Development and Validation of a Novel LC-MS/MS Based Proteomics Method for Quantitation of Retinol Binding Protein 4 (RBP4) and Transthyretin (TTR)

Aprajita S. Yadav[1], Katya B. Rubinow[2], Alex Zelter[3], Aurora K. Authement[1], Bryan R. Kestenbaum[4], John K. Amory[5], Nina Isoherranen[1]

1. Department of Pharmaceutics, School of Pharmacy, University of Washington, Seattle, Washington, USA
2. Division of Metabolism, Endocrinology and Nutrition, Department of Medicine, University of Washington, Seattle, Washington, USA
3. Department of Genome Sciences, School of Medicine, University of Washington, Seattle, Washington, USA
4. Division of Nephrology, Kidney Research Institute, Department of Medicine, University of Washington, Seattle, Washington, USA.
5. Division of Internal Medicine, Department of Medicine, University of Washington, Seattle, Washington, USA

Corresponding Author:
Nina Isoherranen
ni2@uw.edu
1959 Pacific Ave, HSB H-272N, Seattle, WA 98195
(206)543-0829

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List of abbreviations, in order cited:
RBP4: retinol binding protein 4, TTR: transthyretin, ELISA: enzyme linked immunoassay, LC-MS/MS: liquid chromatograph-tandem mass spectrometry, SIL: stable isotope labeled, HSA: human serum albumin, BSA: bovine serum albumin, FBS: fetal bovine serum, DTT: dithiothreitol, TFA: trifluoroacetic acid, QC: quality control, LLOQ: lower limit of quantitation, LQC: low QC, MQC: medium QC, HQC: high QC, CKD: chronic kidney disease, IQR: interquartile range

Abstract:

Background: Retinol binding protein 4 (RBP4) is the plasma carrier of retinol that complexes with transthyretin (TTR). RBP4 is a potential biomarker of cardiometabolic disease. However, RBP4 quantitation has relied on immunoassays and western blots without concurrent measurement of retinol and TTR. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for simultaneous absolute quantitation of serum and plasma RBP4 and TTR is needed to advance the understanding of RBP4 and TTR as biomarkers.

Methods: Surrogate peptides with reproducible, linear LC-MS/MS response were selected for RBP4 and TTR quantitation. Purified proteins were used as quantitation standards and heavy labelled peptides as internal standards. Matrix effects were evaluated for quantitation. The method was validated using pooled human serum and applied to measure inter- and intra-individual variability in RBP4 and TTR concentrations in healthy individuals and in patients with diabetic kidney disease.

Results: The quantitation was linear for the clinically relevant concentration ranges of RBP4 (0.5 – 6 μM) and TTR (5.8 – 69 μM). The assay inter-day variability was <12% and precision within 5%. The inter-individual variability for RBP4 and TTR concentrations was 18 – 26%, while intra-individual variability was similar to assay variability. RBP4 and TTR quantitation correlated with commercially available ELISA assays.

Conclusions: The developed LC-MS/MS method allows simultaneous absolute quantitation of RBP4 and TTR in human serum and plasma with reduced samples

volumes. The method can be applied to clinical biomarker studies, for analysis of nutritional vitamin A status, and measurements of stoichiometry of RBP4, TTR and retinol in circulation.

Introduction:

Retinol binding protein 4 (RBP4) is the blood carrier of retinol (Vitamin A). RBP4 is a 21 kDa protein and is filtered by the kidney (1,2). To prevent filtration, RBP4 forms a complex with transthyretin (TTR) (1), a 55 kDa homotetramer that also binds thyroxine. Binding of RBP4 to TTR stabilizes TTR tetramer formation.(3,4) The complex formation prevents filtration of RBP4 and increases the half-life of RBP4.(5) When glomerular filtration is impaired, plasma RBP4 concentrations are increased while TTR concentrations are unaffected (6). In the absence of nutritional deficiency, the plasma concentrations of retinol (7), RBP4 (2), and TTR (4) have little variability between individuals (8). Data on the intra-individual variability of RBP4 and TTR are, however, limited. Blood RBP4 concentrations are affected by dietary vitamin A status (9,10) as well as by disease processes. As such, there is considerable interest in RBP4 as a biomarker for disease progression (3,11,12). RBP4 has been suggested as a potential biomarker of diabetes progression and cardiovascular disease (13,14). During pregnancy, RBP4 concentrations decrease (15) but higher RBP4 has been proposed as a marker of development of preeclampsia and gestational diabetes (16,17). However, RBP4 is typically measured in isolation as a biomarker, and TTR concentrations along with the formation of RBP4-TTR complex are not considered in the biomarker data interpretation (12).

RBP4 and TTR concentrations have typically been measured by western blots or enzyme linked immunoassays (ELISA), which have shortcomings such as limited dynamic range, variability between assays, and interference from disease states, matrix effects, or anticoagulants (18,19). It has been suggested that RBP4 measurement

methodology may contribute to inconsistent clinical findings (12,19). Whether RBP4-TTR complex formation interferes with antibody-based assays is currently unknown. Contemporary TTR measurements focus on differentiating misfolded TTR through intact proteomics or targeting post-translational modifications rather than quantification of TTR. A specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to measure RBP4 and TTR addresses the analytical issues with current methods.

