The Influence of Proteoforms: Assessing the Accuracy of Total VDBP Quantification by Proteolysis and LC-MS/MS

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Abbreviations:

25(OH)D: 25-hydroxyvitamin D
AAA: amino acid analysis
CV: coefficient of variation
DDA: data-dependent acquisition
DTT: dithiothreitol
ETHcD: electron-transfer/higher-energy collisional dissociation
HCD: higher-energy collisional dissociation
HCl: hydrochloric acid
LC: liquid chromatography
LC-MS/MS: liquid chromatography-tandem mass spectrometry
MRM: multiple reaction monitoring
MS: mass spectrometry
NIH-ODS: National Institutes of Health-Office of Dietary Supplements
NIST: National Institute of Standards and Technology
PPM: parts per million
pVDBP: purified vitamin D-binding protein
QQQ: triple quadrupole
SIL: stable isotope label
SI: International System of Units
SRM: Standard Reference Material®
TCEP: Tris(2-carboxyethyl)phosphine hydrochloride
TFE: 2,2,2-trifluoroethanol
UW: University of Washington
VDBP: vitamin D-binding protein
v/v: volume fraction

Abstract (250 words)

Objectives: Vitamin D-binding protein (VDBP), a serum transport protein for 25-hydroxyvitamin D [25(OH)D], has three common proteoforms which have co-localized amino acid variations and glycosylation. A monoclonal immunoassay was found to differentially detect VDBP proteoforms and methods using liquid chromatography-tandem mass spectrometry (LC-MS/MS) might be able to overcome this limitation. Previously developed multiple reaction monitoring LC-MS/MS methods for total VDBP quantification represent an opportunity to probe the potential effects of proteoforms on proteolysis, instrument response and quantification accuracy.

Methods: VDBP was purified from homozygous human donors and quantified using proteolysis or acid hydrolysis and LC-MS/MS. An interlaboratory comparison was performed using pooled human plasma [Standard Reference Material® 1950 (SRM 1950) Metabolites in Frozen Human Plasma] and analyses with different LC-MS/MS methods in two laboratories.

Results: Several shared peptides from purified proteoforms were found to give reproducible concentrations [≤ 2.7% coefficient of variation (CV)] and linear instrument responses (R2 ≥ 0.9971) when added to human serum. Total VDBP concentrations from proteolysis or amino acid analysis (AAA) of purified proteoforms had ≤ 1.92% CV. SRM 1950, containing multiple proteoforms, quantified in two laboratories resulted in total VDBP concentrations with 7.05% CV.

Conclusions: VDBP proteoforms were not found to cause bias during quantification by LC-MS/MS, thus demonstrating that a family of proteins can be accurately quantified using shared peptides. A reference value was assigned for total VDBP in SRM 1950, which may be used to

standardize methods and improve the accuracy of VDBP quantification in research and clinical samples.

Introduction

VDBP is the primary transporter of vitamin D metabolites to target tissues. There is a recent interest in calculating the unbound or bioavailable fraction of 25-hydroxyvitamin D [25(OH)D], the metabolite typically measured to assess vitamin D status [1]. However, since the majority of vitamin D metabolites are bound to VDBP, an accurate measurement of the VDBP concentration is needed to accurately calculate the concentration of unbound 25-(OH)D [2]. Additionally, determining the amounts of bioavailable 25-(OH)D and VDBP are important when concentration changes are expected such as during pregnancy, trauma, sepsis and inflammatory diseases [3].

We have previously developed multiple reaction monitoring (MRM) LC-MS/MS methods in our laboratories at the University of Washington (UW, Seattle, WA, US) and the National Institute of Standards and Technology (NIST, Gaithersburg, MD, US) for the quantification of VDBP in human serum [4, 5]. At NIST, a quantification method traceable to the International System of Units (SI) was developed in collaboration with the National Institutes of Health Office of Dietary Supplements (NIH-ODS). This method was used to evaluate Standard Reference Material® (SRM) 1949 Frozen Human Prenatal Serum [5] and SRM 1950 [6]. Total VDBP concentrations measured had good repeatability between measurements. However, the reproducibility of quantification was not compared between different methods or laboratories in those studies.

