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A quantitative LC-MS/MS method for insulin-like growth factor 1 in human plasma

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Abstract

Background: Insulin-like growth factor 1 (IGF1) is a biomarker with various applications in medicine and also in doping control.

Methods: A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed that employs ^{15}N -IGF1 as an internal standard. The method features urea-based IGF1/IGFBP-complex dissociation which is directly followed by tryptic digestion. Following solid-phase extraction (SPE) sample clean-up of the digest, IGF1 is detected by means of two signature peptides that enable quantification of total IGF1 as well as discrimination between IGF1 proteoforms with ‘native’ and modified or extended N-terminal sequences.

Results: Our method is capable of measuring plasma IGF1 concentrations over the clinically relevant range of 10–1000 ng/mL and was validated according to regulatory guidelines. Comparison with the IDS-iSYS IGF1 immunoassay revealed good correlation ($R^2 > 0.97$) and no proportional bias between both assays was observed after normalizing the results against the WHO reference standard for IGF1 (02/254). Evaluation of several commercially available IGF1 preparations showed varying responses which were due to inconsistencies in purity and absolute amount of IGF1 present in these products.

Conclusions: Our LC-MS/MS method introduces urea-based dissociation of IGF1/IGFBP-complexes to enable reliable quantification of IGF1 in plasma. Furthermore, the method is able to detect clinically relevant IGF1 levels without an enrichment procedure at the protein-level and thereby minimizes the risk of losing IGF1 proteoforms during sample preparation.

Keywords: insulin-like growth factor 1 (IGF1); liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS); quantification.

Introduction

Insulin-like growth factor 1 (IGF1) is a biomarker with various applications in routine clinical chemistry, including diagnosing and monitoring of treatment for the human growth hormone (GH)-related disorders acromegaly, gigantism and (Laron-type) dwarfism [1], and is also applied in the field of oncology [2, 3]. Furthermore, this peptide hormone is measured to assess the effects of caloric restriction [4, 5] and to detect misuse of GH (and IGF1) in sport [6, 7]. IGF1 has been also addressed in various clinical studies, for example, focusing on diabetes mellitus [8] and chronic kidney disease [9].

Quantification of IGF1 in biological samples is traditionally performed using ligand-binding assays (LBAs) which may show poor reproducibility due to their dependence on immunoreagents (e.g. antibodies) for which considerable batch-to-batch variability can be observed [10–13]. This dependence may result in poor inter-laboratory comparability of LBAs leading to different sets of reference values at different sites or even requiring the adjustment of reference values when new lots of critical reagents are being used. In recent years, the use of liquid chromatography coupled to tandem mass spectrometric detection (LC-MS/MS) has gained popularity as an alternative platform for quantification of proteins [14–16]. As this approach combines efficient chromatographic separation with highly selective mass spectrometric detection of well-defined ions, LC-MS/MS quantification is based on analyte-specific physicochemical properties and is

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thereby less prone to interferences from other proteins than LBAs [17].

In the past decade, several LC-MS/MS-based methods for quantifying IGF1 in human plasma and/or serum have been described [18–28]. Most of these methods [18–24] do not cover the full range of clinically relevant IGF1 levels, which may be as low as 15 ng/mL [29]. To obtain sufficient sensitivity, several strategies for IGF1 enrichment have previously been developed, which are mostly based on immunoaffinity enrichment [28], and precipitation of the larger plasma proteins with an organic solvent while maintaining IGF1 in solution [25, 27], which is possible due to IGF1's hydrophobic characteristics. Moreover, another essential aspect of IGF1 quantification is its release from circulating insulin-like growth factor binding proteins (IGFBPs) to obtain an accurate estimate of total IGF1 levels. This aspect was not taken into account in some of the published methods [23–26], while other methods employed SDS treatment [18, 28] or sample acidification [19–22, 27] for this purpose. However, corresponding publications typically lack data to support the effectiveness of these IGF1 liberation procedures.