Accurate quantitation of RBP4 and its binding partners is critical in understanding their role as biomarkers or etiologic factors in metabolic and chronic diseases. LC-MS/MS based protein quantitation has been developed as an alternative strategy to immunoassays. Plasma proteomic analysis can, however, be confounded by the complexity of the human plasma proteome, abundance of albumin and IgG, and the presence of protease inhibitors which can affect tryptic digest (20,21). Additionally, since RBP4 and TTR are endogenous proteins, the lack of a blank matrix can be problematic.

To address these challenges, we developed a robust and sensitive method for quantifying RBP4 and TTR in human serum and plasma with stable isotope labeled (SIL) peptides as internal standards. Matrix effects were evaluated, and method comparability was assessed in a set of samples from patients with diabetic chronic kidney disease. We also aimed to establish inter- and intra-individual biological variability of RBP4 and TTR in serum and plasma by analyzing multiple matched serum and plasma samples from healthy individuals.

Methods

Complete list of chemicals and materials used is provided in the Supplemental Materials.

Identification of surrogate peptides and optimization of digestion

The amino acid sequences for human RBP4 and TTR in FASTA format were retrieved from UniProtKB database (P02753 and P02766, respectively). RBP4 and TTR were digested *in silico* using Skyline v22 (22) (Seattle, WA) to identify tryptic peptides. Peptides with 7-25 amino acids and predicted m/z range of 50 – 1500 were considered. Nonspecific peptides in human plasma were screened as previously described (23) and excluded. Precursor ions with 2 or 3 charges, fragmentation (b+ and y+ ions), declustering potential and collision energies were predicted in Skyline for method development.

Purified recombinant RBP4 and TTR (400 nM) were digested as previously described (23) to test peptide detection and fragmentation. The trypsin digestion time course (3, 5, 15, and 20 h) was evaluated similarly (23) in replicate (n=3) digestions of 20 µL sample of purified RBP4 (20 nM) from four vendors, purified TTR (200 nM), and serum diluted 100-fold with ammonium bicarbonate repeated on three different days.

Matrix effects and assay specificity were evaluated following 5 h trypsin digestion of RBP4 and TTR spiked into human serum albumin (HSA) and bovine serum albumin (BSA) matrices (0.6 mg/mL in 1% Ringer's in 100 mM ammonium bicarbonate), fetal bovine serum (FBS), mouse serum, and mouse plasma. Isotope labeled internal

standard peptides were added to the samples and peak area ratio of the peptides of interest quantified. The impact of reducing agent and detergent was also evaluated (**Supplemental Methods**).

LC-MS/MS method

Peptides were separated using an Aeris Peptide column (50 x 2.1 mm, 1.7 µm) with a SecurityGuard Ultra C18-peptide cartridge (Phenomenex, Torrance, CA) on an Agilent 1290 LC (Agilent, Santa Clara, CA) (23). Injection volume was 10 µL for RBP4 analysis and 3 µL for TTR analysis. The column oven was set to 40ºC. The mobile phases were water (A) and acetonitrile (B), both with 0.1% formic acid, at flow rate of 0.4 mL/min. Gradient elution was 3% B for 3.5 min, increased linearly to 40% B over 8.5 min, increased to 95% B over 0.5 min, and held at 95% B for 2.5 min before returning to starting conditions.

Peptides were detected using a SCIEX 5500 QTRAP MS (Sciex, Framingham, MA). Isotope labelled peptides were infused to optimize mass spectrometer parameters. The mass spectrometer parameters for the peptides analyzed are summarized in **Supplemental Table 2**. Three MS/MS transitions were selected for each quantitation peptide and summed peak area was used.

Digestion Protocol

40 µL of serum, diluted 100-fold with 100 mM ammonium bicarbonate, standard curves and quality control (QC) samples were aliquoted into 96-well PCR plates with 20 µL of 100 mM ammonium bicarbonate (pH 7.8), 8 µL 100 mM dithiothreitol (DTT), and 2 µL of 8 ng/µL yeast enolase as a process control. Samples were incubated for 20 min at room

temperature to reduce disulfide bonds before addition of 10 µL of 10% sodium deoxycholate in 100 mM ammonium bicarbonate. Proteins were denatured by incubation at 95ºC for 10 min in a ThermoMixer. Under yellow light, 8 µL of 200 mM iodoacetamide was added, and the samples were incubated for 20 min at room temperature. Samples were digested with trypsin at 37ºC for 5 h at 400 rpm in a ThermoMixer. Digestions were quenched with 40 µL of acetonitrile with 8% TFA containing internal standard peptides (25 nM FSGTWYAMAK[$^{13}C_6{}^{15}N_2$] and YWGVASF[$^{13}C_9{}^{15}N$]LQK, and 250 nM GSPAINVAVHVFR[$^{13}C_6{}^{15}N_4$]). The plate was centrifuged at 3,000*g* for 30 min at 4ºC, the orientation of the plate flipped 180º and centrifuged for another 30 min, and supernatants transferred to a new 96-well plate for LC-MS/MS analysis.

Validation

Validation was performed according to Food and Drug Administration guidance (24), including linearity, lower limit of quantification, precision, accuracy, stability of digested peptides, and carryover.

Signal linearity was first confirmed by serial dilution of digested purified protein, equivalent to starting at 80 µM for TTR and parallel serial dilutions of RBP4 starting at 6 and 4 µM. Instrument variance, including autosampler and detection variability, was assessed by calculating coefficient of variance (%CV) from twelve replicate injections. Enolase peptides were monitored as a process control (25).

