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Quantification of the soluble Receptor of Advanced Glycation End-Products (sRAGE) by LC-MS after enrichment by strong cation exchange (SCX) solid-phase extraction (SPE) at the protein level

Frank Klonta, Marc R. Joosten, Nick H.T. Ten Hacken, Péter Horvatovich, Rainer Bischoff

Department of Analytical Biochemistry, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, the Netherlands

Department of Pulmonary Diseases, University Medical Center Groningen, University of Groningen, Hanzeplein 1, 9713 GZ, Groningen, the Netherlands

Highlights

- Serum sRAGE was quantified in the low to sub ng mL\(^{-1}\) range without using antibodies.
- sRAGE was enriched by SCX at pH 10, despite having a calculated protein pI of 8.
- Look beyond a calculated pI when considering ion exchange for protein enrichment.
- Correlation between the SCX-based method and an antibody-based method was moderate.
- Measured protein levels must be seen in the context of the measuring principle.

Abstract

The study of low abundant proteins contributes to increasing our knowledge about (patho)physiological processes and may lead to the identification and clinical application of disease markers. However, studying these proteins is challenging as high-abundant proteins complicate their analysis. Antibodies are often used to enrich proteins from biological matrices prior to their analysis, though antibody-free approaches have been described for some proteins as well. Here we report an antibody-free workflow on the basis of strong cation exchange (SCX) enrichment and liquid chromatography-mass spectrometry (LC-MS) for quantification of the soluble Receptor of Advanced Glycation End-products (sRAGE), a promising biomarker in chronic obstructive pulmonary disease (COPD). sRAGE was quantified in serum at clinically relevant low to sub ng mL\(^{-1}\) levels. The method was validated according to U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines and was compared to an antibody-based LC-MS sRAGE method. The SCX-based method builds upon the bipolar charge distribution of sRAGE, which has a highly basic N-terminal part and an acidic C-terminal part resulting in an overall neutral isoelectric point (pI) of 10.3. The highly basic N-terminal pI\(_{\text{calculated}} = 10.3\) allowed for sRAGE to be enriched by SCX at pH 10, a pH at which most serum proteins do not bind. This study shows...
1. Introduction

The quantification of proteins in blood-based samples is one of the hallmarks of modern laboratory medicine and allows for the diagnosis and staging of disease as well as for monitoring disease progression and treatment efficacy. Blood is, however, a highly complex matrix with only a few proteins (e.g. albumin, immunoglobulins) accounting for most of the total protein content [1]. These high abundant proteins complicate the analysis of many other proteins and often need to be depleted to enable the detection of clinically relevant proteins of lower abundance [2,3].

Antibody-based enrichment procedures are commonly used to enrich low abundant proteins prior to their quantification, yet it has been shown in recent years that antibody-free workflows can quantify proteins in the ng mL$^{-1}$ range as well [4]. Antibody-free workflows are based on specific properties of a target protein that allow it to be separated from most other proteins in a biological sample. For example, some small, hydrophobic proteins can be enriched following protein precipitation procedures in which these smaller proteins remain in solution while larger proteins denature and precipitate [5]. In addition, proteins exhibiting regions rich in histidine, cysteine, or tryptophan residues, which form complexes with transition metal ions (e.g. Zn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, Co$^{2+}$), can be enriched by immobilized metal affinity chromatography (IMAC) [6]. Solid-phase extraction (SPE) furthermore offers a range of options for efficient enrichment of protein subclasses with specific physical-chemical properties. For example, proteins with an elevated net negative or positive charge can be enriched by strong anion exchange (SAX) or strong cation exchange (SCX)-based procedures, respectively [4].