With any IGF1 assay, it is important to realize that there may be several variants (often referred to as 'proteoforms' [30] or 'protein species' [31]) of IGF1 present in our bodies [32]. Besides 'wildtype' IGF1, a 70 amino acid long protein of 7.65 kiloDaltons (kDa), clinical samples may also contain a variant lacking the first three amino acids known as des(1-3)IGF1 [7]. Furthermore, a small subset of individuals carry single nucleotide polymorphisms (SNPs) within the *IGF1* gene leading to substituted amino acids (e.g. Ala67Thr and Ala70Thr) in the mature IGF1 protein of which the clinical relevancies are yet unknown [32, 33]. In addition, two synthetic IGF1 variants may be encountered in blood-based samples, particularly when analyzing IGF1 in the context of doping abuse in sport: R³IGF1, which has a substituted glutamic acid residue at position 3, and LONGTMR³IGF1, which has an additional N-terminal extension [7]. These different proteoforms may have different extraction characteristics or even different activities, and it is thus essential for any IGF1 method that the assay is adequately characterized with respect to the proteoforms that are actually being quantified.

In this work, we present an LC-MS/MS based method for IGF1 over the clinically relevant range of 10–1000 ng/mL in plasma which takes the above-mentioned methodological aspects into account. The method employs an IGF1/IGFBP-complex dissociation strategy based on urea treatment followed by proteolysis with trypsin and does not include a procedure for IGF1 enrichment at the protein-level. IGF1 is analyzed by means

of two tryptic peptides which enable quantification of 'total' IGF1 as well as differentiation between IGF1 proteoforms with a 'native' N-terminal amino acid sequence and variants with a modified N-terminal sequence. Moreover, special attention was paid to the selection of appropriate reference standards for calibration which is essential for the accurate quantification of endogenous IGF1.

Materials and methods

Materials

Recombinant human IGF1 (Cat. No. CYT-216) and fully ¹⁵N-labeled IGF1 (Cat. No. CYT-128) were obtained from Prospec (Ness-Ziona, Israel). Alternative IGF1 preparations were obtained from R&D Systems (Abingdon, UK; Cat. No. 291-G1-200) and Sigma-Aldrich (Zwijndrecht, The Netherlands; Cat. No. I3769). The WHO international IGF1 reference standard (02/254) was purchased from the National Institute for Biological Standards and Control (NIBSC; South Mimms, UK), and an IGF1 reference sample for interlaboratory harmonization was obtained from the Dutch foundation for quality assurance in medical diagnostic laboratories (SKML). Ovalbumin (Cat. No. A5503), urea (Cat. No. 02493) and trypsin (Cat. No. T0303) as well as all other chemicals were from Sigma-Aldrich.

Calibrants and quality control samples

One vial containing 100 µg of IGF1 was reconstituted in 500 µL proxy matrix (2% ovalbumin in 1× PBS) to obtain a 200 µg/mL stock solution. This stock was sequentially diluted to 2 µg/mL in proxy matrix, and the resulting solution was further diluted in proxy matrix to obtain calibrants at 10, 20, 50, 100, 200, 500, 800 and 1000 ng/mL. The internal standard ¹⁵N-IGF1 was reconstituted in the proxy matrix to obtain a 100 µg/mL of stock solution. For preparation of quality control (QC) samples, human plasma from Seralabs (West Sussex, UK) was used either directly as a QC-medium sample, diluted four times with the proxy matrix to prepare the QC-low sample, or fortified with recombinant IGF1 (+700 ng/mL) to obtain the QC-high sample. All stocks, dilutions, calibrants and QC-samples were stored in polypropylene tubes at –20 °C.

Sample treatment

Fifty microliters of (QC-)plasma or calibrant were pipetted into a 2 mL polypropylene microtiter plate (Corning Life sciences, Amsterdam, The Netherlands), and IGF1/IGFBP-complexes were dissociated through the addition of 100 µL of an 8 molar aqueous urea solution containing 200 ng/mL of ¹⁵N-IGF1 followed by 60 s of vortex-mixing. Subsequently, disulfide bonds were reduced with 100 µL 25 mg/mL dithiothreitol (DTT) in 50 mM ammonium bicarbonate (ABC) (30 min; 600 rpm; 60 °C), and cysteines were alkylated in the dark with 100 µL 75 mg/mL iodoacetamide in 50 mM ABC (30 min; 600 rpm; room temperature). Proteolysis was performed for 3 h at 37 °C and 750 rpm