Standard curves and QCs were prepared by spiking purified Bio-Rad RBP4 and TTR into 0.6 mg/mL BSA in 1% Ringer's in 100 mM ammonium bicarbonate. Standard

curves included 8 non-zero points (0.5 – 6 μM RBP4 and 5.8 – 69.3 μM TTR), with four QCs – lower limit of quantitation (LLOQ), low QC (LQC), medium QC (MQC), and high QC (HQC) at 0.6, 0.9, 1.8, 3.6 μM for RBP4 and 6.9, 10.4, 20.8, and 41.6 μM for TTR. A minimum of three QC samples per day were digested and analyzed across nine days to determine inter-day variance. Intra-day variance was calculated from four QC samples per concentration within a day and the mean intra-day variance from nine days is reported. Aliquots of a pooled mixture of serum from ten individuals were prepared and run in triplicate with every run as pooled QCs. Freeze-thaw and autosampler stability of digested peptides was determined by quantifying QC samples that were either frozen at – 20°C for two freeze-thaw cycles or kept at 4°C for 24 h, respectively.

Method comparison

There were two clinical cohorts from which human serum and plasma were obtained. The first was a subset of plasma samples (n=49) from the Seattle Kidney Study, a prospective cohort study of individuals with chronic kidney disease (CKD) (26). All patients provided written informed consent, and the study protocol was approved by the Institutional Review Board at the University of Washington (STUDY00001067). The samples were from patients diagnosed with diabetes, with geometric mean (IQR, interquartile range) age of 58 (55, 70) years, estimated glomerular filtration rate of 48 (35, 64) mL/min/1.73m^2, and 31 (63%) were female. These samples were analyzed by LC-MS/MS and by commercial RBP4 and TTR ELISA kits (Human RBP4 Quantikine Kit, R&D Systems, Minneapolis, MN and Human TTR ELISA Kit, Abnova, Taipei City, Taiwan).

There is no accepted “gold standard” reference ELISA for RBP4 and TTR, thus kits that have been previously employed were selected (6,15). Concentrations were measured according to manufacturer’s recommendations. Samples were diluted 1,000-fold for RBP4 analysis and 100,000-fold for TTR analysis. Absorbance was read on a Tecan Microplate Reader (Männedorf, Switzerland) at 450 nm with a 570 nm correction for optical imperfections. Samples were quantified against a standard curve (0-100 ng/mL for RBP4 and 0-31.25 ng/mL for TTR). Method comparison was examined using both a Bland-Altman plot, and a Deming regression model.

Clinical variance

The second set of human samples came from a study of twelve healthy premenopausal female volunteers enrolled in a study evaluating the role of pregnancy hormones on disposition of dronabinol (ClinicalTrials.gov ID NCT04374773). Baseline samples were collected on two different days to study intra-individual variability. The study was approved by the Institutional Review Board at the University of Washington (STUDY0008064). Blood samples were collected after an overnight fast to serum separator tubes and sodium citrate tubes. Serum and plasma were separated by centrifugation at 3,000*g* at 4°C for 20 min and stored at -80°C until analysis.

Inter-individual variability was calculated as the geometric mean of the coefficient of variance on both baseline days separately for serum and plasma. Intra-individual variability was calculated as the mean of half the absolute difference between baseline visits for each individual divided by the mean measured concentration for all individuals.

Results

Selection of surrogate peptides, optimization of digestion conditions and matrix effects

Seven of the eight predicted tryptic peptides for RBP4 were detected after digestion of purified protein (**Figure 1, Supplemental Figure 1**). Only one of the detected RBP4 peptides, YWGVASFLQK (YWG), did not contain cysteine or methionine residues, or a ragged end (adjacent lysine or arginine residues). However, the YWG peptide was also detected with a missed cleavage, MKYWGVASFLQK (MKY) (**Figure 1E**). Of the remaining detected RBP4 peptides, FSGTWYAMAK (FSG) was considered as a second potential surrogate peptide despite the methionine present. For TTR, three of five predicted peptides were observed (**Supplemental Figure 1**), all of which contained a ragged end at either the C or N terminus (**Figure 1B**). The GSPAINVAVHVFR (GSP) peptide had the highest signal intensity (**Figure 1F**). Based on this data FSG and YWG were considered further as surrogate peptides of RBP4 and GSP of TTR.

When purified RBP4 from different sources was digested, the peak areas and digestion time courses for YWG, MKY, and FSG peptides were not dependent on protein source (**Supplemental Figure 2**). The presence of serum matrix in the digestion also had minimal effect on digestion time course for the majority of RBP4 and TTR peptides (**Figure 1G-L**). The only exception was the RBP4 peptide MKY with the missed cleavage decreasing with digestion time in serum but not purified protein (**Figure 1H**). Peak area of the missed cleavage TTR peptide GSPAINVAVHVFRK, GSP..RK, was less than 1% of the peak area of the fully cleaved peptide (GSP) and decreased substantially by 5 h digest time (**Figure 1J, K**). Based on these data, a digestion time of 5 h was considered optimal for the assay to maximize peptide signal, reduce TTR

missed cleavage, and minimize peptide degradation or potential methionine oxidation. The FSG and GSP peptides were chosen as the surrogate peptides for method development.

