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Quantification of growth hormone in serum by isotope dilution mass spectrometry

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ABSTRACT

Interassay variation of antibody-based routine tests hampers comparability of measurement results for growth hormone (GH) between different laboratories and decision making in clinical practice. Here it is demonstrated that quantification of GH by isotope dilution mass spectrometry (IDMS) constitutes a way to obtain precise and reliable results that can be referred to in evaluation of performance of commercial test kits. With the IDMS method developed, tryptic cleavage products YSFLQNPQTSLCFSESIPTPSNR (T6) and LEDGSPR (T12) of GH are quantified by liquid chromatography tandem mass spectrometry (LC–MS/MS) using isotopically labeled forms of the peptides as internal standards. The GH cleavage fragments are obtained by whole serum tryptic proteolysis and then extracted from the resulting mixture by semipreparative reversed-phase LC followed by strong cation exchange chromatography. Analysis of blank serum spiked with recombinant 22-kDa GH at different concentration levels would result in a mean recovery of 101.6%, a standard deviation (SD) of 2.5%, a combined uncertainty (u_c) of 3.0%, and a limit of quantification (LOQ) of 1.7 μg/L when quantifying T6 as a GH-derived fragment, whereas recovery = 100.7%, SD = 2.4%, u_c = 2.5%, and LOQ = 2.7 μg/L were found with T12. The potential to acquisition of reference values is exemplified by application to serum materials used in a recent quality assessment exercise for routine laboratories.

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Diagnosis of growth hormone (GH)[1] deficiency and excess (acromegaly) is based on determination of circulating concentrations of GH in serum during dynamic tests [1,2]. In spite of efforts for standardization, decision making in clinical practice is still hampered by significant discrepancies in assay results between different laboratories (test methods). The variation is attributed mainly to different specificities of the antibodies used, varying degrees of recognition of GH dimers, differences in recovery of GH associated with growth hormone binding protein (GHBP), and matrix interferences in general [3]. Whereas some of the sources, in order to be eliminated, require a more explicit definition of the measurand (e.g., epitope/isoform to be targeted), material improvement as to the analytical quality of results can be expected by just taking advantage of selectivity and reliability attainable by isotope dilution mass spectrometry (IDMS).

With small molecular diagnostic markers, IDMS has been a well-established reference measurement principle for nearly 40 years [4–6]. However, in spite of earlier model experiments to extend the scope of the technique to proteins [7], it was not until recently that quantification by IDMS of protein markers in body fluids for diagnostic purposes has been reported [8–13]. Measurement of GH by MS has been discussed previously, namely in a feasibility study [14], a discussion of MS as a fast alternative method regarding the time needed for development of antibody-based assays [15], and an investigation about optimal conditions for proteolysis of serum samples [16]. None of the methods was applicable to measurement of samples in clinical practice at the primary level, however, because no use was made of isotopically labeled internal standards and the concentration ranges considered in these examples were far above what is relevant in clinical practice.

The current article introduces an IDMS-based quantification method that is applicable to samples with GH ranging from 3 to 30 μg/L. Adopting a general strategy in quantitative proteomics [17], tryptic cleavage products of GH are quantified as signature fragments in place of GH as a whole. In this way, the measurand (whole) GH is represented by the amount of substance of the selected fragments. Labeled versions of these are added to the samples prior to proteolysis. Sensitive detection of the targeted fragments requires effective separation from the complex proteolysis product. This is achieved by cleanup using two-step chromatography. The potential of the method to provide reference values is demonstrated with a set of sera that recently have been

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[1] *Abbreviations used:* GH, growth hormone; GHBP, growth hormone binding protein; IDMS, isotope dilution mass spectrometry; TPCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone; IAA, iodoacetamide; DTT, dithiothreitol; TFA, trifluoroacetic acid; $CaCl_2$, calcium chloride; HPLC, high-performance liquid chromatography; RP, reversed-phase; SCX, strong cation exchange; LC, liquid chromatography; SPE, solid-phase extraction; LC–MS/MS, LC–tandem MS; ESI, electrospray ionization; MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantification; SI, International System of Units; SD, standard deviation.

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used in an external quality assurance scheme for clinical testing laboratories [18].

Materials and methods

Reagents and chemicals

Bovine trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone [TPCK]), Trizma hydrochloride, Tris base, iodoacetamide (IAA), dithiothreitol (DTT), acetonitrile (Chromasolv grade), and trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Acetic acid (BioChemika Ultra grade) was obtained from Sigma–Aldrich Chemie (Buchs, Switzerland). Formic acid (98–100%, pro analysi), calcium chloride ($CaCl_2$), and methanol (high-performance liquid chromatography [HPLC] grade) were obtained from Merck (Darmstadt, Germany). Recombinant 22-kDa GH (Swiss–Prot database entry: P01241) was obtained from ProSpec–Tany TechnoGene (Rehovot, Israel). Cleavage fragments YSFLQNPQTS(L)CFSESIPTPSNR (amino acids 42–64) and LEDGS(P)R (amino acids 128–134), corresponding to tryptic peptides T6 and T12, were obtained in labeled form from Thermo Electron (Ulm, Germany) using leucine [$^{13}C_6$,^{15}N] in position (L) and proline [$^{13}C_5$,^{15}N] in position (P) of the sequence, respectively. For the purposes of this article, the labeled peptides are referred to as T6* and T12*. Recombinant human growth hormone soluble receptor (GHBP) was obtained from Protein Laboratories Rehovot (Jerusalem, Israel).

GH reference solution and solutions of labeled peptide internal standards

A (stock) reference solution of recombinant 22-kDa GH at 30.8 nmol/g (681 μg/g) in acetonitrile/water (1:1, v/v) was used for preparation of calibration solutions as well as for defined spiking of GH to serum samples used for method validation. Stock solutions of T6* and T12* used for additions of labeled internal standards were of 102.55 and 146.81 nmol/g, respectively, in water. Concentrations of these solutions were determined by amino acid analysis as described in a previous article [19].

