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Abstract

Objectives: Sex hormone binding globulin (SHBG) is a hormone binding protein which plays an important role in regulating the transport and availability of biologically active androgens and estradiol to target cells and used to calculate free testosterone concentrations.

Methods: A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed, featuring an albumin removal step followed by a tryptic digestion. After a reduction step with dithiothreitol and alkylation with iodoacetamide three signature peptides were used for the quantification of SHBG.

Results: The method enables the quantification of serum and plasma SHBG over the clinically relevant range of 200–20,000 ng/mL and was validated according to the most recent guidelines. The LC-MS/MS method correlates well with the Abbott Alinity immunoassay (R^2>0.95), but the LC-MS/MS results are on average 16–17% lower than the immunoassay results, which is consistent for all three signature peptides.

Conclusions: The LC-MS/MS method which includes an albumin depletion step allows quantification of SHBG in serum and plasma without an immunocapture step at clinically relevant SHBG levels, thus contributing to better lab-to-lab consistency of results.

Keywords: albumin depletion; biomarker; human sex hormone binding globulin (SHBG); liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Introduction

The liver-secreted sex hormone binding globulin (SHBG) consists of two identical monomers with 373 amino acids each [1, 2]. The homodimer includes two zinc-binding sites involved in steroid-binding specificity and a calcium-binding site that stabilizes the homodimer. SHBG plays an important role in regulating the availability of biologically active androgens and estradiol and their transport to target cells [3], and binds estradiol and testosterone with a higher affinity than albumin [4]. Measurement of SHBG is relevant for both research and clinical purposes. Low concentrations of SHBG are associated with obesity and the development of type 2 diabetes [5]. In addition, increased free testosterone levels due to decreased SHBG levels may contribute to hirsutism or polycystic ovary syndrome [3, 6]. Variations in SHBG concentration are related to dyslipidemia and cancer [7, 8]. Importantly, circulating SHBG concentrations are used to calculate free testosterone concentrations using mathematical models such as the Vermeulen equation [9]. Quantification of SHBG was traditionally performed using steroid-binding capacity assays while currently automated immunoassays are generally used for patient care [4, 10–13]. Structurally different forms of SHBG, including mutated and glycosylated forms, occur in the circulation [14, 15]. Antibodies against SHBG may vary in their recognition and affinity towards these forms, depending on the way they were raised. This could lead to discrepant results for different immunoassays and eventually even to differences

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in diagnosis and treatment [16, 17]. Antibody-free LC-MS/MS methods have the potential to overcome the issues related to immunoassays and thus generate more consistent results. Up to now, the experience with LC-MS/MS for SHBG quantification has been limited. Two LC-MS/MS methods have been published, both based on SHBG digestion with the enzyme trypsin and quantification of a suitable signature peptide by LC-MS/MS [13]. However, both methods use an antibody-based immunocapture step and are therefore potentially sensitive to the same selectivity issues related to the use of antibodies in immunoassay. To avoid this altogether and improve lab-to-lab comparability of results, we developed an antibody-free LC-MS/MS assay for the quantification of SHBG in serum and plasma using three different signature peptides, two of which are universal and also occur in the known SHBG mutants [14, 15], while the third is unique for wild-type SHBG. The method was validated extensively according to the most recent guidelines [18, 19] and compared with an immunoassay.

Materials and methods

Chemicals and materials

Sex hormone binding globulin (UniProtKB – P04278) (purified native Human SHBG protein, purity: >90%) was purchased from Fitzgerald (North Acton, MA, USA). WHO International Standard SHBG (freeze dried serum obtained from a pool of normal healthy female volunteers) (NIBSC code: 08/266) was obtained from NIBSC (Hertfordshire, UK). Formic acid ≥95%, ammonium bicarbonate BioUltra ≥99.5%, trypsin from porcine pancreas (Cat. No. T0303), DL-Dithiothreitol (DTT), iodoacetamide (IAA), trichloroacetic acid (TCA) and albumin human serum 99% were purchased from Sigma-Aldrich (St Louis, MO, USA). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Thermo Fischer Scientific (Waltham, MA, USA). Gibco Dulbecco's tenfold concentrated phosphate buffered saline (PBS) was purchased from Life Technologies Europe (The Netherlands) and diluted tenfold with water before use, a solution further referred to as PBS. Milli-Q water was prepared using a water purification system from Merck-Millipore (Burlington, MA, USA). Acetonitrile was purchased from Merck (Darmstadt, Germany) and isopropanol, methanol, ethanol and formic acid 99% from Biosolve (Valkenswaard, The Netherlands). Internal standard peptides TSSSFEVR (purity: 99.7%), IALGGLLFPASNLR (purity: 99.8%), and LPLVPALDGC(CAM)LR (purity: 97.2%, further referred to as LPLVPALDGCLR) all with $^{13}C_6{}^{15}N_4$-labeled C-terminal arginine were custom synthesized by Pepscan (Lelystad, The Netherlands). Human serum and rat serum were obtained from BioIVT (West Sussex, UK).