The choice of reducing agent (DTT or tris(2-carboxyethyl)phosphine) did not affect peptide detection (data not shown) and DTT was used for further analyses. In contrast, the choice of detergent had a significant effect on digestion and peptide detection, although the effect was protein and matrix dependent. The detergent had a minor impact on FSG detection from RBP4 (**Figure 2A**). However, deoxycholate was critical for TTR digestion and detection of the GSP peptide. The GSP peptide signal was 88 – 99% lower in the absence of deoxycholate than in its presence (**Figure 2B**).

Sample matrix can impact protein digestion and MS ionization of peptides, and some matrices can contain interfering peptides (23,27). No peaks were observed for the RBP4 peptide FSG in any tested matrix (**Figure 2C**), but a significant peak corresponding to TTR peptide GSP was observed in HSA in Ringer's, suggesting that the HSA purified from human plasma is contaminated with TTR (**Figure 2D**). No contaminating signal for the FSG or GSP peptides was observed in BSA in Ringer's, in FBS, in mouse serum or in mouse plasma. When human RBP4 and TTR were spiked into BSA in Ringer's, mouse serum, or mouse plasma, quantified concentrations of RBP4 and TTR were within 15% of the nominal concentration at low, medium, and high QC levels (**Figure 2E,F**). No discernible differences were observed between the three matrices and hence BSA with 1% Ringers, mouse serum or plasma are considered acceptable surrogate matrices for the assay.

Method Validation

FSG and GSP peptide MS signals were linear for sample concentrations of 0.5 – 6 μM and 2.5 – 80 μM, respectively (**Supplemental Figure 3A,B**). Representative standard curves with QCs for RBP4 and TTR are shown in **Supplemental Figure 3C,D**. The instrument variability was 7.3% for enolase peptide AADALLLK (AAD), 7.0% for FSG, and 7.1% for GSP based on 12 replicate injections of a digested pure enolase, RBP4, and TTR. When the peak area was normalized to the corresponding labeled peptides, the variability decreased to 5.2% for FSG and 3.5% for GSP. Across 96 parallel digestions, enolase AAD peptide peak area had 16% CV (**Supplemental Figure 3E**).

At LLOQ the signal-to-noise was >10, and representative serum sample peaks were greater than LLOQ for all analytes (**Figure 3**). Carryover was assessed based on detection of a peak in an acetonitrile injection after the highest standard. No carryover was observed for either RBP4 or TTR. The intra-day variance for QC samples was <10% at all concentrations and the inter-day variance was <12% including the pooled QC (**Table 1**). Digested peptides for both RBP4 and TTR maintained <15% error at all QC levels following 24 h at 4°C or two freeze-thaw cycles (**Table 1**).

Method Comparison and Application

The “gold standard” method for RBP4 measurement is uncertain, particularly in samples that may be complicated by disease or chronic condition (19). Here, RBP4 and TTR concentrations in 49 samples from individuals with diabetes and CKD were measured by ELISAs previously used in published studies (6,15). The Bland-Altman plot indicated minimal bias for both RBP4 and TTR, around 1%; however, the standard deviation of bias was greater for TTR (31%) compared to RBP4 (18%) (**Figure 4A,B**). The Pearson coefficient was 0.63 for TTR and 0.84 for RBP4. The slope of the Deming regression

was 0.97 (95% CI: 0.63-1.31) for TTR and 1.05 (95% CI: 0.81-1.30) for RBP4, again indicating good overall agreement between the ELISAs and LC-MS/MS methods but a greater variability in TTR measurements compared to RBP4 (**Figure 4C,D**). Geometric mean (interquartile range) concentrations measured by LC-MS/MS were 2.7 (2.4, 3.5) μM for RBP4 and 19 (17, 22) μM for TTR, in agreement with prior measurements by ELISAs in patients with CKD (6,28).

TTR and RBP4 were also quantified in plasma and serum samples collected on two occasions from healthy women (n=12). The measured concentrations in matched plasma and serum samples were within 15% of each other and the average TTR:RBP4 ratio was 12 (**Table 2**). The inter-individual variance for RBP4 and TTR was 18 – 26% in the healthy individuals (**Table 2**). The inter-individual variability of the TTR:RBP4 ratio was about half of this. The observed apparent intra-individual variability was similar in magnitude as the assay intra-day variance, suggesting minimal variability in RBP4 and TTR concentrations from day to day within an individual and reflecting the tight control of RBP4 and TTR in circulation (**Table 2**).

Discussion

Quantifying RBP4 may lend mechanistic insight into numerous clinical conditions or provide a biomarker for disease progression (3,11–14). However, RBP4 forms a complex with TTR, and the complex may be different both functionally *in vivo* and bioanalytically from free RBP4. It is not yet known how RBP4-TTR complex formation impacts the quantitation of RBP4 or TTR by ELISAs. The use of different methods for

RBP4 measurement may be a major source of discordance in clinical studies (12,19). Disease and physiologic dysregulation may similarly confound measurement of TTR by ELISA. We developed and validated an LC-MS/MS based method for the quantitation of RBP4 and TTR in plasma and serum and demonstrated accurate quantitation of RBP4 and TTR in both a healthy population and patients with diabetic CKD.

