Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

Quantification of growth hormone in serum by isotope dilution mass spectrometry

Cristian G. Arsene[a,*], André Henrion[a], Nina Diekmann[a],
Jenny Manolopoulou[b] and Martin Bidlingmaier[b]

Preliminary draft, December 7, 2009

Abstract

Inter-assay variation of antibody based routine tests is hampering 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 precise and reliable results which 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 LC/MS-MS using the 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 semi-preparative reversed phase liquid chromatography followed by strong cation-exchange chromatography. Method validation basing on recovery of recombinant 22 kDa GH, spiked to blank serum in defined amounts covering the intended concentration range 3 μg/L to 30 μg/L, would yield mean recoveries of 101.6% (100.7%), standard deviations (SD) of 2.5% (2.4%) and combined uncertainties (u_c) of 3.0% (2.5%) if quantifying T6 (T12) as GH derived fragments, while the LOQ were 1.7 μg/L (2.7 μg/L). Potential to acquisition of reference values is exemplified by application to serum materials used in a recent quality assessment exercise for routine laboratories.

Keywords: Growth hormone, GH, somatotropin, serum, quantification, standardization, reference measurement, LC/MS-MS, isotope dilution mass spectrometry, IDMS.

*To whom correspondence should be addressed. Phone: ++49 531 592-3124, Fax: ++49 531 592-3015.
E-mail: christian.arsene@ptb.de
[a]Physikalisch-Technische Bundesanstalt (PTB), Braunschweig, Germany
[b]Medizinische Klinik−Innenstadt, Ludwig-Maximilians-University, Munich, Germany

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

1 Introduction

Diagnosis of growth hormone (GH) deficiency and -excess (acromegaly) is based on determination of circulating concentrations of GH in serum during dynamic tests [1, 2]. In spite of efforts to standardization, decision making in clinical practice continues being 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 degree of recognition of GH-dimers, differences in recovery of GH being associated with growth hormone binding protein (GHBP), as well as matrix interferences in general [3]. While part of the sources, in order to be eliminated are requiring 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 almost 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 fast alternative method regarding the time needed for development of antibody based assays [15] and with an investigation about optimal conditions for proteolysis of serum samples [16]. Neither of the methods was applicable to measurement of samples in clinical practice at primary level, however, since no use was made of isotopically labeled internal standards and concentration ranges considered in these examples were far above what is relevant in clinical practice.

The present paper introduces an IDMS based quantification method which is applicable to samples with GH ranging from 3 μg/L to 30 (μg/L). Adopting a general strategy in quantitative proteomics [17], tryptic cleavage products of GH are being quantified as signature fragments in place of GH as a whole. 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 is requiring effective separation from the complex proteolysis product. This is achieved by clean-up using two-step chromatography. The potential of the method to providing reference values is demonstrated with a set of sera which recently have been used in an external quality assurance scheme for clinical testing laboratories [18].

Abbreviations: GH: growth hormone, GHBP: Growth hormone binding protein, IDMS: isotope dilution mass spectrometry, LC: liquid chromatography, LC/MS-MS: liquid chromatography-tandem mass spectrometry, ESI: electrospray- ionization, LOD: limit of detection, LOQ: limit of quantification, TFA: trifluoroacetic acid, RP: reversed phase, SCX: strong cation exchange, SPE: solid phase extraction, MRM: multiple reaction monitoring, Q1, Q3: quadrupole 1, quadrupole 3, SD: standard deviation, TPCK: L-1-Tosylamide-2-phenylethyl chloromethyl ketone.

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

2 Materials and methods

Reagents and chemicals. Bovine trypsin (treated with L-1-Tosylamide-2-phenylethyl chloromethyl ketone, TPCK), TRISMA Hydrochloride, TRIS-base, iodoacetamide (IAA), dithiothreitol (DTT), acetonitrile (CHROMASOLV grade) and trifluoroacetic acid (TFA) were from Sigma-Aldrich (St. Louis, USA), acetic acid (BioChemika Ultra grade) from Sigma-Aldrich Chemie (Buchs, Switzerland). Formic acid (98-100%, p.a.), calcium chloride ($CaCl_2$), and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). Recombinant 22 kDa GH (Swiss-Prot database entry: P01241) was from ProSpec-Tany TechnoGene (Rehovot, Israel). Cleavage fragments YS-FLQNPQTS(L)CFSESIPTPSNR (amino acids 42-64) and LEDGS(P)R (amino acids 128-134), corresponding to tryptic peptides T6 and T12, had been obtained in labeled form from Thermo Electron G.m.b.H. (Ulm, Germany), using leucine [$^{13}C_6$,^{15}N-] in position (L) of the sequence and proline [$^{13}C_5$,^{15}N-] in position (P), respectively. For the purposes of this paper the labeled peptides will be referred to as T6* and T12*. Recombinant human growth hormone soluble receptor (growth hormone binding protein, GHBP) had been obtained from Protein Laboratories Rehovot Ltd. (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 1/1 (v/v), acetonitrile/water, 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 nmol/g and 146.81 nmol/g, respectively, in water. Concentrations of these solutions had been determined by amino acid analysis as described in a previous paper [19].