Preparation of calibration and quality control samples

A 1.00 mg/mL stock solution of sex hormone binding globulin was prepared by dissolving the content of the vial of lyophilized protein (label claim: 1.0 mg) in 1.00 mL of PBS. The stock was divided into 0.1 mL aliquots in Eppendorf (Hamburg, Germany) Protein Lobind tubes and stored at −80 °C. The calibration samples were directly made from the stock solution at 200, 400, 1,000, 2,000, 4,000, 10,000, 16,000 and 20,000 ng/mL in a mixture of rat serum and PBS (1:1, v/v) (proxy matrix). Quality control samples were human serum, containing an endogenous SHBG level (low level), and the same human serum lot spiked with an additional 5,000 (medium level), 12,000 (high level) or 50,000 (integrity of dilution) ng/mL SHBG. For accuracy testing, the content of an ampoule (WHO standard SHBG) (label claim: 180 pmol) was completely dissolved in 1.00 mL of Milli-Q water resulting in a SHBG concentration of 180 nM (17,100 ng/mL). All calibration and quality control samples were stored at −80 °C in polypropylene tubes.

Sample pretreatment

50.0 µL serum or plasma samples were pipetted into 1,000-µL wells of an Eppendorf Protein Lobind 96-well plate and 200 µL of 1% TCA in 2-propanol was added. After vortex-mixing (room temperature, 5 min, 1,200 rpm), proteins were pelleted by centrifugation (10 min, 1,500 g). The supernatant was discarded, and the protein pellets were washed twice with 200 µL methanol by resuspending the pellets in methanol, centrifuging at 1,000 g for 1 min and discarding the supernatant. Next, 200 µL of trypsin (150 µg/mL in 50 mM ABC buffer with 5% acetonitrile) was added to each well, followed by 20 µL of the internal standard peptides (557 ng/mL of stable-isotope labeled (SIL) peptide TSSSFEVR, 881 ng/mL of SIL IALGGLLFPASNLR and 815 ng/mL of SIL LPLVPALDGCLR) in a 70:30 (v/v) mixture of water and acetonitrile. After centrifugation (1 min, 1,500 g), the samples were digested (37 °C, 3 h, 900 rpm) using an Eppendorf Thermomixer® Comfort, which was also used for subsequent incubation steps. Then, 40.0 µL of 32.5 mM DTT in 50 mM ABC buffer was added and the samples were reduced by incubation (37 °C, 30 min, 900 rpm). After addition of 40.0 µL of 75 mM IAA in 50 mM ABC buffer, the samples were alkylated by incubation (25 °C, 20 min, 900 rpm, protected from light). Next, remaining IAA was quenched with 40.0 µL of 32.5 mM DTT in 50 mM ABC buffer (25 °C, 5 min, 900 rpm). The samples were acidified with 20.0 µL 20% formic acid in water and centrifuged (1 min, 1,500 *g*). Finally, the plate was sealed, vortex-mixed for 5 min, centrifuged (5 min, 3,000 *g*) and placed in the autosampler at 10 °C for analysis.

Chromatography and detection

The extracts (2 µL) were injected into an M-class UPLC system, coupled to a Xevo TQ-S triple-quadrupole mass spectrometer (Waters, Milford, MA, USA). Chromatography was performed on a 100 × 1.0 mm Luna Omega C18 column (particle size 1.6 µm, pore size 100 Å) (Phenomenex, CA, USA) at 60 °C. Gradient elution was done at 80 µL/min with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) using the following profile: 0.0–6.0 min: 5–11% B; 6.0–7.0 min: 11–24% B; 7.0–12 min: 24–31.5% B; 12.1–15 min: 95% B; 15.1–17 min: 5% B. The mobile phase was diverted to waste between 0 and 3.5 min and between 12.5 and 17 min after injection. Detection was performed in positive electrospray ionization mode with Waters MassLynx 4.1. Data was processed with TargetLynx 4.1 software. The following general MS settings were applied: capillary voltage: 3,000 V; source offset: 60 V; desolvation temperature: 400 °C; cone gas flow: 150 L/h; desolvation gas flow: 800 L/h; collision gas flow: 0.15 L/min; nebulizer gas flow: 3 bar. Peptide-specific detection parameters are presented in Table 1.

Table 1: Detection parameters for the signature peptides.