In addition, the SRMs were prepared from donated serum or plasma and were found to contain multiple VDBP proteoforms, which include co-localized changes in amino acids and glycosylation. These proteoforms were previously shown to result in bias in an assay using

monoclonal antibodies [4, 7]. The proteoforms were assumed to result in equivalent tryptic proteolysis and instrument response during LC-MS/MS quantification of total VDBP using the shared peptides, but they were not validated in this manner.

To investigate the potential for method bias due to proteoforms, VDBP was purified from homozygous donors for further evaluation. The results demonstrate that a family of protein proteoforms can be accurately quantified using surrogate peptides that the isoforms have in common. These data, along with data from an interlaboratory comparison, were used in assigning a reference (non-certified) value [8] for total VDBP in SRM 1950.

Materials and Methods

SRM 1950 and SRM 1949 were from NIST [ww.nist.gov/srm] [9, 10]. Purified VDBP (pVDBP) from pooled sources was from Athens Research & Technology (Athens, GA, US). Solvents were purchased from Fisher Scientific (Pittsburg, PA, US) or Honeywell Burdick & Jackson (Charlotte, NC, US). Other chemicals used in the method were purchased from Sigma Aldrich (St. Louis, MO, US) unless otherwise noted. At NIST, LC-MS/MS using MRM or data-dependent acquisition (DDA) were performed on a triple quadrupole (QQQ, Agilent 6490, Santa Clara, CA, US) or Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, US), respectively.

VDBP Purification

Blood (100 mL) was collected from human volunteers with homozygous genotypes of VDBP (GC-1f, GC-1s or GC-2) as described by Haste *et al*. [11]. Additional information about the donors is available (www.ClinicalTrials.gov, NCT02258035). VDBP was enriched by affinity chromatography with immobilized anti-VDBP antibodies. For each isolation, 40 mL was incubated overnight with anti-VDBP Sepharose and eluted with 4 mol/L $MgCl_2$. Purification was performed using a Mono Q 5/50 GL column (Pharmacia GE Healthcare Life Sciences, now Cytiva, Marlborough, MA, US) developed with 0 to 500 mmol/L NaCl in 20 mmol/L Tris pH 7.40 (146 min, 0.25 mL/min). Fractions were pooled and buffer exchanged to 20 mmol/L Tris pH 7.40 using Amicon Ultra 3 kDa MWCO centrifugal filters (Millipore Sigma, Burlington, MA, US). The extracted protein (≈8 mL) was injected onto a Superose 6 HR 16/50 column (Cytiva) and eluted with 300 mmol/L NaCl and 20 mmol/L Tris pH 7.40. Fractions were pooled, concentrated and buffer exchanged to PBS using centrifugal ultrafiltration. The final concentration of pVDBP was ≈3 mg/mL. Homozygous pVDBP (and number of donors) was purified as follows: GC-1s (n=3), GC-2 (n=6), and GC-1f (n=1).

Identification of VDBP Proteoforms

Samples of pVDBP, from individual or pooled donor samples, were digested with trypsin as described previously [6]. A protein-denaturing mixture was prepared with 2,2,2-trifluoroethanol (TFE), 50 mmol/L Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) adjusted to pH 8.0, and 50 mmol/L Tris buffer at pH 8.3 (20:1:23.8 volume fraction, v/v).

Samples were prepared with pVDBP (≈5 μg) and 10 μL of the denaturing solution. Samples were heated (60 °C, 45 min) and alkylated with 20 mmol/L iodoacetamide (2 μL) in the dark (45 min). Tris buffer was added to achieve TFE concentrations of < 5% by volume. Five units (≈300 ng) of trypsin (sequencing grade, Promega, Madison, WI, US) was added to each sample vial and proteolysis was performed (19 h, 37 °C). Proteolysis was stopped by adding trifluoroacetic acid (0.5% of the total volume).