Material used in method validation

Pooled human serum (NIST SRM 971, female serum subset [20]) was spiked with different amounts of GH to yield samples at (target) concentrations of 0, 5, 10, 20, and 30 μg/L. A working solution obtained from the GH reference solution by dilution with acetonitrile/water (1:1, v/v) and 0.1 M acetic acid down to 22.12 pmol/g was used to do this. Of this solution, 0, 5, 10, 20, and 30 mg were added to 500-μl aliquots of the serum. Samples were prepared in duplicate at each of the concentration levels, resulting in eight GH-fortified samples to be analyzed. No GH was detectable with the pure serum (i.e., without addition) using the measurement protocol described in this article.

Material used as example for method application

Samples from two serum pools (HP1/08 A and HP1/08 B) were obtained from DGKL (Referenzinstitut für Bioanalytik, Bonn, Germany) [18]. These are pooled human sera fortified with pituitary GH extract. Prior to IDMS analysis, the lyophilized material was reconstituted with 3 ml of water per vial according to the supplier's protocol.

Calibration solutions

Calibration solutions were prepared by adding defined amounts of GH, T6*, and T12* to 500-μl aliquots of water. The amounts to be added were chosen so as to mimic the concentration of GH expected with the serum sample to be analyzed. Additions were made from working solutions obtained by dilution to 22.12 pmol/g of the (stock) GH reference solution and the solutions of the labeled peptide internal standards.

Proteolysis

Here 500 μl of the sample (serum or calibration solution) was added to 12.1 mg of Tris base, 15.8 mg of Trizma hydrochloride, and 2.2 mg of $CaCl_2$ (pH 8.2). To this, 50 μl of a solution containing 20 mg/ml trypsin in acetic acid (50 mM) was added, and the sample was incubated at 37 °C. Another 50-μl aliquot of the trypsin solution was spiked into the reaction vial after 10, 30, 90, 150, and 210 min, respectively. In parallel, 100 μl of acetonitrile was added every 30 min starting at $t = 30$ min, resulting in a final fraction of 58% acetonitrile after 330 min. The reaction was allowed to proceed for a total of 24 h (1440 min). Following reductive cleavage of cysteine links (3.2 mg of DTT, 1 h, at 37 °C), the nonsoluble fraction was removed by centrifugation (15 min at 17,860*g*). The residue was extracted with 1 ml of acetonitrile/water (1:1, v/v) and the extract was added to the filtrate from centrifugation. This was lyophilized and redissolved in 400 μl of water. Again, the nonsoluble residue was removed by centrifugation (15 min at 3809*g*) and the extract of the residue (300 μl) was added to the filtrate. After alkylation (15.7 mg of IAA, incubation for 30 min at room temperature and then addition of 12.9 mg of DTT), 15.3 μl of TFA in 100 μl water was added.

Extraction of T6,T6 and T12,T12* by two-step liquid chromatography*

T6,T6* and T12,T12* were extracted using reversed-phase (RP) chromatography on a Jupiter 300 Å C18 column (10 × 250 mm, Phenomenex, Torrance, CA, USA) followed by strong cation exchange (SCX) chromatography (Luna, 5 μm, SCX 100 Å, 10 × 250 mm, Phenomenex). An Elite LaChrom liquid chromatography (LC) system (VWR–Hitachi, Darmstadt, Germany) consisting of a low-pressure binary pump, an autosampler, a column oven, a variable wavelength detector, and a Foxy Jr. fraction collector (Teledyne Isco, Lincoln, NE, USA) was used for separation and fraction collection. Of the proteolysis product, 5× 170 μl was injected onto the RP column, which was run at a 1.5-ml/min flow rate of the mobile phase. A solvent gradient (A/B) was applied, with 0% B for 5 min, 0–80% B within 40 min, 80% B for 5 min, 80–0% B within 0.5 min, and 0% B for 40 min (A: water [0.1% TFA]; B: acetonitrile [0.1% TFA]). Time windows for collection were 33.4–34.5 min (T6,T6*) and 25.2–26.2 min (T12,T12*). For SCX chromatography, a solvent gradient (C/D) was applied, with 0% D for 3 min, 0–100% D within 37 min, 100% D for 5 min, 100–0% D within 0.1 min, and 0% D for 45 min (C: KH_2PO_4, 5 mM, in acetonitrile/water [1:4, v/v], pH 2.83; D: KH_2PO_4, 5 mM, plus KCl, 0.5 M, in acetonitrile/water [1:4, v/v], pH 2.83). Each collected fraction (one for T6,T6* and one for T12,T12*) from RP chromatography was lyophilized and redissolved in 600 μl of mobile phase C prior to injecting the whole amount (2× 600 μl in two separate runs [see Fig. 2]) onto the SCX column. Collection windows were 34.8–36.5 min in the run with the T6,T6*-containing fraction from RP chromatography and 34.9–36.4 min in the second run with the T12,T12*-containing fraction. After SCX chromatography, the respective fractions were desalted according to a standard protocol using solid-phase extraction (SPE) C18 ec cartridges (Chromabond, 500 mg, Macherey–Nagel, Düren, Germany). After lyophilization, residues were redissolved in 20 μl of water (0.1% formic acid) before analysis by LC–tandem MS (LC–MS/MS).