after the addition of 1 mL 5 µg/mL trypsin in 50 mM ABC, and digestion was stopped by adding 100 µL 2% (v/v) aqueous formic acid following 5 min of centrifugation at 2500×g. Next, sample clean-up was performed by loading 1 mL of sample on 1 mL Oasis HLB solid-phase extraction (SPE) cartridges (Waters, Milford, MA, USA; Cat. No. WAT094225), which had been conditioned with 1 mL methanol and 1 mL 2% (v/v) aqueous formic acid. The cartridges were then washed with 1 mL 20% (v/v) methanol in 100 mM ammonium acetate, pH 4 and peptides were eluted with 1 mL 0.5% (v/v) ammonia in 50% (v/v) aqueous methanol. Eluates were dried at 60 °C under a gentle flow of nitrogen, and peptides were reconstituted in 200 µL 1% (v/v) formic acid in 10% (v/v) aqueous acetonitrile.

LC-MS/MS analysis

Samples were analyzed using a Waters ACQUITY UPLC I-Class liquid chromatographic system and a XEVO TQ-S triple quadrupole mass spectrometer. Twenty-five microliters of peptide extract were injected into a Waters ACQUITY CSH column (1.7 µm particles, 130 Å pore size, 2.1 × 150 mm) kept at 50 °C. Gradient elution was performed at 0.4 mL/min using 0.1% (v/v) formic acid in water as eluent A and 0.1% (v/v) formic acid in acetonitrile as eluent B. With an initial 6-min linear gradient from 10% to 13% eluent B, IGF1's first signature peptide (T-1) was eluted. Subsequently, the percentage of eluent B was increased to 25%, and the second peptide (T-4) was eluted with a 5-min linear gradient from 25% to 28% eluent B. After a 3-min washing step with 90% eluent B, the column was equilibrated at 10% eluent B for 3 min prior to the next injection thereby resulting in a total LC-MS/MS run time of 17 min. Tandem mass spectrometric detection was carried out using positive electrospray ionization (ESI) with an ionization voltage of 3500 V, desolvation temperature of 400 °C, cone voltage of 40 V and while operating in the multiple reaction monitoring (MRM) mode (see Table 1 for MRM transitions and settings).

Method validation and comparison

Method validation for both signature peptides was based on the validation and implementation guidelines as defined by the Dutch Coordinating Commission for Quality Management in Medical Laboratories (CCKL) and the ISO 15189:2012 standard [34] addressing the following criteria: linearity, precision, accuracy, interference by IGF binding protein 3 (IGFBP3), stability of IGF1 in plasma stored at room temperature, −20 °C and after three freeze-thaw cycles, autosampler stability of the signature peptides and stability of IGF1 in stock solution after storage at −20 °C. A detailed overview of the validation approach plus the corresponding acceptance criteria are presented

in Table S1 in the Supplementary Materials. Furthermore, the method was compared with the IDS-iSYS IGF1 immunoassay using 40 clinical samples of patients between 2 and 87 years old.

Results

Signature peptide selection

The selection of the signature peptides which are formed upon trypsin digestion, was based on *in silico* assessment of peptide properties and uniqueness with mMass software (version 5.5.0) and the online Basic Local Alignment Search Tool (BLAST; version 2.2.29), respectively. Trypsin cleaves IGF1 into eight different peptides, four of which are unique for IGF1, meaning that their amino acid sequences do not occur in any other human protein. With the described LC-MS/MS approach, three of these peptides could be detected with a proper peak shape and good sensitivity: GPETLCGAELVDALQFVCGDR (T-1 peptide; Gly1-Arg21), GFYFNKPTGYGSSSR (T-2 peptide; Gly22-Arg36), and APQTGIVDECCFR (T-4 peptide; Ala38-Arg50). In our hands, T-2 was less favorable because of reproducibility issues. T-1 and T-4 were readily released within 3 h of tryptic digestion (see Supplementary Figure 1), and both peptides were selected for IGF1 quantification. As T-1 and T-4 both contain two cysteine residues which are involved in the formation of all three intra-molecular disulfide bridges of IGF1, reduction of IGF1 and subsequent alkylation of the reduced cysteines is needed prior to digestion to break the disulfide bonds and release the signature peptides. Accordingly, a standard reduction and alkylation procedure as described above was found to be adequate.