Samples of proteolyzed pVDBP were analyzed using LC-MS/MS (see Supplemental Table S1). The mass spectrometer was set with positive polarity for DDA (topN, 3 s cycle time) with a dynamic exclusion of 60 s (10 ppm error). Fragmentation was performed using higher-energy collisional dissociation (HCD) or electron-transfer/higher-energy collisional dissociation (EThcD) at a normalized collision energy of 32. Peptides were identified from MS/MS spectra using Sequest HT (Proteome Discoverer 2.3) versus matches to theoretical spectra from the 2022 UniProtKB human database [12].

Quantification by Amino Acid Analysis

AAA was performed on pVDBP and peptides as described elsewhere [6, 13, 14]. Briefly, external calibrants containing six amino acids and their corresponding stable isotope labeled (SIL) forms were prepared in parallel with samples. Labeled amino acids were obtained from Cambridge Isotope Laboratories (Tewksbury, MA). Information about the unlabeled amino acid is provided in Table S2. Glass vials containing external calibrants and samples (spiked with labeled amino acids) were placed into Teflon vessels. Hydrochloric acid (HCl) was added to the bottom and the vessels were tightly sealed. Gas-phase hydrolysis was performed in a chemical

fume hood (120 °C, 48 h). Vessels were cooled to room temperature before opening to avoid exposure to HCl vapor. HCl condensates were removed from hydrolyzed samples using vacuum centrifugation. The samples were solubilized in 0.1% formic acid and injected onto a Primesep 100 column (SIELC Technologies, Wheeling, IL, US, Table S3) prior to MRM (Table S4).

Quantification of Peptides

Stock solutions of VDBP unlabeled and SIL peptides ELPEHTVK, TSALSAK, VLEPTLK, and YTFELSR [GenScript (Piscataway, NJ, US), Biomatik (Wilmington, DE, US) or EZBiolab (Carmel, IN, US)] were solubilized in 50 mmol/L Tris buffer at pH 8.3 and peptide concentrations were determined by AAA. See Figure S1 for the amino acid sequence of VDBP. Five external calibrants (non-matrix matched) were prepared by diluting unlabeled and SIL peptide stocks in 0.1% formic acid in water (v/v). An aliquot of the SIL peptide mixture was added to each proteolyzed pVDBP sample (1.2:3 v/v). Samples were prepared and analyzed over the course of several months by LC-MS/MS with MRM (Table S5, Figure S2) as previously described [6].

Samples were also prepared with pVDBP (concentrations determined by AAA) gravimetrically spiked into human or chicken serum at different concentrations. Spiked samples were proteolyzed with trypsin and analyzed by LC-MS/MS at UW as described below. The mean instrument response ratio of VLEPTLK or ELPEHTVK and the corresponding SIL peptide was plotted versus the peptide concentration.

Interlaboratory Comparison

Seven vials of pooled human plasma (SRM 1950) were randomly selected and quantified at NIST. Two aliquots (10 μL) from each vial were prepared and analyzed as described above with modifications as follows: the protein denaturing solution was prepared with 20:8.8:16 (v/v) of TFE, TCEP and Tris buffer, the iodoacetamide concentration was 300 mmol/L, and 350 units of trypsin was used. The SIL peptide mixture was added to samples following proteolysis (1.3:5 v/v). Two MRM transitions for TSALSAK and VLEPTLK were monitored during LC-MS/MS (Table S5).

Seven vials of SRM 1950 were randomly selected and sent to UW for quantification as previously published [4]. Two aliquots (10 μL) from each vial were spiked with SIL peptides (70 μL), denatured with TFE (70 μL), reduced with 0.5 mol/L dithiothreitol (DTT, 2 μL, 1 h at 65 ºC with agitation), and alkylated in the dark (30 min) with 0.5 mol/L iodoacetamide (8 μL). DTT (2 μL) was added to quench any remaining iodoacetamide. Ammonium bicarbonate (28 mmol/L) was added to achieve TFE <5% by volume. TPCK treated trypsin (Worthington Biochemicals, NJ, US) was used for proteolysis (30 min, 37º C with agitation). Digestions were stopped by adding formic acid. ELPEHTVK and VLEPTLK were quantified using an Acuity UPLC coupled to a Xevo TQMS QQQ (Waters, MA, US). External matrix-matched calibrants were SIL peptides in pooled human serum, with endogenous VDBP, diluted with chicken serum to different concentrations. Reference calibrators (pVDBP prepared in chicken serum with concentrations determined by AAA) were used to assign concentrations of endogenous VDBP.