The use of the purified proteins as calibration standards and SIL peptides as internal standards allowed characterization of digestion and detection efficiency from different matrices and yielded confidence to the absolute quantitation based on the surrogate peptides. The selection and optimization of quantitative peptides in our method development emphasizes the importance of addressing digestion conditions and the need for rigorous assessment of quantitative peptides when limited options are available. For example, TTR missed cleavage peptide behaved as expected with decreasing signal as trypsin digestion time increased but the RBP4 peptide MKY in serum did not. Blood-derived matrices contain protease inhibitors (20), which may inhibit trypsin activity and lead to increased missed cleavages. Yet, the missed cleavage peptide was observed in purified protein, a matrix free of protease inhibitors, regardless of purified protein source or trypsin digest time. The source or expression system of purified RBP4 did not affect the trypsin digestion time course, likely because RBP4 has limited annotated post-translational modifications (11). The impact of detergent on protein digestion and surrogate peptide detection was highly protein specific. Sodium deoxycholate was imperative for TTR peptide signal but had minimal impact on RBP4 peptide detention. The necessity of detergent has been observed previously with a different TTR peptide (29).

The accuracy and precision of the final method met validation criteria both for spiked samples and the pooled QC. The pooled QC sample served as a measure of assay reproducibility in real world samples. Instrument variance may account for over 50% of assay variability observed within a day. The intra-day assay variance ranged from 5.5-9.8% whereas the injection variance for a single sample ranged from 3.5-5.2%. Overall assay variance is likely a major contributor to the observed apparent intra-individual variability in RBP4 and TTR concentrations. The lowest limit of the intra-individual variance in RBP4 and TTR is the intra-day variance defined by the pooled QC. The magnitude of observed intra-individual variability in RBP4 and TTR concentrations in plasma and serum was 6-8.5%, about the same magnitude as the assay variance. Hence it is difficult to differentiate intra-individual variability from inherent method and instrument variance highlighting the homeostatic control of RBP4 and TTR concentrations. The inter-individual variability was about 2-fold the assay's intra-day variance.

There are some limitations to our work. Both RBP4 and TTR have relatively short sequences, 183 and 147 amino acids respectively, resulting in a limited number of tryptic peptides. Only two predicted RBP4 peptides met ideal surrogate peptide selection criteria, lacking methionine, cysteine, or ragged ends, but only one of these (YWG) was observed. However, a missed cleavage (MKY) was observed in digestions of RBP4 for that peptide and thus a different peptide was selected for quantification. Similar surrogate peptide selection was previously reported for RBP4 (30). A limitation for all RBP4 and TTR measurement methods is the lack of certified reference standards and reference methods for RBP4 or TTR. Additionally, intra-individual variance was

determined from two separate visits and in a population limited to reproductive age women. However, our findings are in agreement with a prior analysis of repeated RBP4 ELISA measurements, in which intra-individual variance was not different between men and women (31).

In conclusion, our method is robust and reliable for absolute quantitation of RBP4 and TTR in both serum and plasma. LC-MS/MS and ELISA measurements were overall comparable in samples from patients with diabetic CKD, though there was a larger spread in TTR measurement. It is possible this is due to sample complexity as increased variance in RBP4 measurement has been previously noted in individuals with diabetes compared to healthy volunteers (19). Our LC-MS/MS method offers a robust alternative to ELISAs, which have been shown to be affected by protein conformational diversity, disease states, and lack of concordance (18). Here, the specific peptides used for quantitation are known and the specificity of the method has been tested in multiple matrices. Our method is transferable and represents a candidate reference method for the standardization of RBP4 and TTR measurements, a critical step for the use of RBP4 as a biomarker.

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Tables

Table 1. Method validation data. Accuracy (% error) and precision (% CV) of RBP4 and TTR for quality control (QC) samples across nine separate days. The observed percent error (accuracy) was calculated as (nominal-observed)/nominal for minimum n=3 at each QC level. LLOQ, lower limit of quantitation; LQC, low QC; MQC, middle QC; HQC, high QC.