Analysis by LC–MS/MS

Samples were analyzed on an Agilent 1100 series LC instrument (Agilent Technologies, Waldbronn, Germany) combined with a 4000 Q Trap (Applied Biosystems, Foster City, CA, USA) operated in electrospray ionization (ESI) (positive ion) mode. A Discovery BIO wide pore C18 RP column (2.1 × 150 mm, 3 μm, 300 Å) was used at a 200-μl/min flow rate of mobile phase. The solvent gradient (A/B) applied was as follows: 0% B for 3 min, 0–80% B within 37 min, 80% B for 5 min, 80–0% B within 0.1 min, and 0% B for 15 min (A: water [0.1% formic acid]; B: acetonitrile [0.1% formic acid]). The whole material as resulting from sample cleanup (2× 20 μl) was injected in two separate analytical runs (one with the T6,T6* extract and one with T12,T12*). The mass spectrometer was run in multiple reaction monitoring (MRM) mode with quadrupole mass filters Q1 and Q3 at unit resolution. The transitions monitored were as follows: m/z 892.3 → 671.3 and m/z 894.6 → 671.3 for T6,T6* and m/z 387.4 → 531.3 and m/z 390.4 → 537.3 for T12,T12*, corresponding to $[M+3H]^{3+} \rightarrow y_6$ with T6,T6* and $[M+2H]^{2+} \rightarrow y_5$ with T12,T12*. Fragmentation was induced using nitrogen at a high-pressure setting and at collision energies of 50 eV for T6,T6* and 20 eV for T12,T12*. The ESI source was operated at a 5.5-kV sprayer voltage and a 350 °C dry gas temperature. All other parameters were optimized to obtain maximum signal intensities for T6,T6* and T12,T12*. Under the conditions used, retention times were 24.0 min for T6,T6* and 14.1 min for T12,T12*.

Measuring substance and signal recovery after extraction of T6/T6 and T12/T12**

NIST SRM 971 (female serum), the same material as with the validation experiments, was used as blank matrix. T6* and T12* were used as markers. Solutions used for additions contained 20.2 and 20.6 pmol/g T6* and T12*, respectively, in acetonitrile/water (1:1, v/v) and 0.1 M acetic acid. T6* and T12* were spiked to the blank serum at different stages of the cleanup/extraction process, and the LC–MS/MS response was recorded each time. The amounts spiked were chosen such as to correspond to 10 μg/L of 22-kDa GH. Substance recoveries were obtained by relating the signals observed with spiking of T6* and T12* prior to cleanup by two-step chromatography to those observed with spiking the blank serum after it was subjected to the same procedure. For obtaining signal recoveries, aqueous solutions of T6* and T12*, the same concentrations as before, were used as reference instead. In a second experiment, signal recovery was determined for a reduced cleanup procedure omitting the SCX chromatography step.

Testing for interference by presence of GHBP

T6* and T12* internal standard solutions were spiked to a solution containing GHBP and GH in a molar ratio of 9:1. The final volume was 500 μl, and concentrations were 5.0:0.54:0.55:0.56 nmol/L (GHBP/GH/T6*/T12*). The mixture was incubated at 4 °C overnight and analyzed immediately after. For comparison, another sample was prepared in the same way but missing the addition of GHBP.

Calculation of LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) were calculated from the results of the validation experiment (Table 1) using the linear regression approach [21] following the parameter definitions provided in Ref. [22] setting $\alpha,\beta = 0.01$. By this, LOD is specified as the concentration that, in 50% of cases, will produce a signal that could have been seen with a blank at no more than 1% probability, whereas at the LOQ signals of such abundance are obtained in 99% of cases.

Table 1
Recovery of GH added to blank serum and method performance data derived from this.

GH concentration (μg/L)			Recovery (%)	
By T6	By T12	Expected	By T6	By T12
4.79	4.82	4.63	103.5	104.1
4.45	4.60	4.58	97.2	100.4
10.24	10.49	10.19	100.5	102.9
10.14	10.15	10.25	98.9	99.0
20.28	19.99	19.51	103.9	102.5
19.71	18.70	19.32	102.0	96.8
30.57	29.17	29.39	104.0	99.3
31.24	30.43	30.31	103.1	100.4
Mean recovery (%)			101.6	100.7
Mean bias (%)			1.6	0.7
SD (%)			2.5	2.4
u_c (%)			3.0	2.5

Results

Choice of GH-specific fragments

The cleavage fragments of GH used for quantification (T6 and T12) are shown in Fig. 1. They were selected so as to selectively code for GH in a complex matrix. A BLAST search (BLASTP 2.2.21 database) and MS–Homology (UCSF Protein Prospector) were used for alignment and identification of potentially interfering cleavage products from other proteins. With T6, sequence alignment yielded exact matches exclusively with GH, whereas in the case of T12, a second hit (caspase recruitment domain protein 9, isoform 2) also was returned. In this protein, the T12 sequence is flanked by amino acids other than lysine and arginine, however, and trypsin does not release T12 as a fragment from it.

Apart from approximately 1% O-glycosylation in the sequence range covered by T6, no posttranslational modifications are observed with GH in the regions of T6 and T12 [23], so the mass spectrometric signals monitored correspond to virtually all of the T6 and T12 present after proteolysis.