Data Analysis

Amino acid, peptide, and protein samples were prepared gravimetrically at NIST and analyzed by LC-MS/MS in triplicate. At NIST, MassHunter Qualitative Analysis B.07.00 (Agilent) was used to determine peptide MRM peak areas. Calibration curves were plotted from the instrument response ratio of each peptide and the corresponding SIL form versus the ratio of the masses. Data were fit with a linear regression in Microsoft Excel. For AAA, protein and peptide concentrations were calculated from the mean concentrations of six amino acids. For experiments using trypsin proteolysis, the total concentration of VDBP was calculated from the concentrations of TSALSAK and VLEPTLK.

Reference Value Assignment

The reference value for VDBP in SRM 1950 was the mean concentration of the data measured in the interlaboratory comparison. The error was calculated from $U = ku_c$, where u_c is the combined standard uncertainty and k is a coverage factor (k=2.0 for these data) corresponding to approximately 95% confidence [15]. The uncertainty incorporates the standard error of the values measured with an additional component of uncertainty due to calibrant purity, consistent with the ISO Guide [16].

Results and Discussion

Proteoform Identification

Variations in the amino acid sequence of VDBP result in changes to one tryptic peptide (Figure S1). The most common proteoforms (and tryptic peptides) are designated as follows: GC-1s (LPEATPTELAK), GC-1f (LPDATPTELAK), or GC-2 (LPDATPK). The VDBP genotypes of the donors selected for these analyses were previously identified [11] and are homozygous for one of the three common forms. In this work, proteolyzed pVDBP from the donors was analyzed by LC-MS/MS with DDA. Because neutral losses of the sugars dominate collision-induced dissociation spectra, ETD and EThcD were used to increase fragmentation of the peptide backbone. The resulting spectra, searched versus theoretical spectra from a human protein database containing the three VDBP proteoforms, verified the presence of the expected proteoform-specific peptide for each donor (Table S6).

Sites of O-linked glycosylation in VDBP are located on the tryptic peptide containing the amino acid variations (Figure S1). GC-1f and GC-1s peptides both have two threonines (T) which may be glycosylated. GC-2 has one less potential site of glycosylation due to the substitution of one T for lysine (K). Donor pVDBP analyzed by ETD or ETHcD were found to include partial glycosylation of GC-1f and GC-1s with mono- (GalNAc), di- (GalNAc-Gal) or trisaccharides (GalNAc-Gal-NeuNAc) which is consistent with previous studies [6, 17-20]. GC-2 samples were not glycosylated in these samples which differed from other reports showing the presence of a disaccharide [18-20] and may be a result of differences in donors, sample preparation or instrumentation. Proteoforms in SRM 1950 were previously identified and also

include all three common amino acid sequences with and without the presence of glycosylation [6].

Due to the lack of commercially available glycopeptide standards, relative amounts of each glycopeptide were then determined using the ratio of the LC-MS ion abundances of the glycosylated and unglycosylated tryptic peptides (Table 1). Table S6 shows additional peptides with missed cleavages which were also found. About 8.6% of the ion abundance of the GC-1f sample was due to the full length trisaccharide while the GC-1s samples were calculated to have between 14.4% to 34.0% of the trisaccharide. Less than 5% of the glycopeptide ion abundances in all samples were due to di- or monosaccharides. The relative amounts of glycosylated GC-1s and GC-1f found here are consistent with previous amounts determined from mass spectrometry analyses of intact pVDBP [18-20]. Although the exact quantities are unknown, these data show the commonly observed proteoforms expected in clinical samples are present at different concentrations which may affect quantification of VDBP.

