM-IDMS was monitored in earlier work and determined to be equimolar (14). However, salts and buffers are present as stabilizers in the EP/WHO standards that were not present in the purified IS material, and therefore digestion conditions may differ. Alternative quantification methods were employed to determine whether digestion conditions are comparable for all samples.

Using the purified IS, we performed standard addition experiments to assess the linearity of digestion across the concentration range of interest. Good linearity, with $R^2 > 0.99$, was observed for both peptides. We concluded that total peptide release was not affected by excipients present, and digestion was reproducible between samples with no fall-off in trypsin activity. The results agreed with the EM-IDMS values for both materials (<6%) within the quoted measurement uncertainties (Table 1).

Digestion for standard additions, however, was performed in separate vials (each standard/sample mixed solution digested in a single vial) with the potential for small variations, e.g., different buffers, in the conditions. The relative quantification using ICAT al-
allowed labeling and sample mixing before digestion, with samples then digested collectively under identical conditions. Using this technique to corroborate the standard addition results, EP and WHO materials were quantified relative to the purified somatropin material (IS). The ICAT results were consistent with the EM-IDMS results within the measurement uncertainties, with a consistent negative bias possibly due to labeling variations.

Posttranslational modifications, e.g., deamidation, and the formation of multimers are well documented for pituitary hGH (16). It has also been shown (18) that there is potential for ≤2% sequence variations and modifications to occur in recombinant 22 kDa hGH as expressed by Escherichia coli. All modifications and protein isoforms containing quantification peptides T2/T13 will be accounted for in total protein quantification. However, the absence of modified forms of the standard peptides must be confirmed. In our iTRAQ experiment, labeling was performed after digestion and occurred at each peptide N-terminus, providing coverage across the protein (62%, Fig. 1). This gave quantification data relative to the standard material (IS). These results were consistent, within the measurement uncertainties, with previous results (Table 1). Additionally, iTRAQ can be used to quantify modifications and indicate differential behavior between the materials.

Two desamido forms of hGH have been identified in plasma: Asn-152 and Glu-137 (16, 17). Capillary electrophoresis of impurities has been documented for both standards and, in both cases, a small percentage (1%–3%) of deamidated hGH was identified but the deamidation site not specified (10, 14). Peptide T13 (TGQIFK) contains Glu-137, but no deamidation at this site for the recombinant form has previously been reported or observed during these studies. Asn-152 (T15) deamidation was observed in both materials at similar proportions (Fig. 3).

No naturally occurring isoforms have been reported that contain modifications found in the peptide T2 (LFDNAMLR). However, 2 methionine-14 modifications, an oxidation and amino acid substitution (M→I), have been observed at very low amounts within a specific recombinant form has previously been reported or observed during these studies. Asn-152 (T15) deamidation was observed in both materials at similar proportions (Fig. 3). No formally assigned peptide (T10) was identified by iTRAQ (Fig. 3), with deamidation more prominent in the WHO material. There are 2 Asn residues in T10, and although the exact site of deamidation has not been identified here, deamidation of Asn-99 has been observed as a minor degradation product of somatropin (19) and could potentially indicate a difference in sample stability.

No formal uncertainty estimate was assigned to either standard, so we did not consider variables such as the uncertainty of purity, repeat experiments, etc. Consequently, this lack of the assignment of a formal uncertainty estimate, along with the lack of characterization of all of the influencing quantities in a traceable manner, makes it impossible to determine whether differences observed in routine results when the calibration standard is changed are acceptable. All values assigned by quantitative methods described here had full uncertainty estimates carried out that encompass all potential sources of measurement error. This process provides an estimate of the real variation between results. For comparison, uncertainties shown for the WHO and EP standards represent either the SD of results (WHO) or the moisture content (EP) determined during the collaborative studies, as described by Brustow et al. (10) and Daas et al. (11).

Good agreement of all methods within the measurement uncertainties was observed for both materials. The use of a combined value incorporating the results from independent quantitative methods gave further confidence in the results (Fig. 2), for which a difference from the certified value was consistently observed for the EP but not the WHO material.

The 20- and 22-kDa forms of hGH are differentiated by a series of 15 amino acids. With the use of a peptide specifically chosen such that it includes part of this series, it may be possible in the future to individually quantify these 2 major forms, provided the requirements of complete digestion and peptide stability have been satisfied. This work could further improve assay comparability.
In summary, we quantified WHO and EP somatropin reference standards for total somatropin with the primary ratio method of EM-IDMS using fully characterized peptide standards, traceable to the SI, and not reliant on any preexisting protein standard. The WHO certified value was consistent within the measurement uncertainty, with the combined value determined using 3 independent methods of quantification, whereas a difference was observed for the EP material.

The digestion method was equivalent for all materials, regardless of excipients present, and relative amounts of different modifications were compared. Full measurement uncertainty estimations were undertaken, with the primary contributions due to instrument precision and uncertainty associated with peptide mass fraction. This information can be used to direct efforts for subsequent method improvement to reduce total measurement uncertainty. Using this approach, somatropin reference standards can be accurately and independently quantified in terms of mass or moles.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**References**