Method

The workflow of the method developed is shown in Fig. 2. With each sample, a separate calibration solution is used as prepared by appropriate dilution from the (stock) GH reference solution. The concentration is chosen in a range as close as possible to what is expected in the serum samples to be analyzed (which may require a preliminary analysis run for orientation). GH-specific signature fragments (here T6 and T12) are spiked as internal standards in isotopically labeled form (denoted as T6* and T12*) in equal amounts to both serum sample and calibration solution. Exactly matching [24] the analyte concentration of the sample with the calibration solution is done on purpose to eliminate systematic errors that otherwise would result from uncertainty in knowledge of the degree of isotopic enrichment in the labeled internal standard used [25]. To ensure complete release of T6 and T12 from GH, sample and calibration solution are incubated for 24 h at 37 °C in the presence of 20 mg/L trypsin (water/acetonitrile, 2:3, v/v). Reductive cleavage of cystein–cystein bridges is done after proteolysis with the method as presented here, although the order of these steps is not expected to influence results. Semipreparative RP LC followed by SCX chromatography is used to separate the target peptides from the proteolysis product prior to analysis of the signal ratios T6/T6* and T12/T12* by analytical-scale RP LC–MS/MS on a triple-quadrupole instrument. The amount of GH (two separate results: one using T6 and one using T12) is calculated based on comparison of the signal ratios obtained for that fragment in the

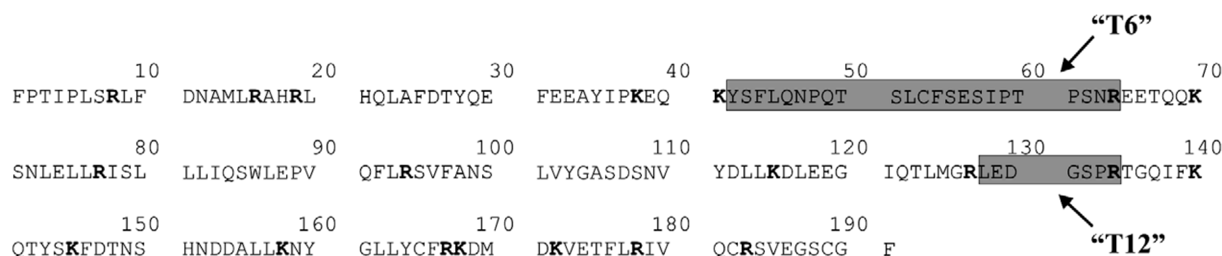

Fig. 1. Amino acid sequence of 22-kDa GH (Swiss–Prot database entry: P01241) and signature fragments (T6 and T12) chosen to represent the amount of GH.

sample and in the calibration solution, corresponding to a comparison of the amount of fragment generated from GH in the sample with the known amount generated from the calibration solution given that equal amounts of labeled internal standard were spiked with both. The detailed protocols for proteolysis, extraction of T6,T6* and T12,T12*, and analysis by MS are provided in the corresponding subsections of Materials and Methods. Ion chromatograms from LC–MS/MS as obtained for a serum sample are shown in Fig. 3.

Substance and signal recovery of signature fragments

Substance recoveries after extraction of T6,T6* and T12,T12* by two-step chromatography, as described, were found to be 61% with T6,T6* and 64% with T12,T12*. At the same time, signal recoveries were 14 and 8%, respectively. Curtailing the cleanup procedure by leaving out the SCX chromatography step would result in a decrease of signal recoveries to 3 and 1%, respectively.

Validation

Method performance was characterized based on evaluation of analytical recovery of defined amounts of GH spiked to a serum material that had been checked not to contain GH at a concentration detectable with the current method. Recombinant 22-kDa GH reference solution was used for the additions, which previously [19] had been value assigned by IDMS-based amino acid analysis. Eight samples were prepared at four different concentration levels ranging from 4.5 to 30.5 μg/L. All samples were analyzed completely independent of one another on different occasions (days). Therefore, the observed deviation from the respective target value for each individual sample contains all random errors occurring in the sample treatment and instrumental analysis, including those between different days. Quantification results are compiled in Table 1. Plots of results by T6 and T12 versus expected GH concentrations (not shown) are of excellent linearity. In particular, $R^2 = 0.999$ for both T6 and T12, $s_{y|x} = 0.22$ for T6 and 0.34 for T12, slope $a = 1.04$ for T6 and 0.99 for T12, and intercept $b = -0.21$ for T6 and 0.10 for T12 were obtained by regression analysis. The LODs calculated from the data were 0.5 and 0.7 μg/L for T6 and T12, respectively, and the LOQs were 1.7 and 2.7 μg/L for T6 and T12, respectively.

Test for interference by GHBP

The effect of the presence of GHBP on quantification results was investigated in a separate model experiment. Two solutions were processed according to the described protocol. Both contained GH at 11.9 μg/L as well as T6* and T12* as internal standards. One of them also was spiked with GHBP in ninefold molar excess. There was no significant difference in signal ratios found in the presence of GHBP and in its absence: 1.00 and 1.00, respectively, for T6/T6* and 0.91 and 0.92, respectively, for T12/T12*.

IDMS measurement of GH in quality control materials

Samples from two serum pools, HP 1/08 A and B, which had recently been used as test materials in an external quality assessment scheme [18] were reanalyzed by IDMS. Results were 7.07 and 6.81 μg/L for pool A with T6- and T12-based quantification, respectively, and 12.33 and 12.28 μg/L for pool B with T6- and T12-based quantification (means of $n = 3$ runs each). The data reported by 174 testing laboratories are visualized as Youden plots in Fig. 4. For reference, the IDMS results are included.

Discussion

GH, as found in vivo, displays considerable heterogeneity. It has been described as consisting of several isoforms and variants resulting from modification of amino acid side chains as well as forming dimers and even complexes of a higher order [23,26]. Clear definition of the measurand, although vital with respect to comparability of measurements, is still subject to ongoing discussion in clinical chemistry. The current report demonstrates the performance of IDMS as an analytical tool given a defined signature fragment(s) that is agreed on to represent that measurand. Note that specification of a cleavage fragment in many situations is similar to selecting an epitope on the surface of the protein. In this study, T6 and T12 were selected as examples. Both are present on 22-kDa GH, the most abundant isoform, as shown in Fig. 1. The suitability of the two fragments to represent GH is further supported by the fact that virtually no posttranslational modification needs to be taken into account, so the mass spectrometric signals monitored should collect all of T6/T6* and T12/T12* present after proteolysis.