Material used in method validation. Pooled human serum (NIST SRM$^{(R)}$ 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), 0.1 M acetic acid, down to 22.12 pmol/g was used to 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 analysed. No GH had been detectable with the pure serum (i.e., without addition) using the measurement protocol being described in this report.

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, D-53127 Bonn, Germany [18]. These were pooled human sera fortified with pituitary GH extract. Prior to IDMS analysis, the lyophilized material was reconstituted with 3 mL water per vial according to the suppliers 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 analysed. 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. 500 μL of the sample (serum or calibration solution) were added to 12.1 mg TRIS base, 15.8 mg TRIS-HCL and 2.2 mg $CaCl_2$ (pH=8.2). To this, 50 μL of a solution containing 20 mg/mL trypsin in acetic acid (50 mM) were added and the sample was incubated at 37 °C. After 10, 30, 90, 150 and 210 min, respectively, another 50 μL aliquot of the trypsin solution was spiked into the reaction vial. In parallel, 100 μL of acetonitrile were added every 30 minutes 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 24h (1440 min). Following reductive cleavage of cysteine links (3.2 mg DTT, 1h at 37 °C) the non soluble fraction was removed by centrifugation (15 min at 17860 g). The residue was extracted with 1 mL of acetonitrile/water, 1/1 (v/v), and the extract added to the filtrate from centrifugation. This was lyophilized and re-dissolved in 400 μL water. Again, the non soluble 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 IAA, incubation for 30 min at room temperature, then addition of 12.9 mg DTT) 15.3 μL TFA in 100 μL water were 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), followed by strong cation exchange (SCX-) chromatography (Luna 5 μm SCX 100 Å, 10×250 mm, Phenomenex). An ELITE LaChrom LC system (VWR-HITACHI, Darmstadt, Germany) consisting of a low pressure binary pump, autosampler, column oven, VWD detector and a Foxy Jr. fraction collector (Teledyne ISCO, Lincoln, NE) was used for separation and fraction collection. Of the proteolysis product, 5 times 170 μL were injected onto the RP column which was run at 1.5 mL/min flow rate of the mobile phase. A solvent gradient was applied of 100% A (0-5 min)→ 80% B (at 45-50 min)→100% A (at 50.5-90 min), where A: water (0.1% TFA) and 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, the gradient was 100% C (0-3 min)→100% D (40-45 min)→ 100% C (45.1-90 min), where C: KH_2PO_4, 5 mM in acetonitrile/water, 1/4, pH=2.83 and D: KH_2PO_4, 5 mM, plus KCl, 0.5 M in acetonitrile/water, 1/4, pH=2.83. Each collected fraction (one for T6,T6*, another one for T12,T12*) from RP chromatography was lyophilized and re-dissolved in 600 μL mobile phase C, prior to injecting the whole amount (2×600 μL in two separate runs) onto the SCX column. Collection windows were 34.8-36.5 min in the run with the T6,T6* containing fraction and 34.9-36.4 min with the T12,T12* containing one. After SCX-chromatography the respective fractions were desalted according to a standard protocol using SPE C18 ec cartridges (Chromabond, 500 mg, Macherey&Nagel, Düren, Germany). After lyophilization, residues were re-dissolved in 20 μL water (0.1% formic acid) before analysis by LC/MS-MS.