The soluble Receptor of Advanced Glycation End-products (sRAGE) is an example of a clinically relevant protein of low abundance, which can currently only be quantified using affinity ligands (e.g. antibodies). This protein is a circulating form of membrane-bound RAGE, an important receptor of the innate immune system that is involved in triggering and sustaining inflammation, in particular in the lungs [7]. sRAGE is formed after proteolytic cleavage of RAGE by metalloproteases (e.g. ADAM10, MMP9) or by alternative splicing of the AGER gene leading to the so-called endogenous secretory RAGE (esRAGE) splice variant. sRAGE is proposed to act as a decoy receptor for various pro-inflammatory RAGE ligands thereby attenuating inflammation [8–11]. Several large-scale clinical studies advanced sRAGE as promising biomarker for chronic obstructive pulmonary disease (COPD), and sRAGE is currently being considered for biomarker qualification by the U.S. Food and Drug Administration (FDA) [12,13].

sRAGE is a member of the immunoglobulin superfamily and consists of three immunoglobulin-like domains, the N-terminal ‘V domain’, the ‘C1 domain’, and the C-terminal ‘C2 domain’, which together result in a fairly neutral isoelectric point (pI) [7,8]. However, the V domain of sRAGE displays a net positive charge at neutral pH due to 16 positively charged amino acids versus 7 negatively charged residues, whereas its C2 domain displays a net negative charge at neutral pH by carrying 10 negatively charged amino acids versus 3 positively charged residues (see Fig. A.1). The bipolar charge distribution of sRAGE thereby provides opportunities for ion exchange-based protein enrichment, which is not obvious when solely considering the average pI of this protein. In this study, we developed an ion-exchange-based enrichment approach for the quantification of sRAGE in human serum by liquid chromatography-mass spectrometry (LC-MS) at clinically relevant low to sub ng mL$^{-1}$ levels. In addition, we identified other low abundant proteins for which ion-exchange-based protein enrichment may represent an alternative to the commonly-used affinity-based strategies for quantification by LC-MS.

2. Materials and methods

2.1. Chemicals and materials

Recombinant human sRAGE (rh-sRAGE; Cat. No. C423; UniProtKB ID ‘Q15109’, Ala23-Ala344 with C-terminal hexa-histidine tag) was purchased from Novoprotein (Summit, NJ, U.S.A.) and stable-isotope-labeled sRAGE peptides (SIL peptides; i.e. IGEPALIK & VLSPOQGGPWDVSAR’$^\#$) were synthesized by Pepscan Presto (Leystad, The Netherlands). TSKgel SP-3PW SCX resin (250 Å; Cat. No. 0021977; this resin was used for all experiments described in this manuscript unless stated otherwise), TSKgel SP-5PW SCX resin (1000 Å; Cat. No. 0043282), and TSKgel SuperQ-5PW SAX resin (1000 Å; Cat. No. 0043283) were supplied by Tosoh Bioscience (Darmstadt, Germany) and empty solid-phase extraction reservoirs (Cat. No. 120–1111-A) were purchased from Biotage (Uppsala, Sweden). Acroniitle (Cat. No. 01200702) was obtained from Biosolve (Valkenswaard, The Netherlands), ethanol (Cat. No. 100983.2500) from VWR (Amsterdam, The Netherlands), ammonia (Cat. No. 1054321000) from Merck (Amsterdam, The Netherlands), and sequencing grade modified trypsin (Cat. No. VS111) from Promega (Madison, WI, U.S.A.). Acetic acid (Cat. No. 1000631000), ammonium acetate (Cat. No. 73594), and all other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

2.2. Serum samples

Pooled human serum from Seralab (West Sussex, U.K.) was used directly as QC-low sample or was fortified with rh-sRAGE at two levels to obtain the QC-medium and QC-high samples. Recovery and spike recovery experiments as well as shotgun proteomics experiments were carried out using six different sources of human serum from healthy subjects (all from Seralab).

2.3. Calibrants and internal standard

rh-sRAGE was dissolved in Milli-Q water to obtain a 200 μg mL$^{-1}$ solution (based on the quantity as declared by the supplier) which was diluted to 100 μg mL$^{-1}$ with 10 mM phosphate buffered saline, pH 7.4. The resulting solution was sequentially diluted to 100 ng mL$^{-1}$ with rat serum, and calibration samples were prepared at 0.2, 0.5, 1.0, 2.0, 5.0, 8.0 and 10.0 ng mL$^{-1}$ in rat serum. The internal standard (IS) stock solution was prepared by mixing equimolar amounts of the two SIL-peptides (supplied as 5 pmol μL$^{-1}$ solutions in 5% ACN) and subsequently diluting these peptides to 5 fmol μL$^{-1}$ with 1% dimethyl sulfoxide (DMSO) in water.
2.4. SCX-based sRAGE enrichment