Peptide	Q1 *m/z*	Q3 *m/z*	Cone voltage, V	Collision energy, V
TSSSFEVR[a]	456.7 $[M + 2H]^{2+}$	637.3 ($y5^+$)	30	15
TSSSFEVR[b]	456.7 $[M + 2H]^{2+}$	724.4 ($y6^+$)	30	15
TSSSFEVR-SIL[a]	461.7 $[M + 2H]^{2+}$	647.3 ($y5^+$)	30	15
TSSSFEVR-SIL[b]	461.7 $[M + 2H]^{2+}$	734.4 ($y6^+$)	30	15
IALGGLLFPASNLR[a]	721.4 $[M + 2H]^{2+}$	804.4 ($y7^+$)	30	25
IALGGLLFPASNLR[b]	721.4 $[M + 2H]^{2+}$	917.5 ($y8^+$)	30	25
IALGGLLFPASNLR-SIL[a]	726.4 $[M + 2H]^{2+}$	814.4 ($y7^+$)	30	25
IALGGLLFPASNLR-SIL[b]	726.4 $[M + 2H]^{2+}$	927.5 ($y8^+$)	30	25
LPLVPALDGC(CAM)LR[a]	662.4 $[M + 2H]^{2+}$	901.5 ($y8^+$)	30	20
LPLVPALDGC(CAM)LR[b]	662.4 $[M + 2H]^{2+}$	1,113.6 ($y10^+$)	30	20
LPLVPALDGC(CAM) LR-SIL[a]	667.4 $[M + 2H]^{2+}$	911.5 ($y8^+$)	30	20
LPLVPALDGC(CAM) LR-SIL[b]	667.4 $[M + 2H]^{2+}$	1,123.6 ($y10^+$)	30	20

[a]Quantifier ion. [b]Qualifier ion.

Validation

The LC-MS/MS method was validated based on the ISO 15189:2012 standard, the C64 guidelines of the Clinical and Laboratory Standards Institute (CLSI) and the guidelines for bioanalytical methods validation of the Dutch Coordinating Commission for Quality Management in Medical Laboratories (CCKL), by assessing linearity, imprecision, accuracy, integrity of dilution, lower limit of the measuring interval (LLMI), comparability of serum and EDTA- and heparin-anticoagulated plasma, carry-over, stability and comparability with an immunoassay (Abbott Alinity). A detailed description of the validation experiments and of the Abbott Alinity immunoassay is given in the Supplementary Material.

Results

Signature peptide selection and LC-MS/MS

An *in silico* trypsin digestion of SHBG was performed using mMass [20], yielding 29 theoretically expected tryptic peptides. The peptides TSSSFEVR (amino acids 40–47), IALGGLLFPASNLR (141–154) and LPLVPALDGCLR (155–166) were selected because of their uniqueness for human SHBG according to the Basic Local Alignment Search Tool (BLAST) [21]. In addition, all three peptides do not occur in the entire rat proteome, which is desirable because of the intention to use rat serum as a proxy matrix to prepare calibration samples. For sufficient separation of the three signature peptides from endogenous matrix components (Figure 1), a slow gradient from 5 to 11% acetonitrile over 6 min was first used to elute peptide TSSSFEVR. The two more hydrophobic peptides LPLVPALDGCLR and IALGGLLFPASNLR were eluted by a quick step from 11 to 24% acetonitrile in 1 min and a step from 24 to 31.5% acetonitrile in 7 min. With a subsequent 3-min column cleaning step at 95% acetonitrile and a 2 min re-equilibration at 5% acetonitrile, the total run time was 17 min. For the three signature peptides, doubly charged protonated precursor ions and singly charged protonated y-fragment ions were selected (Table 1).

Sample pretreatment

SHBG was isolated from the sample by protein precipitation, and the efficacy and selectivity of different precipitation solvents (1% TCA in isopropanol, isopropanol, ethanol, acetonitrile and methanol) were tested by addition of 200 µL of each solvent to 50.0 µL of plasma. Figure 2 shows the effect of precipitation solvent on albumin removal, quantified by the (remaining) peak areas of three tryptic signature peptides, unique for albumin, in the precipitated protein pellet. After treatment with 1% TCA in isopropanol around 84% of the albumin was removed, while with the other solvents typically all albumin remained in the protein pellet. Precipitation with 1% TCA in isopropanol also gave the highest detection response for the two SHBG peptides tested (Figure 3). The protein pellet was isolated and digested using 150 µg/mL trypsin at the optimal digestion conditions of 37 °C and pH 8, and maximum digestion was achieved for all three peptides after 3 h. Since peptide LPLVPALDGCLR contains a cysteine residue, which forms a disulfide bridge within the SHBG molecule, standard reduction (30 min, 5 mM DTT) and alkylation (20 min, 10 mM IAA) steps were included to break and subsequently prevent reformation of this disulfide bridge [22].