Analysis by LC/MS-MS. Samples were analyzed on an Agilent 1100 series LC- instrument (Agilent Technologies Deutschland G.m.b.H., Waldbronn, Germany) combined with a 4000 Q Trap (Applied Biosystems, Foster City, CA) operated in ESI- (positive ion) mode. A Discovery BIO

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

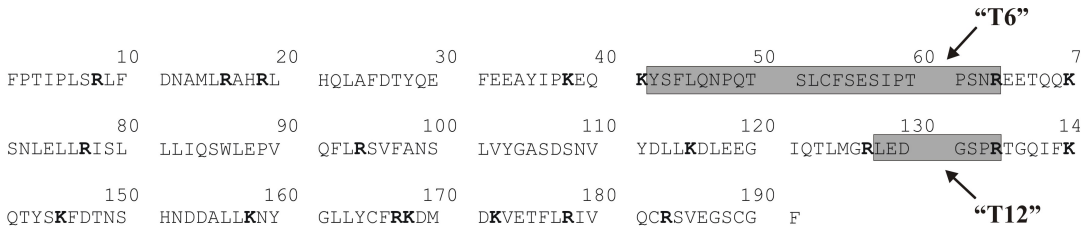

Figure 1: Amino acid sequence of 22 kDa GH (Swiss-Prot: P01241) and signature fragments (T6 and T12) chosen to represent the amount of GH.

wide pore C18 (2.1×150 mm, 3 μm, 300 Å) RP- column was used at 200 μL/min flow rate of mobile phase. The gradient applied was 100% A (0-3 min)→80% B (at 40-45 min)→ 100% A (at 45.1-60 min), where A: water (0.1% formic acid) and B: acetonitrile (0.1% formic acid). The whole material as resulting from sample clean-up (2×20 μL) was injected in two separate analytical runs (one with the T6,T6*-extract, another one with T12,T12*). The mass spectrometer was run in MRM mode with Q1 and Q3 at unit resolution. Transitions monitored were 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*, which corresponds 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 high pressure setting and at collision energies of 50 eV (T6,T6*) and 20 eV (T12,T12*), respectively. The ESI source was operated at 5.5 kV sprayer voltage and 350 °C dry gas temperature. All other parameters had been optimized to obtain maximum signal intensities for T6,T6* and T12,T12*, respectively. 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$^{(R)}$ 971 (female serum), same material as with validation experiments, was used as blank matrix. T6* and T12* were utilized as markers. Solutions used for additions, contained 20.2 pmol/g (T6*) and 20.6 pmol/g (T12*) in acetonitrile/water 1/1 (v/v), 0.1 M acetic acid. T6*(T12*) were spiked to the blank serum at different stages of the clean-up/ 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 22 kDa GH. Substance recoveries were obtained by relating the signals observed with spiking of T6*(T12*) prior to clean-up by two-step chromatography to those observed if spiking the blank serum after it had been subjected to the same procedure. For obtaining signal recoveries, an aqueous solution of T6*(T12*), same concentration as before, was used as reference instead. In a second experiment, signal recovery was determined for a reduced clean-up 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 molar ratio 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, just missing out addition of GHBP.

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

3 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 complex matrix. 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) additionally is 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 about 1% glycosylation in the sequence range covered by T6, no posttranslational modifications are observed with GH in the regions of T6 and T12 [21], so the mass spectrometric signals monitored virtually correspond to all of 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. Exact matching [22] the analyte concentration of the sample with the calibration solution is 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 [23]. In order to ensure complete release of T6 and T12 from GH, sample and calibration solution are incubated for 24h at 37 °C in presence of 20 mg/L trypsin (water/acetonitrile, 2/3, v/v). Semi-preparative reversed phase liquid chromatography (RP-LC) followed by strong-cation-exchange (SCX) chromatography is used to separate the target peptides from the proteolysis product prior to analysis of 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, another one for T12), is calculated based on comparison of the signal ratios obtained for that fragment in the sample and in the calibration solution, which corresponds to comparison of the amount of fragment generated from GH in the sample to the known amount generated from the calibration solution, since equal amounts of labeled internal standard had been spiked with both. The detailed protocols for proteolysis, extraction of T6,T6* and T12,T12* as well as analysis by mass spectrometry 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 the signature fragments. Substance recoveries after extraction of T6,T6* and T12,T12* by two-step chromatography, as described, were found to be