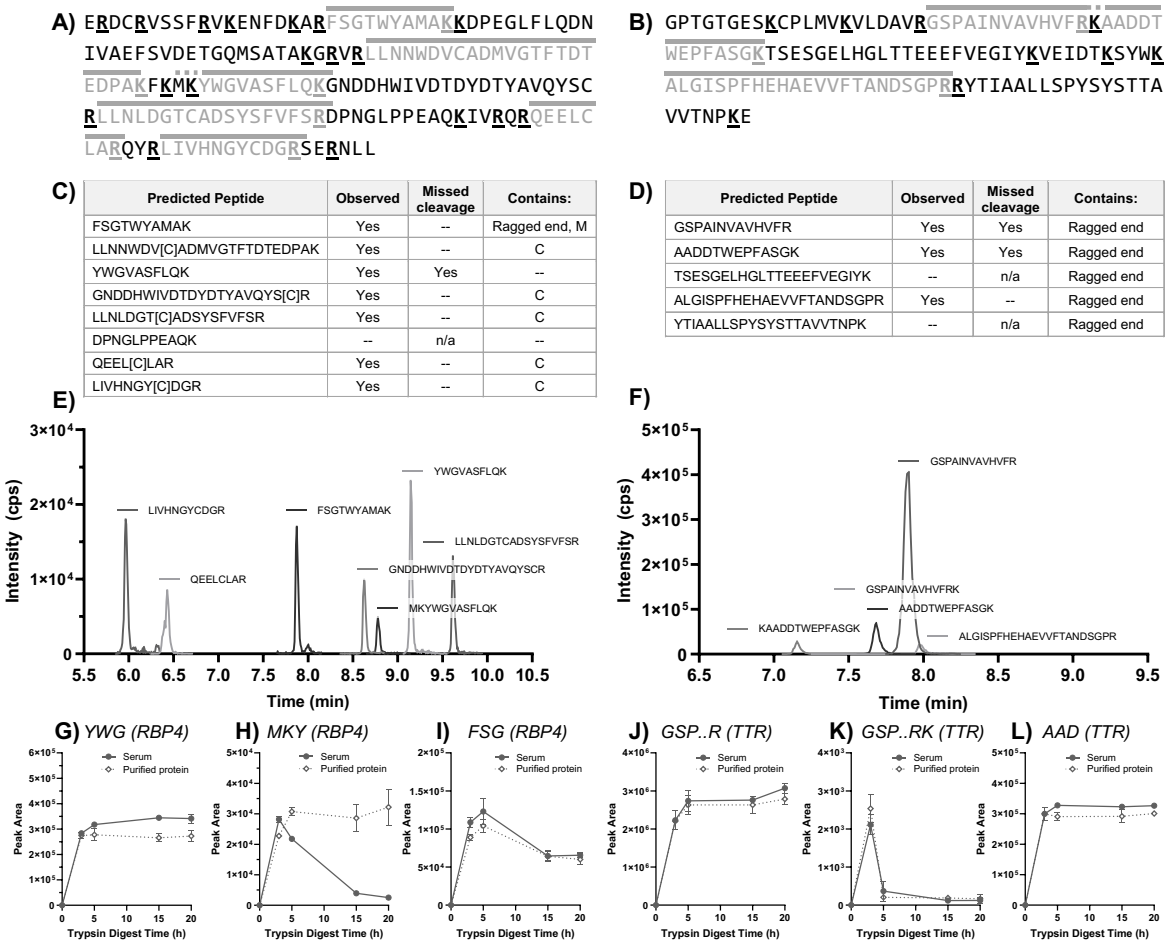
Peptide (Protein)	QC	Nominal Conc. (nM)	Inter-day		Intra-day variance (%CV)[a]	Freeze-thaw error (%)[b]	Autosampler error (%)[c]
			% error	% CV			
FSG (RBP4)	LLOQ	6	1.5	11	5.6	5.6	11
	LQC	9	1.3	8.8	5.9	2.6	4.1
	MQC	18	- 1.4	7.3	5.5	3.0	5.1
	HQC	36	- 4.9	8.7	5.6	7.5	9.4
	Pooled QC[d]	–	–	12	6.6	–	–
GSP (TTR)	LLOQ	69	- 3.8	9.8	9.8	9.4	8.1
	LQC	104	- 2.2	8.6	8.3	1.9	4.9
	MQC	208	- 4.7	7.6	6.2	2.8	-1.9
	HQC	416	- 1.0	8.7	8.0	10.7	-0.9
	Pooled QC[d]	–	–	12	6.6	–	–

[a]Intra-day variance was calculated from five replicate analyses within a day and the average intra-day %CV for each QC level from four days is reported. [b]Stability of quantitative peptides determined following two freeze-thaw cycles at -20°C. [c]Stability of quantitative peptides determined following 24 h at 4 °C in autosampler. [d]Pooled QC was prepared as a mixture of serum from ten individuals.

Table 2. Measured serum and plasma concentrations and inter- and intra-individual variability of RBP4, TTR, and TTR:RBP4 ratio in healthy female volunteers. The RBP4 and TTR concentration in one sample was identified as an outlier using Grubbs' test (α=0.01) and excluded from the analysis.

Analyte	Measured concentration geometric mean (range)		Inter-individual variability (%CV)		Intra-individual variability %	
	Serum	***Plasma***	***Serum***	***Plasma***	***Serum***	***Plasma***
RBP4	1.5 (0.9 – 2.4) μM	1.3 (1.0 – 2.1) μM	26%	19%	8.1%	8.5%
TTR	19 (13 – 29) μM	16 (12 – 23) μM	21%	18%	7.6%	6.1%
TTR:RBP4	12 (9.6 – 18)	12 (9.1 – 15)	13%	9.4%	6.3%	5.9%

Figures



Predicted Peptide	Observed	Missed cleavage	Contains:
FSGTWYAMAK	Yes	--	Ragged end, M
LLNNWDV[C]ADMVGTFTDTEDPAK	Yes	--	C
YWGVASFLQK	Yes	Yes	--
GNDDHWIVDTDYDTYAVQYS[C]R	Yes	--	C
LLNLDGT[C]ADSYSFVFSR	Yes	--	C
DPNGLPPEAQK	--	n/a	--
QEEL[C]LAR	Yes	--	C
LIVHNGY[C]DGR	Yes	--	C

Predicted Peptide	Observed	Missed cleavage	Contains:
GSPAINVAVHVFR	Yes	Yes	Ragged end
AADDTWEPFASGK	Yes	Yes	Ragged end
TSESGELHGLTTEEEFVEGIYK	--	n/a	Ragged end
ALGISPFHEHAEVVFTANDSGPR	Yes	--	Ragged end
YTIAALLSPYSYSTTAVVTNPK	--	n/a	Ragged end

Figure 1. Identification and selection of RBP4 (left) and TTR (right) surrogate peptides. Amino acid sequences (A, B) with observed peptides in grey and key peptide characteristics (C, D). Representative summed chromatograms of observed peptides (E, F) following 15 h of trypsin digestion of purified protein. Trypsin digest time course for potential surrogate peptides from Bio-Rad purified proteins (G-L).