Reproducibility of proteolysis

Peptides shared by the common proteoforms [ELPEHTVK, TSALSAK, VLEPTLK, and YTFELSR] were quantified in proteolyzed pVDBP from homozygous donors. Analyses of pVDBP from pooled human donors and endogenous VDBP in plasma (SRM 1950) or serum (SRM 1949, non-pregnant) were also performed. ELPEHTVK and VLEPTLK (normalized to TSALSAK) concentrations were found to be 0.960 (2.7 % CV) and 0.966 (1.3 % CV), respectively, between all 6 samples (Figure 1). These data show that VDBP proteolysis resulted in similar release of the peptides and bias is not apparent between the different proteoforms or

matrices expected for clinical samples. The time course of proteolysis performed also indicated complete release of these peptides at 20 hours of proteolysis (Figure S3A).

Peptides ELPEHTVK and YTFELSR showed some instability during the long proteolysis time used at NIST (Figure 3B) and so were not selected for use in quantification. This likely resulted in the lower mean concentration for YTFELSR (0.880 with 9.0 % CV) measured for all 6 samples (Figure 1). The slower release of YTFELSR from VDBP purified from serum may be due to a change in the tertiary structure affecting cleavage between the two C-terminal arginines. ELPEHTVK may also undergo a decrease in concentration over time due to N-terminal pyroglutamate formation [21].

Reproducibility of LC-MS/MS

AAA was used to determine the absolute total protein concentration of the pVDBP proteoforms from homozygous donors. Because AAA lacks specificity, analysis of VDBP present in serum or plasma were not performed. Intact pVDBP samples were hydrolyzed in triplicate on three different days and were quantified using external calibrants. Concentrations of pVDBP proteoforms (Table 2) were found to vary between 5.08 μmol/kg and 10.86 μmol/kg. The concentrations determined for replicate measurements of each sample had good agreement with ≤ 2.66% CV.

Total pVDBP concentrations from the homozygous donors were then determined from trypsin proteolysis and MRM LC-MS/MS of TSALSAK and VLEPTLK. The concentrations of the individual peptides determined by AAA are shown in Figure S4. Total pVDBP was found to be between 5.09 μmol/kg and 10.86 μmol/kg and have CVs ≤ 6.64% (Table 2). The slightly

higher CVs for LC-MS/MS of the peptides compared to AAA of the intact protein are likely a result of adding the labeled peptides following the proteolysis and the additional steps in the method affecting quantification accuracy. Concentrations determined by both AAA and LC-MS/MS of the peptides had good agreement with ≤ 1.92% CV for each of the proteoforms. These data demonstrate that tryptic digestion and quantification of total pVDBP from the shared peptides result in reproducible results regardless of the proteoform analyzed. Therefore, accurate quantification of total pVDBP from heterozygous donors or other pooled sources is possible using LC-MS/MS.

Quantification reproducibility using external calibrants

The reproducibility of quantification using external calibrants was determined following gravimetric addition of pVDBP from homozygous donors to pooled human serum at different concentrations. The mean instrument response ratio was then calculated for two peptides versus the corresponding SIL form and plotted versus the concentration (Figure 2). The data for each of the proteoforms, GC-1f, GC-1s and GC-2, resulted in linear fits with $R^2 \geq 0.9971$. The slopes and y-intercepts were also similar between the different proteoform samples with mean values of 0.00523 (5.67% CV) and 1.21 (1.72% CV), respectively. Because the original pooled serum sample had VDBP present prior to addition of the proteoforms, the linear fits for each set of data have y-intercepts above zero. Similar data are shown in Figure S5 for the pVDBP proteoforms added to chicken serum. These data demonstrate that external calibration using shared peptides results in reproducible VDBP concentrations regardless of the proteoform present.