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

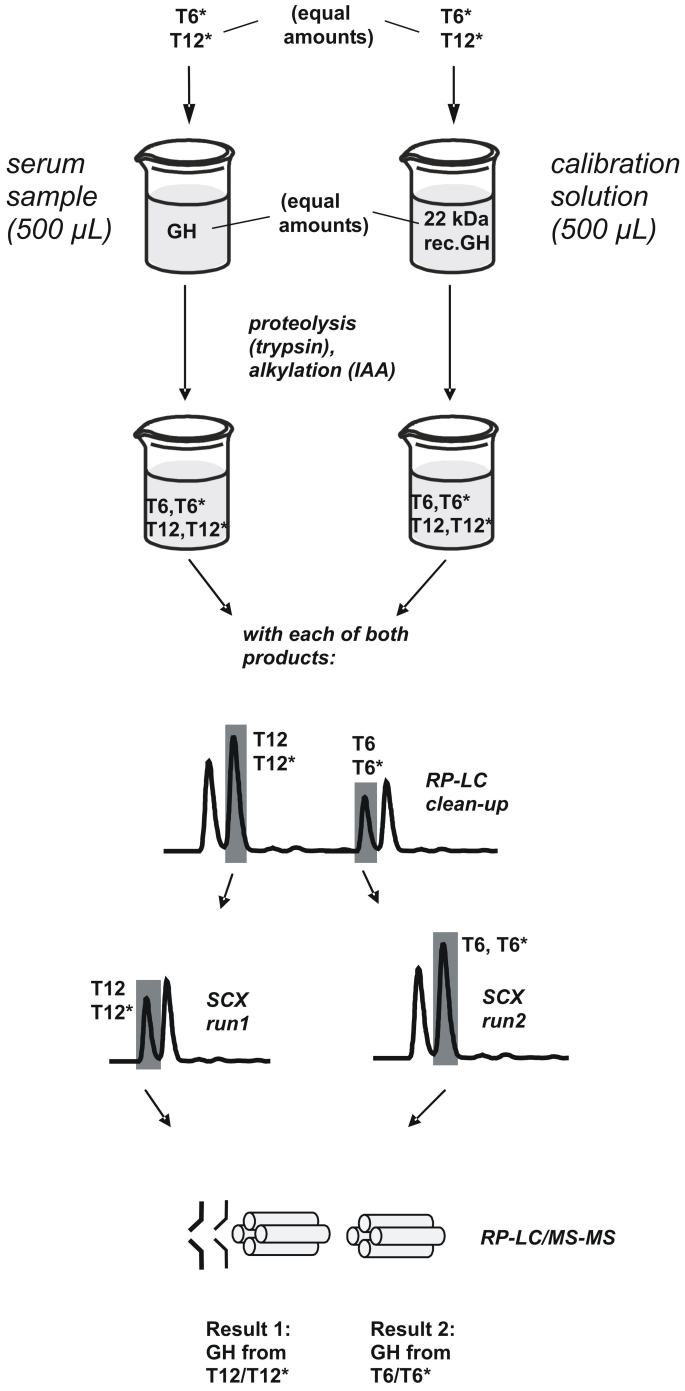

Figure 2: Workflow of the method for quantification of GH by isotope dilution mass spectrometry (IDMS). Tryptic cleavage fragments of GH (T6 and T12) are quantified using 22 kDa GH as reference substance of known concentration and the isotopically labeled analogues (T6* and T12*) as internal standards. 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 semi preparative reversed phase liquid chromatography (RPLC) followed by strong cation-exchange (SCX) chromatography.