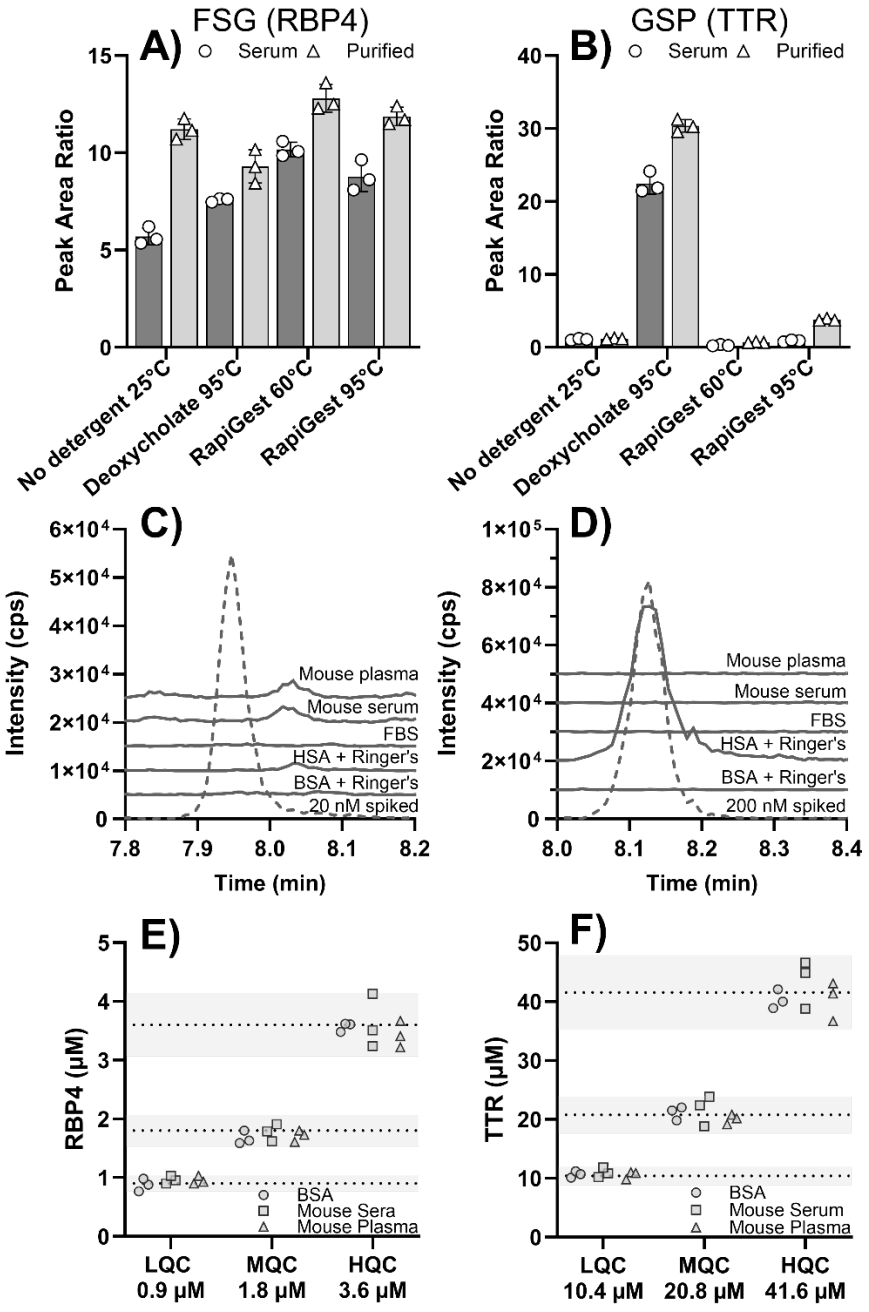


Figure 2. Optimization of digestion conditions for RBP4 and TTR. Detergent effect on peptide signal in serum or purified protein digestions (A , B). Chromatograms from digestions of different matrices and purified reference protein for C) FSG and D) GSP. Concentrations of E) RBP4 and F) TTR calculated in different matrices spiked at QC concentrations. The shaded area represents 15% above or below the nominal concentration. One representative day of three is shown.