The IDMS method reported is applicable to T6- and T12-based serum measurement of GH in clinically relevant concentrations. By measurement principle, IDMS has the potential to provide unbiased and reliable results at the primary level of traceability to the International System of Units (SI) [27]. This is supported by the performance data given in Table 1. Method precision is characterized by the standard deviations (SDs, 2.5 and 2.4%, respectively). Each of the analyses was run on a different day, so a component descriptive of method robustness against variation of measurement and environmental conditions is contained but not separately determined. Bias turns out not to be significant (SD > mean bias in both cases T6 and T12); therefore, the method is found to produce accurate results. Combining both components (precision and formally calculated bias), the uncertainties would be approximately 3% (u_c in Table 1), and when making allowance for another 3% uncertainty in value assignment to the calibration material used, an overall estimate of $u_c \approx 5\%$ may be considered as reasonable for the type of measurement.

The measurement of GH, because it is present in serum only at the trace concentration level, takes particular care in sample preparation so as to obtain instrumental responses of abundances enabling precise quantification. Owing to suppression of analyte signals by competition with matrix ions present at the same time

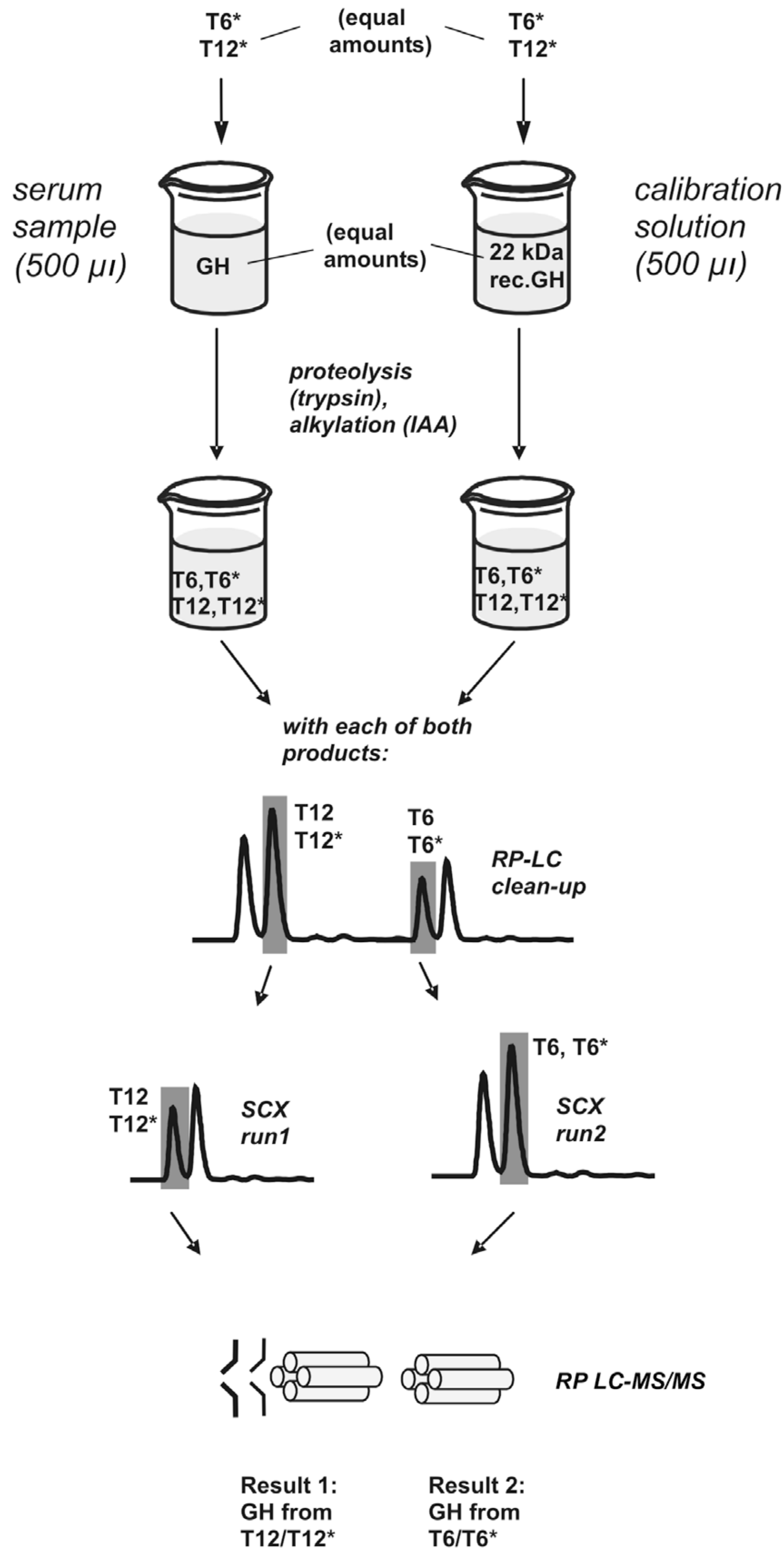

Fig. 2. Workflow of the method for quantification of GH by IDMS. Tryptic cleavage fragments of GH (T6 and T12) are quantified using recombinant 22-kDa GH (rec. GH) as reference substance of known concentration and the isotopically labeled analogues (T6* and T12*) as internal standards. Isolation of T6,T6* and T12,T12* from samples prior to measurement by LC–MS/MS is done by a two-step procedure consisting of semipreparative RP LC followed by SCX chromatography.