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

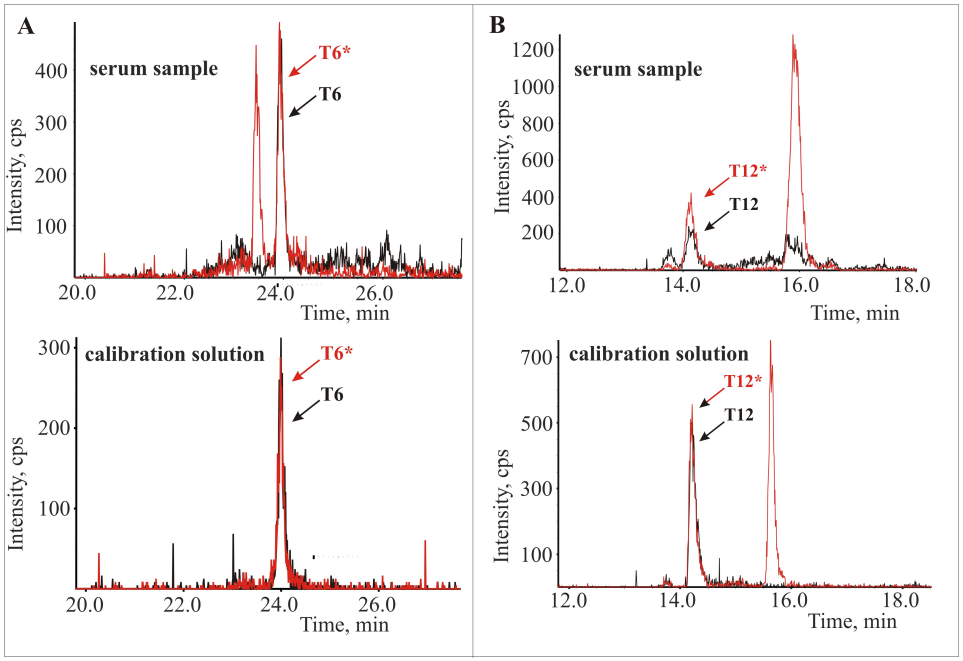

Figure 3: Instrumental responses obtained for GH signature fragments (T6, T12), black graphs, compared to those of the labeled internal standards (T6*,T12*), red, in analysis of quality control serum HP1/08 B. The ion chromatograms shown were monitored for analyte-specific MS-MS transitions. (A) T6-based quantification. (B) T12-based quantification.

61% with T6,T6* and 64% with T12,T12*. At the same time, signal recoveries were 14% (8%). Curtailing the clean-up procedure by leaving out the SCX-chromatography step would result in a decrease of signal recoveries to 3% (1%).

Validation. Method performance was characterized based on evaluation of analytical recovery of defined amounts of GH spiked to a serum material which had been checked not to contain GH at a concentration detectable with the present 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 4.5-30.5 μg/L. All samples were analyzed completely independent of one another on different occasions (days) using a separate calibration solution freshly prepared from the GH reference solution alongside each sample. Therefore, the observed deviation from the respective target value for each individual sample is containing all random errors occurring in sample treatment and instrumental analysis, including those between different days. Quantification results are compiled in Table 1. Plots of results by T6 (T12) vs. expected GH concentrations (not shown) are of excellent linearity. In particular, $R^2 = 0.999$ (0.999), $s_{y\cdot x} = 0.22$ (0.34), slope $a = 1.04$ (0.99), intercept $b = -0.21$ (0.10) were obtained by regression analysis. LOD calculated from the data using the linear-regression

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**

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

approach [24] was 0.5 μg/L (0.7 μg/L) and LOQ=1.7 μg/L (2.7 μg/L). Combination of mean bias, though not significant, and standard deviation (Table 1) is resulting in estimates of 3.0% (2.5%) as combined standard uncertainty (u_c) [25] attributable to the IDMS method.

Test for interference by GHBP. Effect of presence of GHBP on quantification results was investigated in a separate model experiment. Two solutions were processed according to the described protocol. Both were containing GH at 11.9 μg/L as well as T6* and T12* as internal standards. One of them additionally had been spiked with GHBP in 9-fold molar excess. There was no significant difference in signal ratios found in presence of GHBP and without: 1.00 (1.00) for T6/T6* and 0.91 (0.92) for T12/T12*.

IDMS measurement of GH in quality control materials. Samples from two serum pools, HP1/08 A and B, which recently had been used as test materials in an external quality assessment scheme [18] were re-analyzed by IDMS. Results were 7.07 (6.81) μg/L for pool A and 12.33 (12.28) μg/L for pool B with T6- (T12-) based quantification (means of n=3 runs, each). The data reported by 174 testing laboratories are visualized as Youden plots in Fig. 4(a-f). For reference, the IDMS results are included.