The supplied suspension of the SCX resin was homogenized by agitation and 100 μL of this suspension was transferred to a microcentrifuge tube. The resin was washed with 800 μL ethanol and then with 800 μL 20 mM ammonium acetate, pH 10 (SCX buffer) by gentle vortex-mixing after which the resin was pelleted by centrifugation (3000 × g; 2 min), and the washing solution was discarded. Next, the resin was resuspended in 750 μL SCX buffer and 50 μL of serum (or calibrator) was added. After 15 min of incubation on a rotating wheel, the sample was briefly centrifuged to spin down droplets from the lid, and the resin was loaded into an empty SPE reservoir which was placed in a Varian Cerenix™ SPE processor (positive pressure was applied to let solvents pass through the SPE reservoirs in a dropwise manner) and which had been rinsed with 1 mL water. The resin was subsequently washed with 1 mL SCX buffer, and sRAGE was eluted with 250 μL of a 1% aqueous ammonium solution and then collected in low binding tubes (Eppendorf; Cat. No. 525–0133) containing 50 μL of a 15% aqueous acetic acid solution. After drying the eluate in a vacuum centrifuge (10 min; 20,000 g; 30°C), the residue was reconstituted in 50 μL Digestion Buffer (100 fmol μL⁻¹ SIL-peptides in 100 mM ammonium bicarbonate (ABC)). Next, disulfide bonds were reduced with 10 mM dithiothreitol (DTT; 5 μL 110 mM DTT in ABC) for 30 min (60°C; 600 RPM), and thiols were alkylated with 20 mM iodoacetamide (IAM; 5 μL 240 mM IAM in ABC) for 30 min in the dark (room temperature). After quenching the excess of IAM with a 0.5 M excess of DTT (5.5 μL of 110 mM DTT), 100 ng trypsin was added to each sample, and proteins were digested overnight (37°C; 600 RPM). The digests were acidified by adding 5 μL of 25% formic acid (FA), and 20 μL of sample was analyzed by LC-MS in the multiple reaction monitoring (MRM) mode.

2.5. Targeted analysis by LC-MRM/MS

Targeted analyses were performed on a Waters Ionkey/MS system using an ACQUITY M-Class UPLC and a XEVO TQ-S mass spectrometer (Milford, MA, U.S.A.). Chromatographic separation was achieved on a C18-bonded Waters iKey HSS T3 Separation Device (1.8 μm particles, 100 Å pore size, 150 μm × 100 mm; Cat. No. 186007261) at 20°C using 0.1% FA in H2O as mobile phase A and 0.1% FA in ACN as mobile phase B. 20 μL of sample was loaded onto a Dionex Acclaim PepMap100C18 trap column (5 μm particles, 100 Å pore size, 300 μm × 5 mm; Cat. No. 160454) for 4.5 min with 3% B at 20 μL min⁻¹. Peptides were subsequently separated on the analytical column at 3 μL min⁻¹ with a 10 min linear gradient from 3 to 33% B, after which the column was cleaned (0.6 min at 60% B and 2.1 min at 95% B) and equilibrated (4.3 min at 3% B). Mass spectrometric detection was performed using the following conditions: ESI positive, capillary voltage 3.5 kV, source temperature 120°C, cone gas (argon) flow 150 L h⁻¹, sheath (nano)flow (argon) flow 0.2 bar, and collision gas (argon) flow 0.15 mL min⁻¹. MRM transitions and settings for IGEPV/LVK (selected for quantification) and VLSPOQQCPPDSVAR (selected for confirmation) are presented in Table A1. The Ionkey/MS system was operated under the Waters MassLynx software suite (version 4.1), and the TargetLynx module of this package was used for data processing.