Characterization of the stock solution

The SHBG concentration of the stock was established by completely digesting it and comparing the LC-MS/MS response of the resulting peptides TSSSFEVR and IALGGLLFPASNLR with the response of their stable-isotope labeled versions, added equimolarly to the digest. The mean

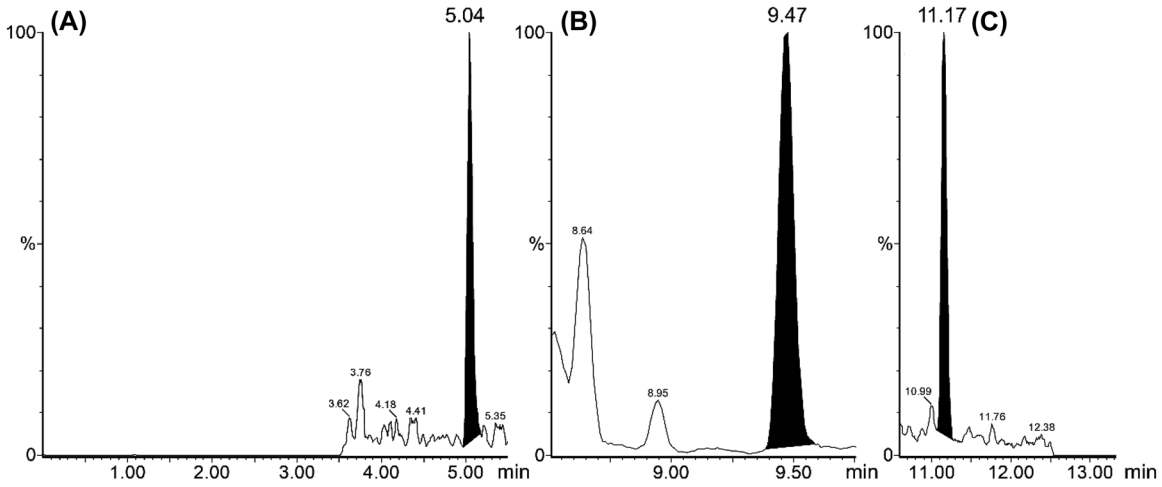

Figure 1: LC-MS/MS chromatograms of the SHBG peptides TSSSFEVR (A), LPLVPALDGCLR (B) and IALGGLLFPASNLR (C) for a human serum sample with an endogenous concentration of 300 ng/mL. Relative intensity is shown on the y axis, and retention time on the x axis.

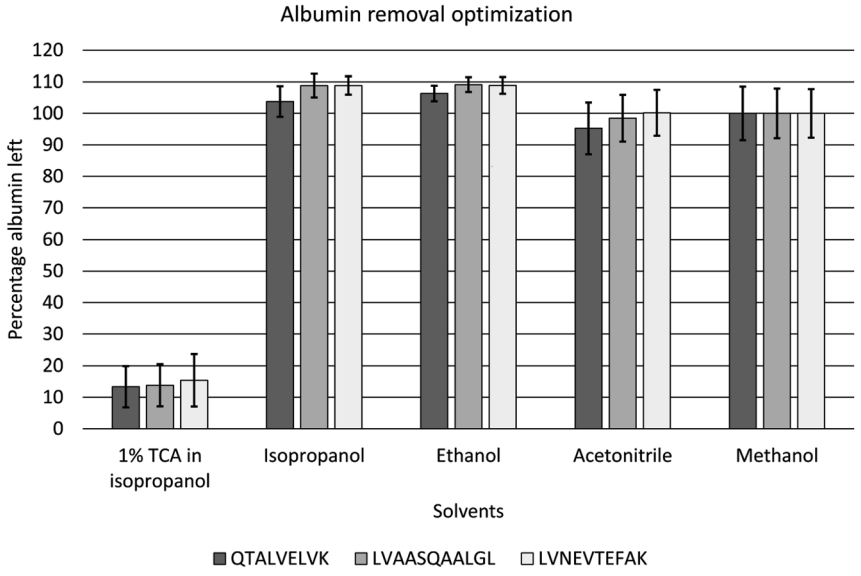

Figure 2: Albumin removal potential of different solvents. Percentages of remaining albumin in the protein pellet are shown for the albumin tryptic peptides QTALVELVK, LVAASQAALGL and LVNEVTEFAK as an average of three measurements. Methanol was used as the reference.

peak area ratio was 1.02 for both signature peptides, confirming the stock concentration of 1.00 mg/mL.