Interlaboratory comparison

SRM 1950, containing multiple proteoforms, was used to investigation the reproducibility of total VDBP quantification between two laboratories using different sample preparation and quantification methods. At NIST, two aliquots from seven vials of SRM 1950 were proteolyzed with trypsin and quantified using LC-MS/MS in triplicate on three different days. Extracted MRM chromatograms and repeatability of LC-MS/MS runs are shown in Figures S2 and S6, respectively. Following sample proteolysis on different days, the concentrations of TSALSAK and VLEPTLK were similar with 1.22 % CV (Table S7) which was well below the 15% recommended limit [22, 23]; therefore, the mean value of these two peptides were used to calculate total VDBP concentrations. The mean of two aliquots (from the same vial) proteolyzed and quantified on the same day was used to calculate the total VDBP concentration and ranged from 2.99 to 3.45 μmol/kg (Table 3). The % CVs between aliquots from the same vial were ≤ 3.81 (Figure S7, vial 4, day 1) indicating homogeneity of the material. The % CVs for mean VDBP concentrations from the same vial (three days, n=6) or day (seven vials, n=14) were ≤ 5.64 or ≤ 3.87, respectively, indicating good repeatability of the method. For all 14 aliquots measured in triplicate on different days, the mean VDBP concentration was 3.17 μmol/kg (3.44% CV). Concentrations of VDBP in SRM 1950 were also found to be stable (3.16 % CV) at the recommended storage temperature (< -60 ºC) over a period of about 8 months (Table S8) which show the reproducibility of quantification over time.

The mean VDBP concentrations for two aliquots from each vial of SRM 1950 proteolyzed and measured by LC-MS/MS at UW is shown in Table 4. Units were initially measured in mg/L (Table S9) and were converted to μmol/kg (Table 4). Mean VDBP

concentrations of two aliquots from the same vial ranged between 3.35 to 3.68 μmol/kg. The % CV between the aliquots from the same vial was ≤ 4.42 (Figure S7, vial 3, day 1) which indicates homogeneity of VDBP in the material. The % CVs for mean VDBP concentrations from the same vial (three days, n=6) or over three days (seven vials, n=14) were ≤ 3.23 or ≤ 2.80, respectively, indicating good repeatability of the method at UW. For all 14 aliquots digested and quantified in triplicate on different days, the mean VDBP concentration was 3.50 μmol/kg (2.68% CV).

When comparing data collected between the two laboratories, the VDBP concentrations determined at NIST were lower than those measured at UW despite both having validated methods for proteolysis and LC-MS/MS. Samples were prepared and quantified using common approaches; however, differences between the laboratories which include the MS instrument and the calibration system, possible differences in proteolysis efficiency (trypsin types, digestion buffers) or timing of the SIL peptide addition [24] may contribute to variation in the final VDBP concentration measured. Despite differences between the laboratories, VDBP concentrations were found to have acceptable agreement with 7.05% CV.

The data presented show that quantification of VDBP is not significantly affected by the presence of different proteoforms and reproducible measurements were made between laboratories. Therefore, a reference (non-certified) value was assigned for VDBP in SRM 1950. The final values, calculated in two different units, were 3.33 μmol/kg ± 0.33 μmol/kg and 175 mg/L ± 18 mg/L (Table 5). The uncertainties for the concentrations in mg/L incorporate components related to estimation of the plasma density and the molecular mass which was conservatively modeled as a uniform distribution with width equal to the difference between the highest and lowest molecular masses of the proteoforms identified in SRM 1950 [6].

Conclusions

Clinical samples may have different proteoforms present [25-28], some of which have been found to have a significant effect on biochemical and immunogenic assays [4, 29-32]. While information on sequence variations and post-translational modifications is available for many proteins [12], differences in enzymatic proteolysis or instrument response between proteoforms are largely not considered in quantitative LC-MS/MS methods. As part of the joint mission of NIST and NIH-ODS to develop reference procedures and materials to improve the measurement of vitamin D, the reproducibility of quantification and potential sources of bias were investigated for VDBP purified from homozygous human donors (GC-1f, GC-1s, or GC-2) or present in plasma (SRM 1950).