[28], effective sample cleanup prior to measurement is crucial for quantification by ESI–MS. Extraction of the proteolytic fragments from the sample by specific antibodies [8,9,29,30] is a promising approach to this. However, with the current method, a chromatog-

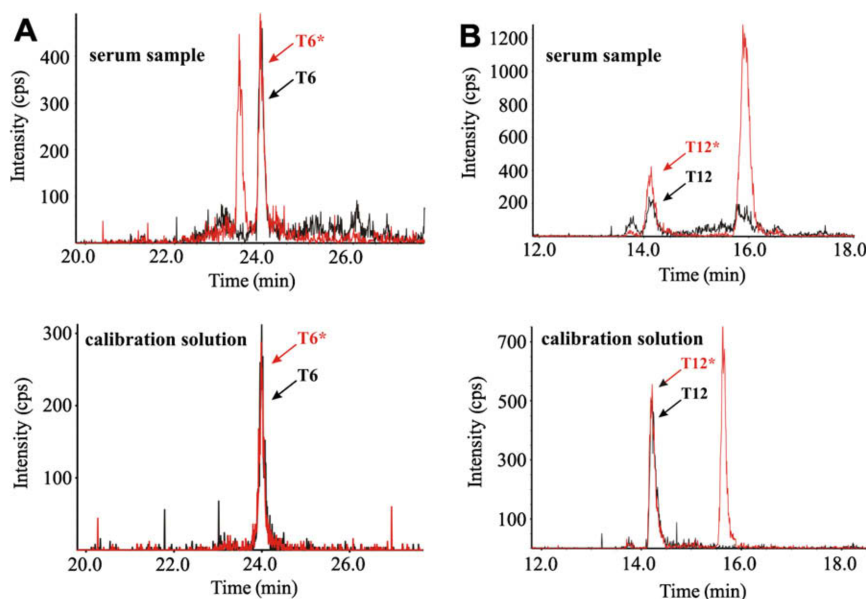

Fig. 3. Instrumental responses obtained for GH signature fragments (T6 and T12, black) compared with those of the labeled internal standards (T6* and T12*, red) in analysis of quality control serum HP 1/08 B. The ion chromatograms shown were monitored for analyte-specific MS/MS transitions. (A) T6-based quantification. (B) T12-based quantification.

raphy-based alternative is used and seems to be quite efficient while reducing time and expense for production of antibodies. Performance data, in particular the LOQ (<3 μg/L), are at least as good as what has been reported with antibody-based cleanup.

Using two-step chromatography (RP LC plus SCX) as described, signal recoveries were approximately 14 and 8% of what would have been obtained in the complete absence of matrix-derived ions with T6,T6* and T12,T12*, respectively. Substance recoveries, at the same time, were 61 and 64%, respectively. Therefore, the effect of matrix suppression can be roughly estimated to be a factor of 4–8 even after cleanup. Basically, improvement in substance recovery would be expected if leaving out one of the chromatography steps. However, the gain in substance recovery is outweighed by the accompanying increase in matrix suppression, as becomes manifest from the drop in signal recovery to 3 and 1% with T6,T6* and T12,T12*, respectively, if reducing analyte extraction to just RP LC. Obviously, both steps are required for the procedure to achieve the reported performance (Table 1) and, in particular, the LOD.

Interference by GHBP and formation of GH–GH complexes are considered as significant sources for disagreement of results between different immunoassays. No such interference is expected to occur if applying fragment-based quantification, as done here, unless proteolysis can be obstructed by complex formation. However, this is not the case, as demonstrated with the GHBP interference test that yielded the same result in the presence of GHBP as in its absence. Obviously, the IDMS method is capable of providing a measure of "total" GH.

Considering the results obtained by the testing laboratories for the samples investigated in the external quality assurance exercise (Fig. 4), the need for improvement is becoming evident. In both cases, pool A and pool B, a significant part of the whole range in which GH concentrations are observed in clinical practice is covered by the spread of data. This would make the clinical decision very difficult without reference to method-dependent cutoff values if these were real patient sera. Referring to the results by IDMS, the most striking discrepancy is the one to the values obtained by laboratories that used the Siemens Medical Solutions assay (Fig. 4A). A good deal of this presumably is caused by the lack of SI traceability of the measurement; either many of these laboratories appear not to have calibrated against the recommended international standard (WHO IS 98/574), which has been SI traceably value assigned [31], or wrong factors have been used in converting mU/L to the required unit (μg/L). Apart from improper calibration, interferences by complex formation, and recognition of nonrepresentative epitopes by the antibodies used, cross-selectivity toward nontarget antigens is among the potential causes for the deviations observed. In this situation, by the outstanding selectivity of MS if compared with the detection principles used with immunochemical tests, combined with reliability of IDMS in providing precise and SI-traceable results, the described method has the potential to produce target values for the routine assays that eventually might be considered for recalibration of their results. In the current example, the DiaSorin test (Fig. 4B) seems to agree best with what is obtained by IDMS.

Although sufficient for GH quantification in materials currently used in quality control schemes, and also in clinical samples seen during stimulation tests, improvement of the LOQ by approximately one order of magnitude would be required to extend the applicability to measure samples in patients during glucose suppression tests, where GH concentrations less than 1 μg/L are expected [32]. In terms of signal recovery, gaining a factor of 4–8 might be expected through complete elimination of ion suppression effects by further improvement of the cleanup procedure. However, a more promising option would consist of targeted enhancement of the analyte signals by appropriate conjugation of amino acid side chains so as to increase their basicity. For instance, a sixfold increase in response factors has been reported on modifying cysteines with quaternary ammonium compounds [33]. Therefore, improvement of method sensitivity to enable application to samples from suppression tests appears to be possible as well.