2.6. Method validation and comparison

The method was validated according to EMA and FDA guidelines by addressing the following criteria: selectivity (e.g. spike recovery), accuracy & precision, recovery, calibration curve, and stability (e.g. 6 days benchtop, 5 × freeze-thaw, and 6 days autosampler (10°C)) [14,15]. Samples were fortified with 5 ng mL⁻¹ sRAGE either before or after SCX-SPE to obtain the pre-SCX-SPE spiked and post-SCX-SPE spiked samples for recovery assessment, respectively. For the spike-recovery experiments, samples were processed either directly or were fortified with 5 ng mL⁻¹ sRAGE (before SCX-SPE) to obtain the non-spiked and spiked samples, respectively. Furthermore, the method was compared with a previously developed immunoaffinity-LC-MS method for sRAGE quantification [16]. For this purpose, 40 serum samples were analyzed from a cross-sectional study (NCT00807469) within the University Medical Center Groningen (UMCG) [17]. Ethical approval for this study had been granted by the UMCG’s review board (METC 2008/136), and the study adheres to the Declaration of Helsinki. Blood samples were collected as described previously [16].

2.7. Shotgun proteomics

Proteins enriched by SCX at pH 10 (see ‘SCX-Based sRAGE Enrichment’ paragraph above) or SAX at pH 4 (SPE was performed following the same steps as described in the ‘SCX-Based sRAGE Enrichment’ paragraph above, however, with the following solutions: loading and washing with 20 mM ammonium acetate, pH 4; elution with 250 μL 3% aqueous acetic acid; and eluate collection in 50 μL 5% aqueous ammonia) were identified on the basis of six serum samples. For these experiments, TSKgel Super-5PW SAX and TSKgel SP-5PW SCX resins were used, and a trichloroacetic acid (TCA) precipitation step was included after SPE-enrichment to concentrate the samples. Briefly, the SPE eluates were mixed 1:1 (v/v) with a 12% aqueous TCA solution, incubated for 5 min at 60°C, placed on ice for at least 30 min, vortexed-mixed, and centrifuged (10 min; 20,000 × g; 4°C). After discarding (most of) the supernatant, 400 μL ice-cold acetone was added, the samples were vortex-mixed and centrifuged (5 min; 20,000 × g; 4°C), and (most of) the supernatant was removed after which the pellets were left to dry in air. Shotgun proteomics analyses were performed using an Ultimate 3000 RSLC UHPLC system connected to an Orbitrap Q Exactive Plus mass spectrometer operating in the data-dependent acquisition (DDA) mode. A sample volume of 6 μL was injected onto an Acclaim PepMap100C18 trap column (see above) using μL-pickup with 0.1% FA in H2O at 20 μL min⁻¹. Peptides were separated on an Acclaim PepMap RLSC C18 analytical column (2 μm particles, 100 Å pore size, 75 μm × 500 mm; Cat. No. 164540) at 40°C using a 60 min linear gradient from 3 to 50% eluent B (0.1% FA in ACN) in eluent A (0.1% FA in H2O) at a flow rate of 200 nL min⁻¹. For DDA, survey scans from 300 to 1650 m/z were acquired at a resolution of 70,000 (at 200 m/z) with an AGC target value of 3 × 10⁶ and a maximum ion injection time of 50 ms. From the survey scan, a maximum number of 10 of the most abundant precursor ions with a charge state of 2⁺ to 6⁺ were selected for fragmentation by higher energy collisional dissociation (HCD). Fragment ion spectra were acquired between 200 and 2000 m/z at a resolution of 17,500 (at 200 m/z) with an AGC target value of 1 × 10⁶, a maximum ion injection time of 110 ms, a normalized collision energy of 28%, an isolation window of 1.6 m/z, an intensity threshold of 1 × 10⁵, and the dynamic exclusion parameter set at 15 s. Shotgun proteomics data were processed using PEAKS Studio software (version 8.5) [18], and peak lists were searched against the UniProtKB Homo sapiens 'UP000005640' reference proteome (canonical; 40,424 entries; downloaded on August 22, 2017) with trypsin selected as protease (≤3 missed cleavages), cysteine carbamidomethylation as fixed modification, methionine oxidation as variable modification, and allowing ≤5 modifications per peptide. <10.0 ppm precursor mass deviation (using monoisotopic mass), ≤0.2 Da fragment ion mass deviation, and ≤0.1% false discovery rates (FDR) for peptide-spectrum matches (PSMs), peptides, and proteins, as well as
requiring at least two unique peptides for protein identification.