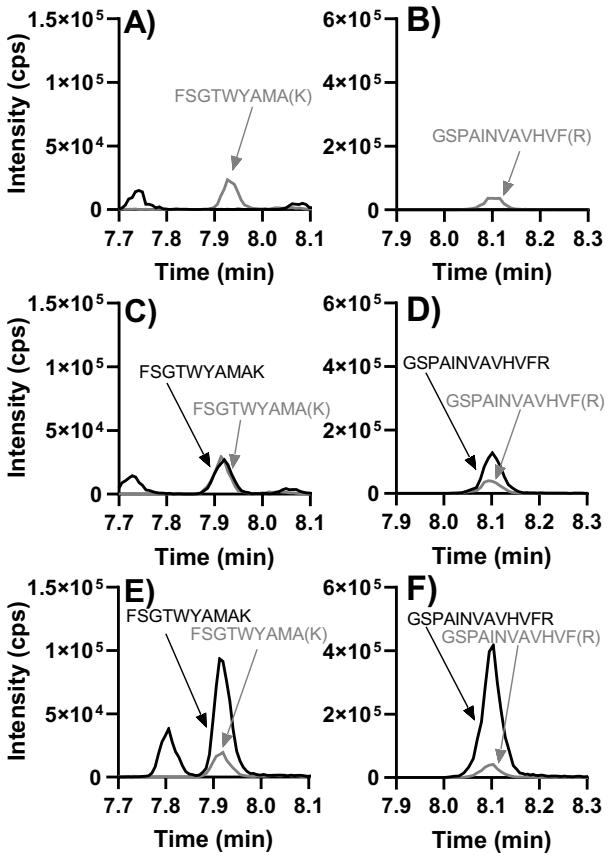


Figure 3. Representative chromatograms. The isotope labeled (K[$^{13}C_6{}^{15}N_2$] or R[$^{13}C_6{}^{15}N_4$]) internal standard peptides for RBP4 peptide (A, C, E) and for TTR peptide (B, D, F) are in grey and the corresponding analyte peptides are in black. Matrix with isotope labeled internal standard (A, B), spiked samples at lower limit of quantitation (C, D), and representative serum sample (E, F).

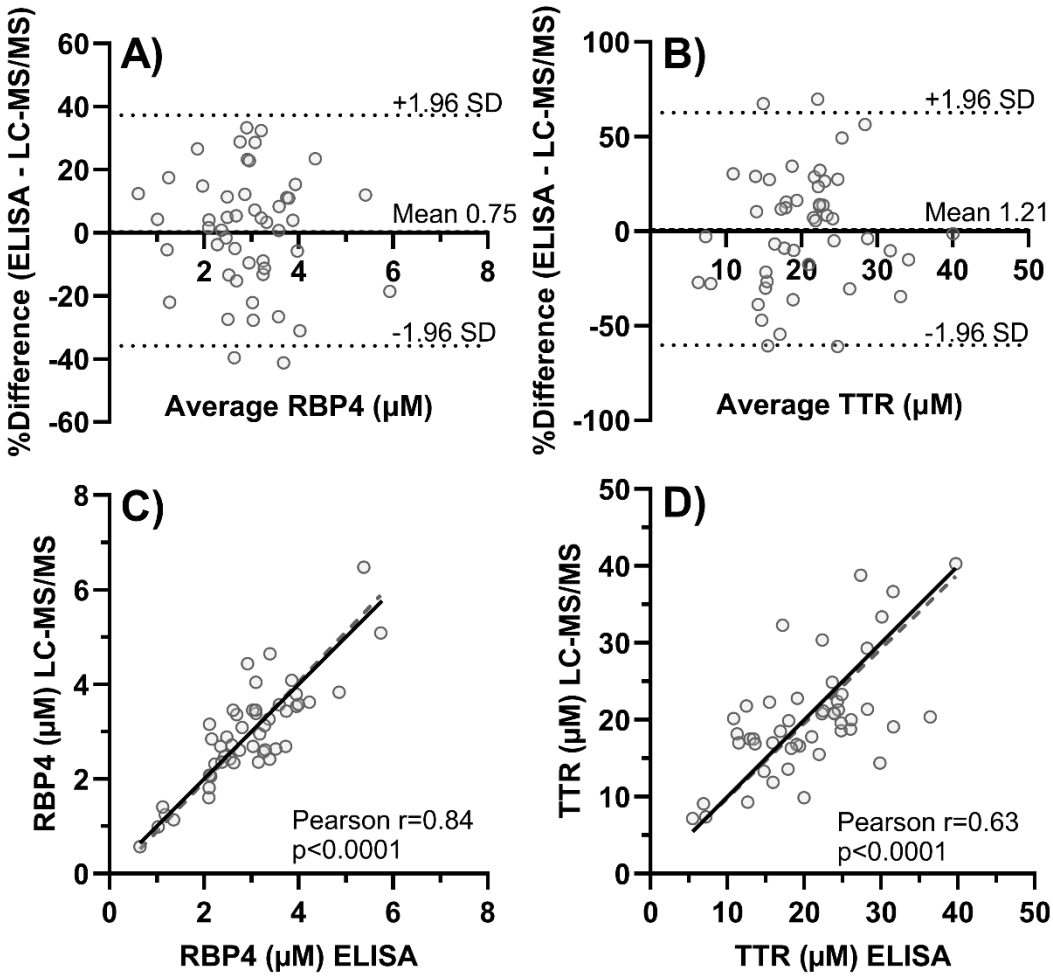


Figure 4. Comparison of LC-MS/MS and ELISA methods in individuals (n=49) with chronic kidney disease (CKD). Bland-Altman plot comparing percent difference of A) RBP4 and B) TTR measurements from LC-MS/MS and ELISA. Dotted lines: 95% CI, dashed line: mean percent bias. Deming regression for C) RBP4 ([LC-MS/MS] = 1.05*[ELISA]-0.16) and D) TTR ([LC-MS/MS]=0.94*[ELISA]+0.78), dashed line: regression, solid black line: equivalence.