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

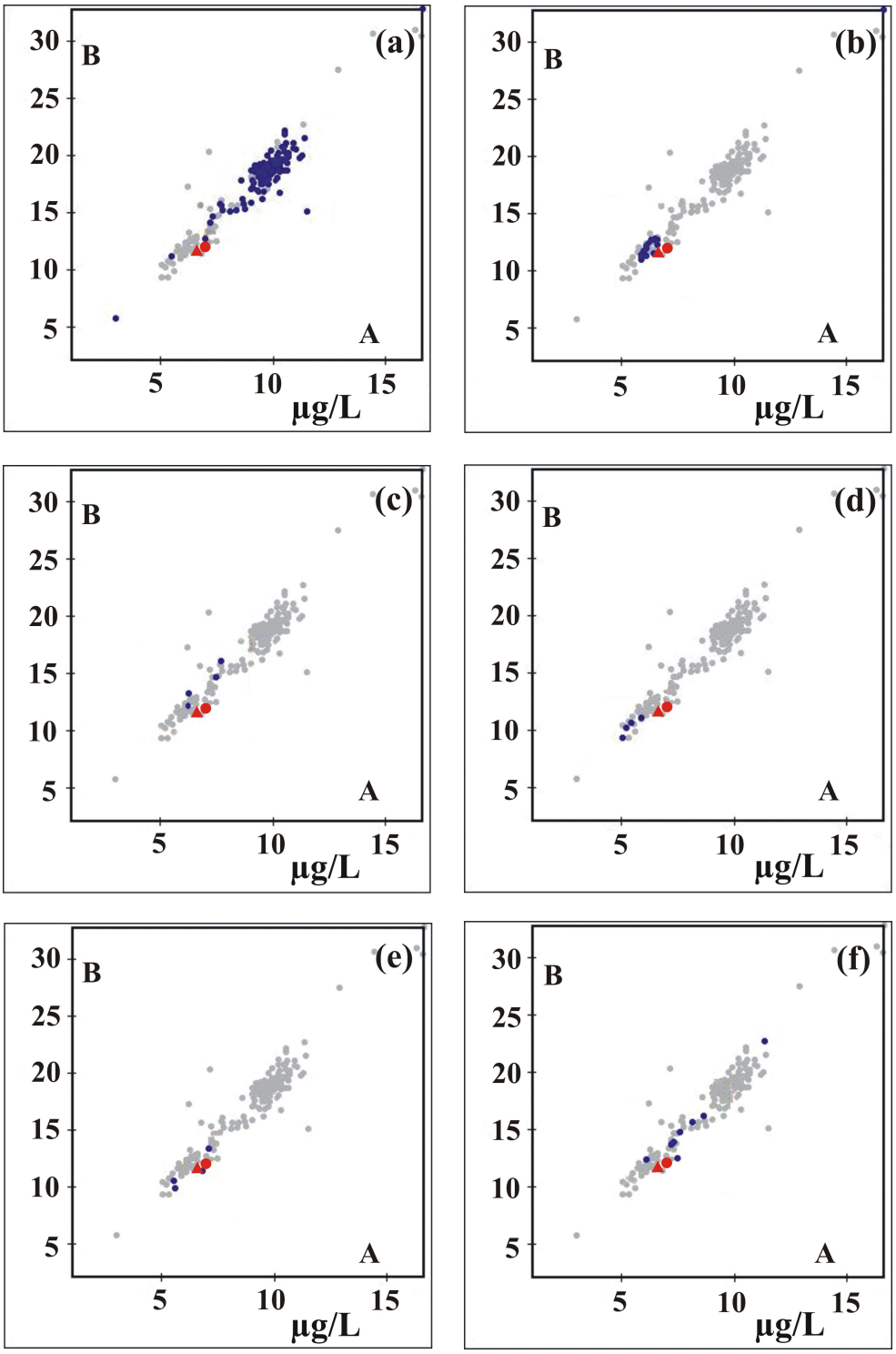

Figure 4: IDMS results obtained for GH in comparison to those reported by testing laboratories in an external quality assessment exercise. Red circle: T6-based result, red triangle: T12 based result. All panels contain the same data, results by different test kits (manufacturers) being highlighted with each one: (a) Siemens Medical Solutions (DPC/Biermann), (b) DiaSorin (c) Schering (CIS Diagn.), (d) Mediagnost, (e) DSL, (f) PerkinElmer/Wallac (Delfia).

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

4 Discussion

GH as found in vivo is displaying considerable heterogeneity. It has been described to consist of several isoforms and variants resulting from modification of amino acid side chains, as well as forming dimers and even aggregates of higher order [21, 26]. Clear definition of the measurand, though vital with respect to comparability of measurements, is still subject to ongoing discussion in clinical chemistry. The present report is demonstrating the performance of IDMS as an analytical tool, given defined signature fragment(s) that are agreed upon 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. It may be anticipated therefore, that IDMS results correlate with those of antibody-based measurements depending on which epitope is being recognized by the antibody. In this study, T6 and T12 were selected as examples. They are both present on 22 kDa GH, the most abundant isoform, as shown in Fig. 1. 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 virtually all of T6,T6* and T12,T12* present after proteolysis.