Conclusion

The results reported here have demonstrated that, by using IDMS, the road is paved to acquisition of reference values for GH in serum at the primary level of precision and reliability, which are expected to be reproducible not only between different

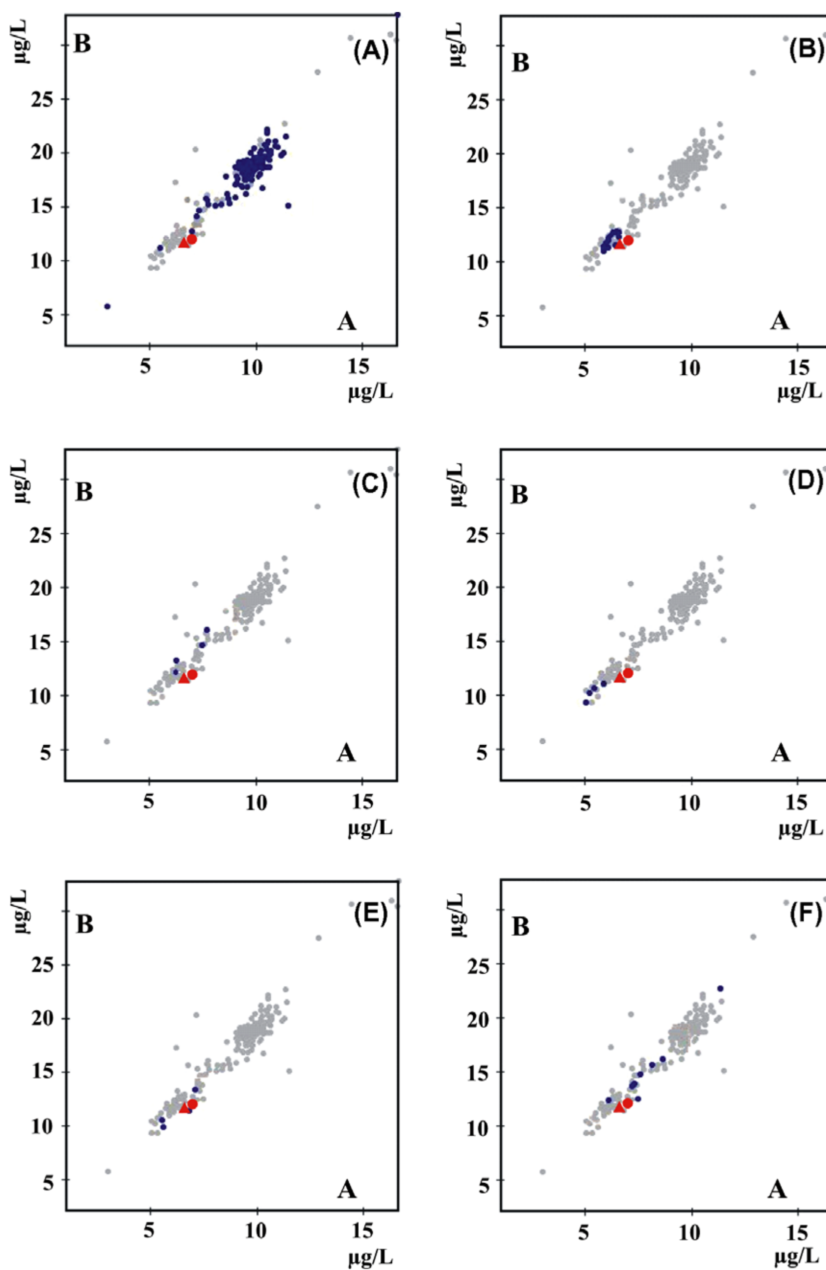

Fig. 4. IDMS results obtained for GH in comparison with those reported by testing laboratories in an external quality assessment exercise. Red circle: T6-based result; red triangle: T12-based result. All panels contain the same data, with results by different test kits (manufacturers) being highlighted with each one: (A) Siemens Medical Solutions (DPC/Biermann); (B) DiaSorin; (C) Schering (CIS); (D) Mediagnost; (E) DSL; (F) PerkinElmer/Wallac (DELFIA). (Adapted with permission from DGKL [18].)

laboratories but also if another GH reference preparation should be used for calibration. Although IDMS is not likely, at its current stage, to replace antibody-based assays in routine, it can be used as a tool for providing backup in the context of quality control of clinical measurements.

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References

[1] M. Bidlingmaier, P.U. Freda, Measurement of human growth hormone by immunoassays: current status, unsolved problems, and clinical consequences, Growth Horm. IGF Res. 20 (2010) 19–25.
[2] L. Cazabat, J.C. Souberbielle, P. Chanson, Dynamic tests for the diagnosis and assessment of treatment efficacy in acromegaly, Pituitary 11 (2008) 129–139.
[3] M. Bidlingmaier, Problems with GH assays and strategies toward standardization, Eur. J. Endocrinol. 159 (Suppl. 1) (2008) S41–S44.
[4] L. Siekmann, H.O. Hoppen, H. Breuer, Zur gaschromatographisch-massenspektrometrischen Bestimmung von Steroidhormonen in Körperflüssigkeiten unter Verwendung eines Multiple Ion Detektors (Fragmentographie), Z. Anal. Chem. 252 (1970) 294–298.
[5] H. Breuer, L. Siekmann, Mass fragmentography as reference method in clinical steroid assay, J. Steroid Biochem. 6 (1975) 685–688.
[6] A. Cohen, H.S. Hertz, J. Mandel, R.C. Paule, R. Schaffer, L.T. Sniegoski, T. Sun, M.J. Welch, E.T. White, Total serum cholesterol by isotope dilution/mass spectrometry: a candidate definitive method, Clin. Chem. 26 (1980) 854–860.
[7] J.R. Barr, V.L. Maggio, D.G. Patterson Jr., G.R. Cooper, L.O. Henderson, W.E. Turner, S.J. Smith, W.H. Hannon, L.L. Needham, E.J. Sampson, Isotope dilution–