3. Results and discussion

3.1. SCX-based protein enrichment

The bipolar charge distribution of sRAGE, which has an average pl of 7.8 (calculated using Expy’s ProtParam tool [19] for the extracellular domain of RAGE, Ala23-Ala342), formed the basis for protein enrichment based on ion-exchange solid-phase extraction. On the one hand, the C-terminal C2 domain of sRAGE with a pl of 4.6 (Pro227-Ser317) was allowed for binding to the SAX material down to pH 6 (see Fig. 1a), while the N-terminal V domain with a pl of 10.3 (Ala23-Arg116) bound to the SCX material up to pH 10 (see Fig. 1b). The latter strategy was selected for quantitative method development given that most human proteins are acidic in nature [20].

The SCX-based protein enrichment method was developed using highly cross-linked polyacrylate beads functionalized with sulfopropyl groups. Resin was transferred into an empty SPE cartridge after incubation with serum under alkaline conditions (pH 10). Loading and washing were performed using a 20 mM ammonium acetate buffer, pH 10 (see Fig. A.2), sRAGE was eluted from the SCX material with 1% aqueous ammonia (pH approx. 11.5), and eluates were directly neutralized by collecting them in tubes containing 15% aqueous acetic acid (see Fig. A.3). The eluate was subsequently dried to reduce the sample volume and to remove most of the ammonium acetate. Including a TCA precipitation step after SCX enrichment instead of drying the eluates was also tested and resulted in a faster protocol and cleaner samples. However, the corresponding protein pellets were fragile and detached easily from the walls of the tubes leading to outliers that were incompatible with a validated quantitative bioanalytical method. The TCA precipitation procedure was, however, used for the shotgun proteomic analyses (see protocol outlined in the ‘Shotgun proteomics’ paragraph of the experimental section and corresponding data in the ‘Ion exchange-based protein enrichment for targeted protein bioanalysis by LC-MS’ paragraph below).

3.2. Quantitative assay development

sRAGE was detected by means of two unique tryptic peptides, rIGEPLVLKc (30–37) was selected as quantifier peptide, since this peptide performed best in terms of accuracy and precision. k.VLSPQGGGPWDSVAR.v (63–77) was selected as qualifier peptide due to its deamidation-prone ‘QG’ sequence motif and due to a single nucleotide polymorphism (SNP) leading to an arginine-to-cysteine substitution (i.e. rs116828224) with an expected frequency of 0.6% in the general population (based on data from the ExAC Browser [21]). Both peptides are derived from the V domain and thereby reflect the fraction of sRAGE forms (so-called ‘proteoforms’ or ‘protein species’) [22,23] to which most ligands will bind [78]. Tryptic peptides from the C2-domain could not be analyzed in parallel, as this domain only gives rise to two tryptic peptides consisting of 41 and 43 amino acids, respectively, as well as a C-terminal peptide containing an unknown proteolytic cleavage site as a result of RAGE shedding. The amino acid sequence of the C1-domain should yield detectable tryptic peptides (with an approximate maximal length of 20 amino acids), yet these peptides were not detected in clinical samples possibly due to incomplete digestion, low ionization efficiency, and/or loss of peptides during sample preparation or LC-MS analysis.

3.3. Method validation

Table 1 summarizes the validation results. A full overview of all validation results is given in Tables A.2 to A.10. Stable-isotope-labeled (SIL) peptides were added after the SCX enrichment procedure, since a SIL version of sRAGE was not available. Variability originating from the enrichment procedure could thus not be compensated for, and sRAGE recovery after SCX enrichment was accordingly evaluated during method validation. Recovery was found to be 74% (see Table A.2) with a CV of 4% showing that sRAGE enrichment by SCX was reproducible according to the FDA guidelines for bioanalytical method validation. The recovery of a previously developed antibody-based enrichment procedure was 83% with a CV of 4% thereby being somewhat higher but equally reproducible [16].