Selection of internal standard and proxy matrix

A ¹⁵N-labeled version of human IGF1 was employed as the internal standard. This protein was added to all samples as the first step in the protocol thereby enabling adequate

Table 1: MRM transitions and collision energies (CE).

Peptide	Sequence	Precursor, <i>m/z</i>	Fragment, <i>m/z</i>	CE
T-1	GPETLCGAELVDALQFVCGDR	[M + 3] ³⁺ (769.7)	y6 ¹⁺ (753.3)	24 V
SIL T-1	GPETLCGAELVDALQFVCGDR ^a	[M + 3] ³⁺ (778.2)	y6 ¹⁺ (762.3)	24 V
T-4	APQTGIVDECCFR	[M + 2] ²⁺ (776.8)	y7 ¹⁺ (985.4)	27 V
SIL T-4	APQTGIVDECCFR ^a	[M + 2] ²⁺ (785.3)	y7 ¹⁺ (995.3)	27 V

^aSIL-peptides are ¹⁵N-labeled.

Figure 1: LC-MS/MS chromatograms of the IGF1 T-4 (eluting at 5.0 min) and T-1 (9.8 min) peptides recorded for a 10 ng/mL calibrant in an ovalbumin-based proxy matrix (upper pane), a blank ovalbumin-based proxy matrix (middle pane) and a blank BSA-based proxy matrix (lower pane).

For each peptide, the three chromatograms are scaled to the same intensity.

compensation for any variability arising from both the sample preparation and the LC-MS/MS detection procedure. For preparation of standard curves, a proxy matrix consisting of 2% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) was initially tested; however, significant interferences were observed for both the T-1 and T-4 peptides (see Figure 1) which were likely caused by contamination of the BSA preparation (i.e. BSA enriched from bovine serum) with bovine IGF1 which is identical to human IGF1. Subsequently, the applicability of 2% (w/v) ovalbumin from chicken egg white in PBS was assessed. Potential contamination of ovalbumin with chicken IGF1 will not yield an interfering T-4 peptide due to sequence variation, though chicken IGF1 may give rise to an interfering T-1 peptide as the corresponding amino acid sequence is identical to that of its human variant. However, contamination was not observed (see Figure 1), and the applicability of this proxy matrix was studied further. To this end, human plasma with an endogenous IGF1 level around 200 ng/mL was fortified with 100, 200, 300 and 600 ng/mL recombinant human IGF1, and IGF1 in these samples was quantified using a calibration curve

prepared in the proxy matrix. The IGF1 levels for each of the fortified samples corresponded to the expected levels (endogenous + added IGF1) with an average bias of 3.7% for T-1 and 7.1% for T-4 thus supporting the suitability of ovalbumin in PBS as the proxy matrix for our method.

Reference material comparison

For comparison of reference materials, the WHO reference standard for IGF1 (02/254), the IGF1 calibrant of the IDS-iSYS IGF1 immunoassay as well as three commercially obtained IGF1 preparations were reconstituted and diluted in the proxy matrix at 450 ng/mL (based on the manufacturer's declared protein quantity), and processed according to the validated workflow for IGF1 quantification. IGF1 levels obtained for the different materials were related to that of the WHO reference standard which was set at 100% (see Figure 2). Following these experiments, a bias of -10% was observed for the immunoassay calibrant whereas biases between -30% and -40% were observed for three commercially obtained preparations.

Figure 2: Responses of different IGF1 reference materials relative to the response of the WHO reference standard (02/254). Error bars represent the relative standard deviations from the repeated measurements.