Despite previous work showing difficulty in obtaining reproducible results from assays for other proteins with multiple proteoforms present [29, 30, 33], the proteolysis, MS response and quantification of VDBP do not appear to be significantly affected by the presence of different proteoforms. VDBP quantification in two laboratories using different, but common LC-MS/MS approaches gave similar results (7.05% CV). Therefore, a reference (non-certified) value for total VDBP in plasma was assigned to SRM 1950. The results demonstrate that a family of protein proteoforms can be accurately quantified using the shared surrogate peptides. This approach may also be used to validate quantification methods for other protein proteoforms. Rigorously validated methods, standardized with reference materials, are important in the absolute quantification of protein families in research and clinical samples.

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Tables

Table 1: Relative amounts of peptides containing O-linked glycosylation (mono-, di-, or trisaccharide) from pVDBP from human donors. Values were calculated from ratio of the abundances of the glycosylated to unglycosylated isoform-specific tryptic peptides identified during LC-MS/MS with DDA. Glycosylated peptides were not identified in the GC-2 samples.

Sample	Proteoform	Relative % of Saccharide		
		Mono	Di	Tri
1	GC-1s	1.2	4.9	34.0
3	GC-1s	1.7	1.4	12.7
4	GC-1s	2.0	1.8	14.4
10	GC-1f	1.9	1.0	8.6

Table 2: Concentrations of pVDBP from homozygous donor serum using AAA following acid hydrolysis of the protein and LC-MS/MS with MRM following tryptic proteolysis. Mean values and % CVs for each method were determined from quantification on three different days. The mean concentrations and % CVs between the two methods (in bold) are also shown for each proteoform.

Sample	Proteoform	AAA (μmol/kg)	%CV	LC-MS/MS (μmol/kg)	%CV	Mean (μmol/kg)	%CV
1	GC-1s	7.29	1.53	7.29	1.77	**7.29**	**0.01**
3	GC-1s	6.56	1.38	6.52	2.53	**6.54**	**0.43**
4	GC-1s	5.08	2.56	5.09	2.03	**5.08**	**0.10**
5	GC-2	7.72	1.43	7.52	6.64	**7.62**	**1.92**
6	GC-2	7.33	1.08	7.35	2.35	**7.34**	**0.18**
7	GC-2	10.86	1.58	10.86	3.24	**10.86**	**0.03**
8	GC-2	6.32	2.66	6.17	2.79	**6.25**	**1.73**
9	GC-2	9.41	1.40	9.59	4.29	**9.50**	**1.33**
12	GC-2	6.91	1.73	6.94	2.77	**6.92**	**0.21**
10	GC-1f	7.01	1.62	6.97	3.84	**6.99**	**0.43**

Table 3: VDBP concentrations (μmol/kg) in SRM 1950 measured at NIST. The mean concentration from two aliquots prepared and quantified on the same day is shown for each vial. Data are shown for aliquots prepared on three different days. Mean values and % CVs for each vial (n=6), day (n=14) and all measurements (in bold, n=42) are also shown.

VIAL #	DAY 1	DAY 2	DAY 3	VIAL MEAN	%CV
1	3.12	3.15	3.12	3.13	0.73
2	3.27	3.15	3.14	3.19	2.59
3	3.11	3.20	3.09	3.13	1.95
4	3.27	3.20	3.05	3.17	3.63
5	3.45	3.25	3.05	3.25	5.64
6	3.18	3.19	2.99	3.12	3.26
7	3.31	3.15	3.05	3.17	3.81
DAILY MEAN	3.25	3.18	3.07	**3.17**	
%CV	3.87	1.26	1.66	**3.44**	

Table 4. VDBP concentrations (μmol/kg) in SRM 1950 analyzed at UW. The mean concentration from two aliquots prepared and quantified on the same day is shown for each vial. Data are shown for aliquots prepared on three different days. Mean values and % CVs for each vial (n=6), day (n=14) and all measurements (in bold, n=42) are also shown.