Method validation and comparison

All individual validation results are included in the Supplementary Material (Tables S1–S19 and Figures S1–S8). A summary is shown in Table 2 for all three signature peptides. The calibration curves were linear across the calibration range of 200–20,000 ng/mL with correlation coefficients (R^2) >0.99. The method performed well for all three signature peptides in terms of carry-over and inter-day (n=2 on 10 different days) and intra-day (n=10 on one day) imprecision, both for low endogenous serum SHBG, (average of 1,409 ng/mL) after spiking with an additional 5,000 or 12,000 ng/mL SHBG and after ten-fold dilution with proxy matrix of a sample spiked at 50,000 ng/mL. The lower limit of the measuring interval was very close to the lowest calibration level at 282 ng/mL, 299 ng/mL and 293 ng/mL for TSSSFEVR, IALGGLLFPASNLR, and LPLVPALDGCLR, respectively. Mean spike recovery, as a measure for method accuracy, ranged from 93 to 102%. The WHO SHBG standard had a negative bias of up to 33% (n=2 on 5 days) for all signature peptides, indicating a discrepancy in SHBG content between the used reference standard and the WHO material. The equivalence of serum and heparin plasma was demonstrated, but for EDTA plasma the correlation is less favorable, especially for peptide LPLVPALDGCLR (Figures S3–S8). SHBG was sufficiently stable in all relevant solutions and in

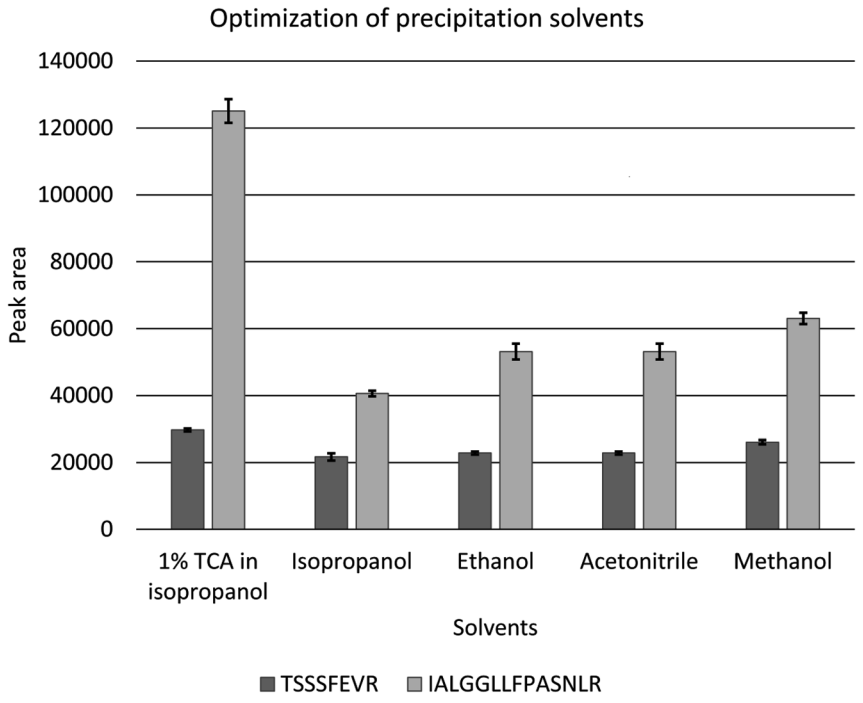

Figure 3: Effect of different precipitation solvents on the detection response of SHBG peptides. Peak areas are shown for TSSSFEVR and IALGGLLFPASNLR as an average of three measurements.

serum as well as in proxy matrix. The LC-MS/MS method and the Abbott Alinity immunoassay, both normalized towards the WHO standard, were compared by analyzing 79 clinical serum samples. Passing–Bablok regression data (Figure 4) and Bland–Altman plots (Figure 5) show that both methods correlate well (R^2>0.95), but that the LC-MS/MS results are on average 16–17% lower than the immunoassay results, which was consistent for all three signature peptides.

Discussion

Signature peptide selection and LC-MS/MS

The objective of this study was to develop an antibody-free method for the quantification of SHBG in human serum and plasma at the clinically relevant concentrations between 200 and 20,000 ng/mL, to avoid inconsistencies related to the variable quality of antibody-based reagents [23, 24]. Currently, the most promising approach to achieve this is so-called bottom-up LC-MS, in which the protein analyte is enzymatically digested and one or more unique signature peptides are quantified by LC-MS/MS as a measure for the intact protein. We preferred trypsin as the digestive enzyme because of its wide availability for a reasonable price and its ability to cleave protein chains after a lysine and an arginine [25], generating positively charged peptides which often have a good MS detection sensitivity. For a good coverage of the SHBG structure, we used the peptides TSSSFEVR and IALGGLLFPASNLR, from different parts of the molecule, for the quantification of SHBG. A third peptide, LPLVPALDGCLR, was selected because it contains two amino acids which are substituted in case of two nucleotide polymorphisms (SNPs) which have an altered degree of steroid binding. These SNPs are rs6258 (proline at position 156 in the SHBG sequence is replaced by a leucine) and rs145273466 (leucine at position 165 is changed to a methionine) [14, 15]. Only a wild-type form of SHBG will therefore show a signal for this third signature peptide. The three signature peptides showed sufficient separation from endogenous interferences in a runtime of 17 min which we consider reasonable for monitoring three tryptic peptides, and which is almost two-fold faster than previously reported [13]. With this method, over 60 patient samples can be analyzed per day. Some further optimization of the chromatographic conditions, such as a higher flow rate, will likely improve sample throughput, if this is needed for routine clinical use.