Besides a quantitative comparison of the reference materials, IGF1 as present in the reference materials was also assessed qualitatively on the basis of pseudo-MRM measurements with Q1 and Q3 set at the same m/z values, which correspond to the eight-fold positively charged intact IGF1 molecule. For these experiments, vials with IGF1 were reconstituted and diluted in PBS at 5 $\mu\text{g/mL}$ with and without treatment with DTT to reduce the disulfide bonds. Non-treated IGF1 standards yielded chromatograms with a single peak, with the exception of the material from supplier 3 which gave rise to two peaks, one at the same retention time as seen for the other materials and one at a different retention time (see Supplementary Figure S2-A and S2-C). This indicates that two different forms of IGF1 were present in the material of supplier 3. Upon DTT reduction, single peaks were obtained for the materials of all suppliers, all at yet another retention time and at an m/z value corresponding to reduced IGF1 (see Supplementary Figure S2-B and S2-D). As reduction with DTT breaks the three intramolecular disulfide bonds of IGF1, it is likely that the material obtained from supplier 3 contains two different IGF1 proteoforms with different disulfide bond configurations that were both converted to the same reduced form upon DTT treatment.

Method validation and comparison

A complete overview of results for all validation experiments is included in Supplementary Tables S2–S13 while

Table 2: The maximum observed total bias and CV values for each validation experiment.

Validation item	Maximum bias, %	Maximum CV, %
Accuracy and precision (10 runs, in three-fold)	14.7	17.2
Accuracy and precision (one run, in 10-fold)	9.8	10.4
Linearity	−2.3	7.5
Interference of IGFBP3	−13.8	9.4
Bench-top stability (96 h)	−14.8	14.9
Freeze-thaw stability (−20 °C; three cycles)	−6.5	9.7
Stock stability (−20 °C; 195 days)	−5.4	2.6
Autosampler stability (10 °C; 72 h)	11.6	11.9

Table 2 provides a concise summary of these experiments featuring the highest total bias and CV per experiment. The method allows accurate and precise IGF1 quantification over the clinically relevant range of 10–1000 ng/mL when using either the T-1 or T-4 peptide for IGF1 quantification. Assessment of within- and between-run accuracy and precision furthermore showed acceptable biases ($\pm 15\%$) and CVs ($\leq 15\%$) for all QC-samples, and stability assessments indicated that the analyte is sufficiently stable upon storage on the benchtop, in the autosampler, and following three freeze-thaw cycles. Interference testing by treating samples with a 10-fold molar excess of insulin-like growth factor binding protein 3 (IGFBP3), the most relevant binding protein for IGF1 [35], showed acceptable biases ($\pm 15\%$) and CVs ($\leq 15\%$), and thus did not pose a relevant challenge for the assay.

The LC-MS/MS method was compared with the IDS-iSYS IGF1 immunoassay using plasma samples from 40 patients of both genders with ages ranging from 2 to 87 years (see Figure 3). Results obtained with the different approaches correlate well throughout the entire calibration range ($R^2 \geq 0.97$), and no relevant differences (proportional bias of 1%) were observed between the IGF1 levels obtained by the immunoassay and data for the T-4 peptide. Levels for the T-1 peptide were, however, slightly different from those obtained by the immunoassay and the T-4 peptide leading to approximately 10% higher IGF1 levels reported for the T-1 peptide.

Discussion

Here we present a validated LC-MS/MS method for quantification of IGF1 in human plasma over the entire range

Figure 3: Comparisons between the IDS-iSYS IGF1 immunoassay and the quantitative LC-MS/MS method for IGF1 on the basis of data obtained for both the T-1 and T-4 peptides using (A) Passing-Bablok regression and (B) Bland-Altman plots.

Statistical analyses were carried out using Analyse-it (version 4.81.6) and plots were constructed using GraphPad Prism (version 5.00).