The IDMS method reported is applicable to T6- (T12-) based serum measurement of GH in clinically relevant concentration. By measurement principle, IDMS has the potential to provide unbiased and reliable results at primary level of traceability to the SI [27]. This is supported by the performance data given in Table 1. Standard deviations (2.5% and 2.4%, resp.) are as low as customarily attained in quantitative LC/MS, and, bias appears not to be significant (SD>mean bias in both cases, T6 and T12). Combining both components (repeatability and formally calculated bias), the uncertainties would be about 3% and 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 reasonable for the type of measurement.

Measurement of GH, since present in serum only at 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 [28] effective sample clean-up 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 present method a chromatography-based alternative is used which seems to be quite as efficient while curtailing time and expense for production of antibodies. Performance data, in particular the limit of quantification (LOQ<3 μg/L) are at least as good as what has been reported with antibody-based clean-up.

Using two-step chromatography (RP-LC plus SCX-) as described, signal recoveries were about 14% (8%) of what would have been obtained in complete absence of matrix-derived ions. Substance recoveries, at the same time, are 61% (64%). The effect of matrix suppression can be roughly estimated to be a factor 4-8 therefore, even after clean-up. 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 down to 3% (1%) 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 growth hormone binding protein, GHBP, as well as formation of GH-GH complexes are considered 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 be obstructed by complex formation. However, this is not the case, as demonstrated with the GHBP interference test which yielded the same result in presence of GHBP as in absence. The IDMS method is capable of providing a measure of 'total' GH, obviously.

Considering the results obtained by the testing laboratories for the samples investigated in the external quality assurance exercise (Fig. 4) need for improvement is becoming evident. In both cases, pool A and pool B, 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 clinical decision very difficult without reference to method dependent cut-off 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 were using the Siemens assay (Fig. 4a). A good deal of this presumably is caused by lack of SI-traceability of the measurement: Many of these laboratories appear either 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 non representative epitopes by the antibodies used cross selectivity towards non target antigens is among the potential causes for the deviations observed. In this situation, by the outstanding selectivity of MS if compared to 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 which eventually might be considered for re-calibration of their results. In the present example, the DiaSorin (Fig. 4b) test seems to be best in agreement with what is obtained by IDMS.

Though sufficient for GH quantification in materials presently used in quality control schemes and also in clinical samples seen during stimulation tests, improvement of the LOQ by about 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 clean-up 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 6-fold increase in response factors has been reported on modifying cysteines with quaternary ammonium compounds [33]. Increase of method sensitivity to enable application to samples from suppression tests appears to be possible therefore too.

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

5 Conclusion

The results reported are demonstrating that, by using IDMS the road is paved to acquisition of reference values for GH in serum at primary level of precision and reliability which are expected to be reproducible not just between different laboratories but also if another GH reference preparation should be used for calibration. The type of measurement is rendering a powerful tool for providing backup to antibody based measurements.

Acknowledgements.

The authors wish to thank Lothar Siekmann and Rolf Kruse from the Referenzinstitut für Bioanalytik (Bonn, Germany) for supplying the quality control materials (HP1/08 A and B) as well as for discussion of results.

This research receives funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 217257.

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

References

[1] M. Bidlingmaier and P.U. Freda, Measurement of human growth hormone by immunoassays: Current status, unsolved problems and clinical consequences. Growth Horm IGF Res (2009).

[2] L. Cazabat, J.C. Souberbielle and P. Chanson, Dynamic tests for the diagnosis and assessment of treatment efficacy in acromegaly. Pituitary 11 (2008) 129-39.

[3] M. Bidlingmaier, Problems with GH assays and strategies toward standardization. Eur J Endocrinol 159 Suppl 1 (2008) 41-4.

[4] L. Siekmann, H.O. Hoppen and H. Breuer, Zur gaschromatographisch- massenspektrometrischen Bestimmung von Steroidhormonen in Körperflüssigkeiten unter Verwendung eines Multiple Ion Detektors (Fragmentographie). Z Anal Chem (1970) 294-298.

[5] S.L. Breuer H, Mass Fragmentography as Reference Method in Clinical Steroid Assay. J Steroid Biochem (1975) 685-686.

[6] A. Cohen, H.S. Hertz, J. Mandel, R.C. Paule, R. Schaffer, L.T. Sniegoski, T. Sun, M.J. Welch and E.T. White, Total serum cholesterol by isotope dilution/mass spectrometry: a candidate definitive method. Clin Chem 26 (1980) 854-60.