mass spectrometric quantification of specific proteins: model application with apolipoprotein A–I, Clin. Chem. 42 (1996) 1676–1682.
[8] A.N. Hoofnagle, J.O. Becker, M.H. Wener, J.W. Heinecke, Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry, Clin. Chem. 54 (2008) 1796–1804.
[9] E. Kuhn, T. Addona, H. Keshishian, M. Burgess, D.R. Mani, R.T. Lee, M.S. Sabatine, R.E. Gerszten, S.A. Carr, Developing multiplexed assays for troponin I and interleukin-33 in plasma by peptide immunoaffinity enrichment and targeted mass spectrometry, Clin. Chem. 55 (2009) 1108–1117.
[10] E. Kuhn, J. Wu, J. Karl, H. Liao, W. Zolg, B. Guild, Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and ^{13}C-labeled peptide standards, Proteomics 4 (2004) 1175–1186.
[11] J.C. Seegmiller, D.R. Barnidge, B.E. Burns, T.S. Larson, J.C. Lieske, R. Kumar, Quantification of urinary albumin by using protein cleavage and LC–MS/MS, Clin. Chem. 55 (2009) 1100–1107.
[12] K. Van Uytfanghe, D. Rodriguez-Cabaleiro, D. Stockl, L.M. Thienpont, New liquid chromatography/electrospray ionisation tandem mass spectrometry measurement procedure for quantitative analysis of human insulin in serum, Rapid Commun. Mass Spectrom. 21 (2007) 819–821.
[13] D.K. Williams, D.C. Muddiman, Absolute quantification of C-reactive protein in human plasma derived from patients with epithelial ovarian cancer utilizing protein cleavage isotope dilution mass spectrometry, J. Proteome Res. 8 (2009) 1085–1090.
[14] S.L. Wu, H. Amato, R. Biringer, G. Choudhary, P. Shieh, W.S. Hancock, Targeted proteomics of low-level proteins in human plasma by LC/MS$_n$: using human growth hormone as a model system, J. Proteome Res. 1 (2002) 459–465.
[15] Z. Yang, M. Hayes, X. Fang, M.P. Daley, S. Ettenberg, F.L. Tse, LC–MS/MS approach for quantification of therapeutic proteins in plasma using a protein internal standard and 2D solid-phase extraction cleanup, Anal. Chem. 79 (2007) 9294–9301.
[16] F. Li, C.M. Schmerberg, Q.C. Ji, Accelerated tryptic digestion of proteins in plasma for absolute quantitation using a protein internal standard by liquid chromatography/tandem mass spectrometry, Rapid Commun. Mass Spectrom. 23 (2009) 729–732.
[17] V. Brun, C. Masselon, J. Garin, A. Dupuis, Isotope dilution strategies for absolute quantitative proteomics, J. Proteomics 72 (2009) 740–749.
[18] DGKL, Referenzinstitut für Bioanalytik, DGKL, Bonn, Germany, 2009. Available from: http://www.dgkl-rfb.de.
[19] C.G. Arsene, R. Ohlendorf, W. Burkitt, C. Pritchard, A. Henrion, G. O'Connor, D.M. Bunk, B. Guttler, Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy, Anal. Chem. 80 (2008) 4154–4160.
[20] National Institute of Standards and Technology, Standard Reference Material SRM 971: Certificate of Analysis, NIST, Gaithersburg, MD, 2008.
[21] A. Hubaux, G. Vos, Decision and detection limits for linear calibration curves, Anal. Chem. 42 (1970) 849–855.
[22] Deutsches Institut für Normung, DIN 32645, Chemische Analytik: Nachweis-, Erfassungs-, und Bestimmungsgrenze, Beuth Verlag, Berlin, Germany, 1994.
[23] M. Kohler, A. Thomas, K. Puschel, W. Schanzer, M. Thevis, Identification of human pituitary growth hormone variants by mass spectrometry, J. Proteome Res. 8 (2009) 1071–1076.
[24] W.I. Burkitt, C. Pritchard, C. Arsene, A. Henrion, D. Bunk, G. O'Connor, Toward Systeme International d'Unite-traceable protein quantification: from amino acids to proteins, Anal. Biochem. 376 (2008) 242–251.
[25] A. Henrion, Reduction of systematic errors in quantitative analysis by isotope dilution mass spectrometry (IDMS): an iterative method, Fresenius J. Anal. Chem. 350 (1994) 657–658.
[26] G. Baumann, Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins, Endocr. Rev. 12 (1991) 424–449.
[27] M. Milton, R. Wielgosz, Uncertainty in SI-traceable measurements of amount of substance by isotope dilution mass spectrometry, Metrologia 37 (2000) 199–206.
[28] T.M. Annesley, Ion suppression in mass spectrometry, Clin. Chem. 49 (2003) 1041–1044.
[29] N.L. Anderson, A. Jackson, D. Smith, D. Hardie, C. Borchers, T.W. Pearson, SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device, Mol. Cell. Proteomics 8 (2009) 995–1005.
[30] J.R. Whiteaker, L. Zhao, H.Y. Zhang, L.C. Feng, B.D. Piening, L. Anderson, A.G. Paulovich, Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers, Anal. Biochem. 362 (2007) 44–54.
[31] C. Pritchard, M. Quaglia, C. Mussell, W.I. Burkitt, H. Parkes, G. O'Connor, Fully traceable absolute protein quantification of somatropin that allows independent comparison of somatropin standards, Clin. Chem. 55 (2009) 1984–1990.
[32] H. Markkanen, T. Pekkarinen, M.J. Valimaki, H. Alfthan, R. Kauppinen-Makelin, T. Sane, U.H. Stenman, Effect of sex and assay method on serum concentrations of growth hormone in patients with acromegaly and in healthy controls, Clin. Chem. 52 (2006) 468–473.
[33] D. Ren, S. Julka, H.D. Inerowicz, F.E. Regnier, Enrichment of cysteine-containing peptides from tryptic digests using a quaternary amine tag, Anal. Chem. 76 (2004) 4522–4530.