Sample pretreatment

To avoid immunochemical materials, we wanted to isolate SHBG from the sample using generically available reagents, preferably with a minimum co-extraction of endogenous proteins. In such a case, protein precipitation with an organic solvent is typically applied prior to digestion, but it has the potential drawback of co-precipitating almost the entire serum protein fraction. As an alternative, we tested

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

Peptide	TSSSFEVR		IALGGLLFPASNLR		LPLVPALDGCLR	
Validation item	**Maximum bias, %**	**Highest CV, %**	**Maximum bias, %**	**Highest CV, %**	**Maximum bias, %**	**Highest CV, %**
Method characteristics						
Linearity	+4.0	5.8	+2.5	1.6	+1.8	2.4
Imprecision inter-assay	NA	3.9	NA	1.9	NA	1.8
Imprecision intra-assay	NA	3.7	NA	1.8	NA	1.5
Intergrity of dilution	+2.7	1.3	+4.4	0.3	+3.7	0.3
LLMI	NA	11.9	NA	4.6	NA	4.6
Accuracy using WHO 08/266	−33.0	1.5	−30.3	1.2	−30.1	1.0
Stability of SHBG in proxy matrix						
Storage stability −80 °C (20 days)	+5.2	4.5	+4.6	0.5	−5.1	3.6
Freeze-thaw −80 °C (3 cycles)	+3.4	1.1	+4.6	2.8	+3.8	1.7
Bench-top stability (20 h)	−4.2	+5.8	−4.9	1.9	+4.1	1.7
Stability of SHBG in human serum						
Storage stability −20/−80 °C (14 days)	−7.7	4.1	+5.6	2.3	−4.3	2.9
Freeze-thaw −20/−80 °C (3 cycles)	−8.9	4.1	+4.3	3.2	−7.1	5.5
Bench-top stability (24 h)	−4.8	8.0	4.1	1.7	−5.7	2.0
Storage stability +4 °C (24 h)	−8.5	3.7	−5.4	12.6	−4.2	2.8
Stability of the signature peptides in final extract						
Autosampler 10 °C (95 h)	+3.6	2.7	+1.4	1.3	+1.5	1.0
Stability of SHBG in stock solution						
Frozen storage −80 °C (292 days + 5 F/T) (batch 1)	−1.2	0.8	−1.9	1.1	−2.1	0.6
Frozen storage −80 °C (21 days + 3 F/T) (batch 2)	−1.7	1.7	−0.4	1.7	−1.1	1.2
Frozen storage −80 °C (21 days + 1 F/T) (batch 2)	−4.4	0.7	−2.2	0.9	−2.9	0.8

Peptide	TSSSFEVR		IALGGLLFPASNLR		LPLVPALDGCLR	
Validation item	**Mean spike recovery, %**	**CV, %**	**Mean spike recovery, %**	**CV, %**	**Mean spike recovery, %**	**CV, %**
Method characteristics						
Spike recovery QC med	93	2.4	97	1.9	98	1.5
Spike recovery QC high	97	2.0	101	1.3	102	1.8