VIAL #	DAY 1	DAY 2	DAY 3	VIAL MEAN	%CV
1	3.49	3.68	3.58	3.58	3.23
2	3.44	3.56	3.52	3.51	2.40
3	3.52	3.51	3.45	3.49	2.98
4	3.35	3.47	3.51	3.44	3.09
5	3.46	3.52	3.50	3.49	2.24
6	3.50	3.51	3.48	3.50	2.53
7	3.46	3.45	3.52	3.47	1.38
DAILY MEAN	3.46	3.53	3.51	**3.50**	
%CV	2.51	2.80	2.53	**2.68**	

Table 5: NIST reference values for VDBP concentrations in SRM 1950.

Concentration	Units
3.33 ± 0.33	μmol/kg
175 ± 18	mg/L

Figures

Figure 1: Comparison of normalized pVDBP peptide concentrations determined by LC-MS/MS at NIST for different proteoforms (GC-1f, GC-1s, or GC-2) prepared in buffer. Peptide concentrations are shown as a ratio to TSALSAK. For comparison, pooled samples containing all 3 genotypes are shown which include pVDBP (prepared in buffer), plasma (SRM 1950), serum (SRM 1949, non-pregnant). Error bars show ± one standard deviation between samples which were prepared in the following numbers: 1f (n=3), 1s (n=9), 2 (n=18), pVDBP (n=3), plasma (n=5), serum (n=3).

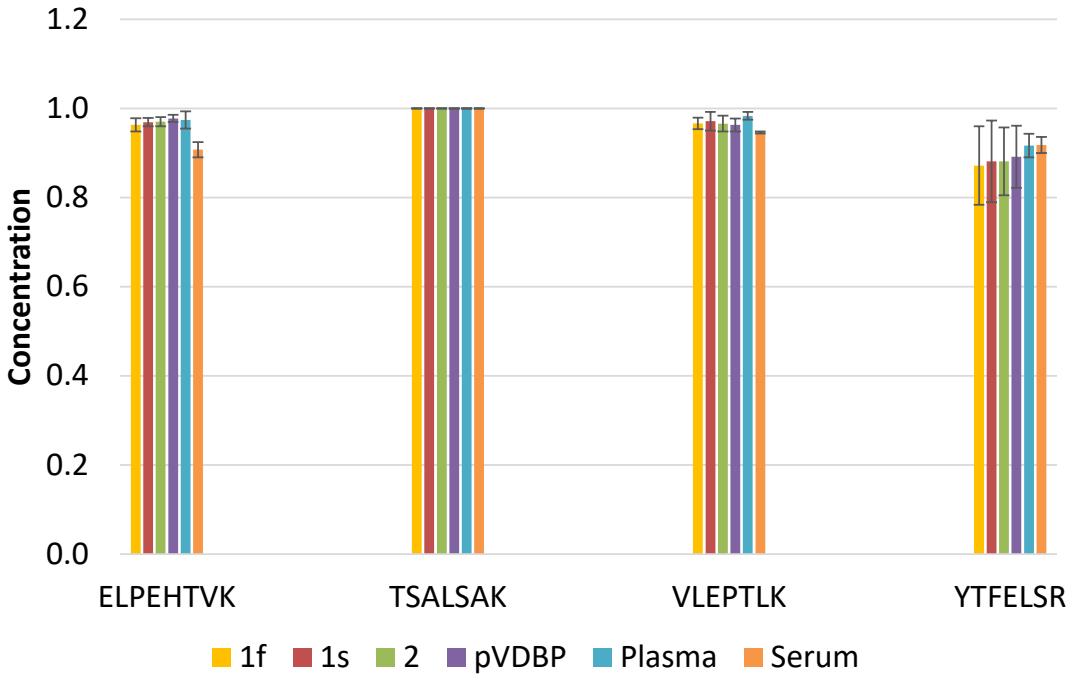

Figure 2: Homozygous pVDBP purified from donors gravimetrically added to human serum at different concentrations. The mean response ratio of ELPEHTVK and VLEPTLK to their corresponding SIL peptide is shown versus the concentration.