[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 and E.J. Sampson, Isotope dilution-mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. Clin Chem 42 (1996) 1676-82.

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009

[8] A.N. Hoofnagle, J.O. Becker, M.H. Wener and J.W. Heinecke, Quantification of thyroglobulin, a low-abundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. Clin Chem 54 (2008) 1796-804.

[9] E. Kuhn, T. Addona, H. Keshishian, M. Burgess, D.R. Mani, R.T. Lee, M.S. Sabatine, R.E. Gerszten and 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) 1108-17.

[10] E. Kuhn, J. Wu, J. Karl, H. Liao, W. Zolg and B. Guild, Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and 13C-labeled peptide standards. Proteomics 4 (2004) 1175-86.

[11] J.C. Seegmiller, D.R. Barnidge, B.E. Burns, T.S. Larson, J.C. Lieske and R. Kumar, Quantification of urinary albumin by using protein cleavage and LC-MS/MS. Clin Chem 55 (2009) 1100-7.

[12] K. Van Uytfanghe, D. Rodriguez-Cabaleiro, D. Stockl and 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) 819-21.

[13] D.K. Williams and 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) 1085-90.

[14] S.L. Wu, H. Amato, R. Biringer, G. Choudhary, P. Shieh and W.S. Hancock, Targeted proteomics of low-level proteins in human plasma by LC/MSn: using human growth hormone as a model system. J Proteome Res 1 (2002) 459-65.

[15] Z. Yang, M. Hayes, X. Fang, M.P. Daley, S. Ettenberg and 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) 9294-301.

[16] F. Li, C.M. Schmerberg and 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) 729-32.

[17] V. Brun, C. Masselon, J. Garin and A. Dupuis, Isotope dilution strategies for absolute quantitative proteomics. J Proteomics 72 (2009) 740-9.

[18] DGKL, Referenzinstitut für Bioanalytik, D-53127 Bonn, www.dgkl-rfb.de.

[19] C.G. Arsene, R. Ohlendorf, W. Burkitt, C. Pritchard, A. Henrion, G. O'Connor, D.M. Bunk and B. Güttler, Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy. Anal Chem 80 (2008) 4154-60.

[20] Standard Reference Material SRM$^{(R)}$ 971, Certificate of Analysis, National Institute of Standards and Technology (NIST), 19 December 2008.

[21] M. Kohler, A. Thomas, K. Puschel, W. Schanzer and M. Thevis, Identification of human pituitary growth hormone variants by mass spectrometry. J Proteome Res 8 (2009) 1071-6.

[22] W.I. Burkitt, C. Pritchard, C. Arsene, A. Henrion, D. Bunk and G. O'Connor, Toward Systeme International d'Unite-traceable protein quantification: from amino acids to proteins. Anal Biochem 376 (2008) 242-51.

[23] A. Henrion, Reduction of systematic errors in quantitative analysis by isotope dilution mass spectrometry (IDMS): an iterative method. Fresenius J Anal Chem 350 (1994) 657-8.

[24] A. Hubaux and G.Vos, Decision and Detection Limits for Linear Calibration Curves. Anal Chem 42 (1970) 849 - 855.

[25] International Organisation for Standardization, Guide to the Expression of Uncertainty of Measurement, Geneva, 1993.

[26] G. Baumann, Growth hormone heterogeneity: genes, isohormones, variants, and binding proteins. Endocr Rev 12 (1991) 424-49.

[27] M. Milton and R. Wielgosz, Uncertainty in SI-traceable measurements of amount of substance by isotope dilution mass spectrometry. Metrologia (2000) 199-206.

[28] T.M. Annesley, Ion suppression in mass spectrometry. Clin Chem 49 (2003) 1041-4.

[29] N.L. Anderson, A. Jackson, D. Smith, D. Hardie, C. Borchers and 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, and 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, and G. O'Connor, Fully Traceable Absolute Protein Quantification of Somatropin that Allows Independent Comparison of Somatropin Standards. Clin Chem (2009).

[32] H. Markkanen, T. Pekkarinen, M.J. Valimaki, H. Alfthan, R. Kauppinen-Makelin, T. Sane and 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) 468-73.

[33] D. Ren, S. Julka, H.D. Inerowicz and F.E. Regnier, Enrichment of cysteine-containing peptides from tryptic digests using a quaternary amine tag. Anal Chem 76 (2004) 4522-30.

Nature Precedings : hdl:10101/npre.2009.4050.1 : Posted 7 Dec 2009