For preparing the calibration curve, we found that a surrogate matrix of sufficient complexity (e.g. serum from other organisms) was required and that a simple, artificial matrix consisting of bovine serum albumin (BSA) in PBS gave very low sRAGE recovery. We reasoned that BSA will be depleted following SCX at pH 10, as this protein has a calculated pl of 5.6, and that the sample after SCX-SPE will have a very low remaining protein concentration thereby likely inducing adsorption of sRAGE after the enrichment procedure. Complex matrices, in turn, are expected to lead to co-enrichment of other proteins, which serve as carrier proteins preventing sRAGE adsorption. Rat serum was found to be a suitable surrogate matrix, as demonstrated by an average spike-recovery bias of 10% (see Table A.3), which is well within acceptable limits (±15%).

Accurate quantification of sRAGE at clinically relevant levels between 0.2 and 10 ng mL

−1 was demonstrated for a 1/x weighted linear calibration model using 7 non-zero standards. Reaching the desired sensitivity was, however, challenging and considerable chemical background was observed in the chromatograms of the calibrants (see Fig. A.4a), though acceptable biases and CVs within 10% were found for all calibrants (see Table A.4). In comparison with an immunoaffinity-LC-MS method for sRAGE quantification [16], chemical background was somewhat higher for samples that were prepared using the SCX-SPE method, as became apparent from selected ion chromatograms of six serum samples prepared with both methods (see Fig. A.5a and A.5b). TCA precipitation after SCX enrichment reduced the chemical background (see Fig. A.5c) illustrating the added benefit of incorporating such a step in the protocol, provided that this procedure can be performed in a reproducible manner without producing outliers.
Accuracy of the method was high with biases ranging from \(-7\%\) to \(7\%\) for the three QC-samples, and precision was acceptable with CVs below or equal to \(15\%\) (see Tables A.5 to A.7). Stability assessments indicated that storing samples for 6 days on the benchtop or subjecting them to 5 freeze-thaw cycles did not affect sRAGE levels (see Tables A.8 and A.9). Stability of the final peptide digests was furthermore assessed for up to 6 days of storage in the autosampler at \(10\,^\circ\text{C}\) and corresponding biases and CVs were well within the acceptance limits (see Table A.10).

### 3.4. Method comparison

Agreement between the SCX-based LC-MS sRAGE method and a previously developed antibody-based LC-MS sRAGE method [16] was assessed on the basis of 40 clinical samples using linear regression and Bland-Altman plots (see Fig. 2). Comparison between the two methods revealed moderate correlation \((R^2 = 0.48)\) and an increasingly positive bias for the SCX-based method with increasing sRAGE concentrations relative to the antibody-based method. Different sRAGE levels have been reported before when comparing the antibody-based LC-MS method with a commercial ELISA (i.e. the Human RAGE Quantikine ELISA kit from R&D Systems), which represents the most frequently used sRAGE assay in clinical biomarker studies [16]. The ELISA was found to report 84\% lower sRAGE levels compared to the antibody-based LC-MS method, while correlation between these assays was rather good with an \(R^2\) of 0.79. These differences indicate that the measured sRAGE concentration strongly depends on methodology even though all three methods represent validated assays. It is, for example, conceivable that the methods capture a different subset of sRAGE proteoforms or sRAGE-containing complexes. sRAGE molecules are known to bind to each other as well as to several other proteins [7], and the binding sites of antibodies may thereby become inaccessible. In addition, some proteoforms lack certain regions or feature specific posttranslational modifications which might interfere with an enrichment strategy. With respect to the ELISA and the antibody-based LC-MS method, a lack of binding capacity of the antibodies may furthermore be a reason for the lower levels reported by these assays. In fact, an insufficient binding capacity would affect higher protein levels disproportionally, in agreement with the increasingly positive bias for the SCX-based method shown in Fig. 2. However, it should be noted that the antibody-based LC-MS method was validated over a much wider concentration range than strictly needed for the 40 samples included in the method comparison. The increasingly positive bias of the SCX-based method relative to the immunoaffinity-based method might also be due to a lower affinity of sRAGE for the SCX material, which would affect lower concentrations to a greater extend. The linear response of rh-sRAGE spiked in rat serum for the calibration samples did, however, not indicate such an effect. A further caveat is that data based on rh-sRAGE may not be representative for

### Table 1

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\(^{a}\) An extensive summary of the validation results is presented in Tables A.2 to A.10.