of clinically relevant levels between 10 and 1000 ng/mL. Although the quantitation of intact IGF1 is possible using, e.g. MALDI-TOF mass spectrometry [32], such instrumentation may be less accessible in clinical laboratories than triple quadrupole MS. As intact IGF1 cannot easily be measured on a triple quadrupole because of its lack of fragmentation [18], we decided to follow the approach of enzymatic digestion and subsequent LC-MS/MS quantitation of tryptic peptides. Although analysis of protein digests has become a widely accepted methodology, an inherent disadvantage is that it does not give direct information about the intact protein. To increase coverage of the IGF1 molecule, we therefore included two peptides (T-1 and T-4), which together account for over 50% of the protein. This approach also generates complementary information, as T-1 represents the unmodified N-terminal peptide of IGF1 which is not present in des(1-3)IGF1, R³IGF1, and LONGTMR³IGF1 [18], and T-4 is preserved in all known IGF1 proteoforms (taking into account SNPs with a prevalence greater than 0.0001, as based on data from the ExAC Browser). T-4 will thus yield a total IGF1

concentration, and T-1 will allow discriminating between IGF1 proteoforms with modified and with non-modified N-terminal sequences.

In the current method, IGF1/IGFBP-complex dissociation was achieved by treating samples with 8 molar urea followed by trypsin digestion. By cleaving all sample proteins into peptides, this strategy completely and irreversibly breaks all protein-protein interactions and thus represents an effective alternative to SDS [18, 28] and acidification-based [19–22, 27] IGF1 liberation procedures. For our method, the efficacy of the urea-based approach was demonstrated by the accuracy of IGF1 quantification in the presence of a 10-fold molar excess of the binding protein IGFBP3.

In contrast to other published methods [18–23, 25–28], we omitted an enrichment step at the protein level in order to minimize the risk of losing IGF1 proteoforms. Instead, the digested plasma sample was subjected to SPE which recovered both signature peptides close to 100% while also eliminating interfering peptides released from other matrix proteins. Consequently, we were able to quantify

IGF1 levels in plasma down to 10 ng/mL which is not possible with most of the available mass spectrometric IGF1 assays [18–24].

A good internal standard is essential for protein quantification by LC-MS/MS [36, 37]. Stable-isotope labeled forms of the signature peptides [19, 23–25] provide excellent normalization of all post-digestion variability, but do not correct for sample handling before and during digestion. Protein-based internal standards are therefore to be preferred, and structural analogues such as oxidized rat IGF1 [27], R³IGF1 [19], LONGTMR³IGF1 [28], des(1-3)IGF1 [20] and ¹⁵N-labeled IGF1 [21, 22, 32] have previously been used in quantitative IGF1 workflows. For our method, we decided to use ¹⁵N-IGF1 which provides the optimal correction for all steps of the analytical workflow and is furthermore commercially available at a reasonable price.

When comparing our LC-MS/MS method with the commonly-used IDS-iSYS IGF1 immunoassay, good correlation was observed between the two methods as well as a high level of agreement when calibrating both assays against the WHO reference standard for IGF1. Furthermore, good inter-peptide correlation was observed between the T-1 and T-4 peptides, although slightly higher IGF1 levels (about 8%) were reported for the T-1 peptide. Further research is needed to elucidate on whether this observation can be attributed to methodological variability or whether it has clinical relevance. Still, it does appear that N-terminally modified IGF1 proteoforms did not constitute a significant portion of the plasma IGF1 content in the samples that were studied, as lower levels were not reported for the T-1 peptide compared to the T-4 peptide.

With our validated method, we assessed the IGF1 content of reference standards obtained from several commercial sources, and we found differences up to 40% between the different materials. Corresponding discrepancies can obviously be corrected for by calibrating IGF1 assays against a universal reference standard (e.g. WHO reference standard 02/254), however, in one of the supplied materials we also observed an IGF1 proteoform with an alternative disulfide bond configuration which may be more problematic. In particular for IGF1 assays employing an enrichment procedure at the protein level, such a disulfide-scrambled impurity may exhibit a different extraction behavior due to its altered three-dimensional structure thus affecting the method's accuracy. Therefore, it is essential that reference standards are properly characterized (both quantitatively and qualitatively) prior to their application, yet attention should also be paid to the successive batches of such standards that are employed for routine assays.

In summary, our method enables accurate quantification of IGF1 at clinically relevant plasma levels and takes all important aspects of IGF1 determination into account. Furthermore, the method allows distinguishing between IGF1 wildtype and N-terminal sequences which may yield valuable information not only for doping control purposes but potentially also in a clinical setting.

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