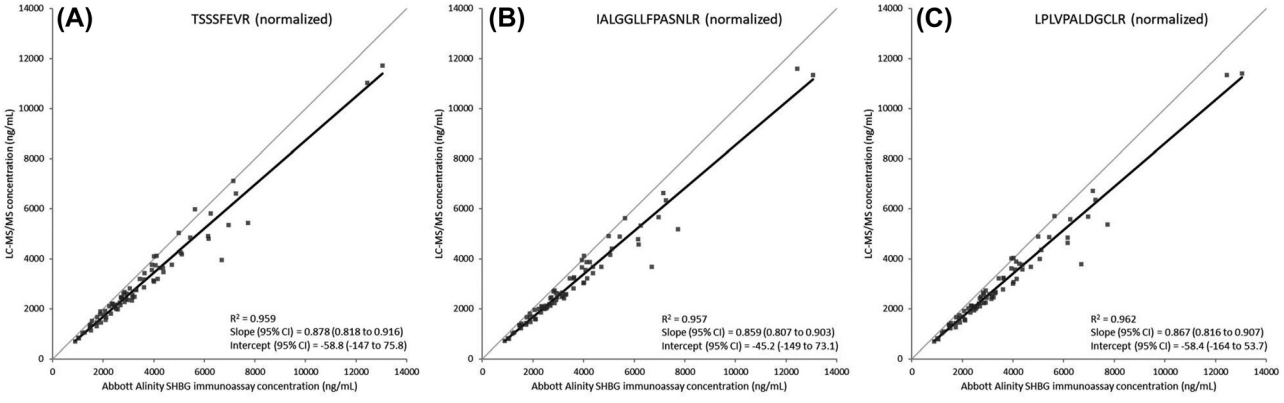

Figure 4: Passing–Bablok results for the comparison of the LC-MS/MS method for SHBG with the Abbott Alinity immunoassay for the peptides TSSSFEVR (A), IALGGLLFPASNLR (B) and LPLVPALDGCLR (C). The LC-MS/MS results were normalized using the WHO standard of SHBG.

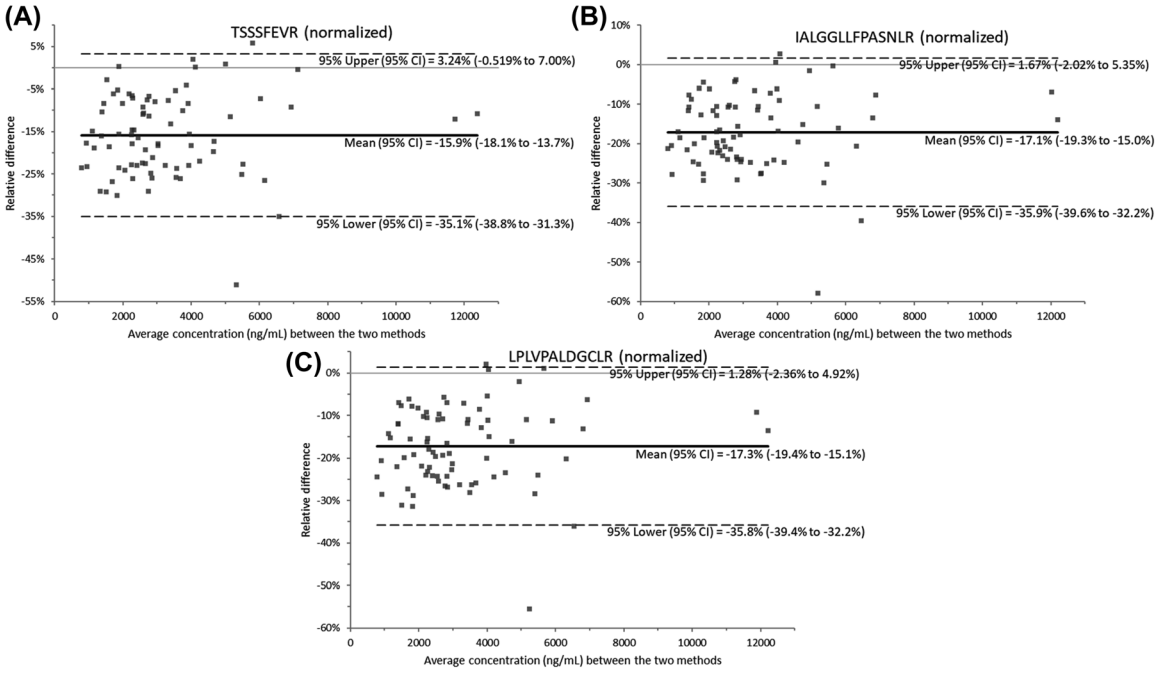

Figure 5: Bland–Altman plots for the comparison of the LC-MS/MS method for SHBG with the Abbott Alinity immunoassay for the peptides TSSSFEVR (A), IALGGLLFPASNLR (B) and LPLVPALDGCLR (C). The LC-MS/MS results were normalized using the WHO standard of SHBG.

the addition of 1% TCA in isopropanol, because TCA forms a complex with the most abundant serum protein, albumin, which has an excellent solubility in alcohols and therefore does not end up in the protein precipitate [26]. In our hands, precipitation with 1% TCA in isopropanol removed around 84% of albumin, which resulted in a much higher detection signal for the signature peptides. This is probably because albumin accounts for about 60% of total serum protein and its removal reduces the complexity of the precipitate and thus leads to fewer peptides in the subsequent digest and finally to fewer compounds competing with the SHBG signature peptides for ionization in the mass spectrometer. Complete SHBG digestion was found after 2 h and with a trypsin concentration of 150 µg/mL (enzyme:protein (E:P) ratio of 1:80), but for maximum robustness a digestion time of 3 h was selected. An E:P ratio of 1:20 is typically applied to untreated serum and plasma, based on a protein concentration of ~60 mg/mL in plasma and serum [27–29]. We lowered this to an E:P of 1:80 because of the depletion of most of the albumin. Since the peptide LPLVPALDGCLR is followed by another arginine in the SHBG protein, there is a chance that trypsin only cleaves after the second arginine, resulting in the undesired peptide LPLVPALDGCLRR, a phenomenon referred to as “missed cleavage”. Under the optimized conditions, complete digestion of SHBG to the signature peptide LPLVPALDGCLR was achieved, without any remaining missed cleavage. The reduction and alkylation procedures are normally applied to serum or plasma before digestion, but after the albumin depletion step the protein pellet did not dissolve in the reduction solution, so in our workflow they were performed after digestion. There is a theoretical risk that, in such a case, the digestion step is hindered by the still existing intramolecular disulfide bonds, but in our approach the cysteine-containing peptide LPLVPALDGCLR also showed complete digestion efficiency after 3 h. Altogether, the method allowed quantification down to 200 ng/mL in serum and plasma, which is sufficient to cover relevant endogenous concentrations.

Method validation and comparison

The method was successfully validated using the most recent guidelines in terms of: linearity, imprecision and stability, demonstrating its general applicability for SHBG quantification. For samples spiked with the WHO standard we found negative biases ranging from −33.0 to −30.1% for the three signature peptides. Because of the consistency for the different peptides, it is very likely that there is an actual difference in SHBG concentration between the reference standard that was used to prepare the calibration standards and the WHO standard, the latter containing about 30% less SHBG according to mass spectrometric analysis than its label

claim. A reason for the discrepancy could be that the concentration of the WHO standard, according to its certificate of analysis, was established by immunoassays and binding assays as the average of the results of 11 laboratories in five countries. Because of the fundamentally different analytical principles, it is not uncommon that differences are found between the results of LC-MS and binding assay platforms, even for relatively well-defined reference standards. For better comparison of results, it is therefore desirable to normalize the results towards the WHO standard. Heparin plasma and serum showed equivalent behavior as the sample matrix for all three signature peptides, but for peptide LPLVPALDGCLR nonlinearity was observed when comparing EDTA plasma with serum, the SHBG concentration found for this peptide being lower for EDTA plasma. Since the alanine within peptide LPLVPALDGCLR binds to a Ca^{2+} ion to stabilize the SHBG homodimer, EDTA might form a complex with peptide LPLVPALDGCLR by chelating the Ca^{2+} ion. This could eventually result in a lower detection response for this peptide, either because of a decreased digestion efficiency or some other effect. Reduced SHBG levels have also been reported in EDTA plasma when quantified by immunoassay [30, 31], which is attributed to its instability upon chelation of the calcium ion. The LC-MS/MS method presented here, on the other hand, does perform well for EDTA plasma when using the other two peptides for detection. A good correlation was found for SHBG between the LC-MS/MS method and the Abbott Alinity immunoassay with correlation coefficients above 0.95. Although both methods were normalized towards the WHO standard of SHBG, there was a small but consistent difference in the obtained concentrations, with the LC-MS/MS results for all three signature peptides being between 15.9 and 17.3% lower than for the immunoassay. This seems to imply that, relative to the spiked calibration standards, endogenous SHBG results in a lower response with LC-MS/MS and/or in a higher response for the immunoassay. It can be ruled out that the lower LC-MS/MS response is caused by a lower amount of the endogenous SHBG being precipitated or digested than of the spiked SHBG in the calibration standards. This was demonstrated with SHBG reference standard by spike recovery experiments, in which results for SHBG spiked to human serum and spiked to proxy matrix were similar. In addition, a parallelism test was performed whereby three different human serum samples with high concentrations of endogenous SHBG concentrations were diluted 2-, 4-, 8- and 16-fold with proxy matrix and, after correction for the dilution factor, good correspondence with undiluted concentrations was found for all signature peptides in all three serum samples. Finally, digestion time curves were recorded for four different human serum samples with endogenous SHBG concentrations. No difference in response was observed between 2 and 5 h of digestion for all three signature peptides, which indicates that endogenous SHBG was completely digested in the method with its digestion time of 3 h. The presence of mutated forms of SHBG as a root cause can also be excluded since all three signature peptides gave the same result for SHBG (Figures S1 and S2). Possibly, the immunoassay signal for endogenous SHBG is slightly higher than for the same concentration of spiked SHBG standard because of e.g. different binding characteristics, or other protein forms might occur in the serum samples that show some binding in the immunoassay, but do not contain the signature peptide sequences and hence give no signal in the LC-MS/MS assay. Altogether, this shows the advantage of LC-MS/MS, which is typically better able to provide an analyte-specific response and to distinguish different closely related proteins, while for immunoassays it often remains unclear what the result exactly represents and how the potentially varying quality of reagents impacts the detection signal [32–35].

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