\(^{b}\) The average value of measured concentrations during the precision and accuracy experiments was used as nominal concentration.

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**Fig. 2.** Comparison between the SCX-based LC-MS method (SCX) and a previously developed antibody-based LC-MS method (Antibody) [16] using (a) linear regression and (b) Bland-Altman plots.
endogenous sRAGE pointing to a general problem in biomarker research, namely the lack of well-characterized authentic protein standards.

3.5. Ion exchange-based protein enrichment for targeted protein bioanalysis by LC-MS

Unlike affinity ligands which enrich their targets from a biological matrix based on well-defined binding sites, ion exchange-based enrichment procedures yield a series of proteins with common physical-chemical properties with respect to number of charges and charge distribution under a given set of experimental conditions. Several other proteins are therefore expected to be co-enriched by the SCX-based method, and we aimed to identify more proteins by shotgun proteomics in the SCX eluate at pH 10. We extended these experiments to the enrichment of serum proteins on an SAX resin at pH 4 to investigate whether the principle holds for both types of ion exchangers. As expected, SCX-based enrichment led to considerably less complex samples compared to SAX, as reflected by 3 times higher total ion current and base peak chromatograms and twice as many identified proteins in the SAX-enriched samples.

Table A.11 gives an overview of proteins that were identified in the SCX- or SAX-enriched fractions of six different serum samples after removing the top 20 high abundant blood proteins [3] and common contaminants such as keratins from the results. Many of the identified proteins were medium to high abundant blood proteins such as apolipoproteins, coagulation-related proteins or proteins of the complement system, yet some clinically relevant, lower abundant proteins were identified as well. When viewing these results, it must be kept in mind that MRM-based targeted protein analyses are more sensitive than shotgun analyses due to the stochastic nature of DDA. It is thus not surprising that sRAGE, the protein biomarker that we quantified in the SCX fraction, was not identified in any of the shotgun analyses.

Fig. 3 shows the calculated pl values of the proteins included in Table A.11 plotted against their concentrations in the circulation, as retrieved from the Plasma Proteome Database [24]. Based on the scatter observed in both plots we concluded that the (calculated) average pl is rather uninformative when aiming to predict the ion exchange behavior of proteins. The SCX fraction, for example, contained the low µg mL⁻¹ insulin-like growth factor binding protein (IGFBP3) (UniProtKB ID ‘P17936’) and the ng mL⁻¹ IGFBP5 (‘P24593’) and IGFBP7 (‘Q16270’), which all have an average pl between 8 and 9. In addition, A disintegrin and metalloprotease with thrombospondin motifs 13 (ADAMTS13; ‘Q76LX8’) and intercellular adhesion molecule 2 (ICAM2; ‘P13598’) were identified in the SAX fraction and hepatocyte growth factor activator (HGFA; ‘Q04756’) was identified in both the SAX and SCX fractions, in spite of their calculated average pl of 7.0, 7.1, and 7.0, respectively. These proteins likely contain charged domains, such as the 4 kDa short chain of HGFA featuring a pl of 11.7 which might explain its binding to the SCX resin at pH 10. These results thus open up new opportunities for enriching proteins by SAX at acidic or by SCX at basic pH. Still, it is important to assess the charge distribution in a protein rather than its calculated average pl when evaluating whether ion exchange SPE might be suitable approach for enrichment. With any prediction it must, however, be realized that post-translational modifications may alter binding to ion exchangers thereby stressing the significance of a thorough analysis of the physical-chemical properties of endogenous proteins. Furthermore, it should be taken into account that conditions under which SAX and SCX are performed can affect protein structure or stability. Protein stability should therefore be evaluated and monitored closely when developing quantitative methods for intact proteins, whereas proteotypic peptide selection and evaluation should be done diligently when developing quantitative methods targeting proteotypic peptides.

4. Conclusions

Protein enrichment is a prerequisite for many biological, biomedical, and clinical studies. The availability of good quality antibodies and other affinity ligands have opened up and expanded opportunities to study proteins for researchers working in biological and medical sciences. Methods that do not rely on affinity ligands are, however, also suitable to enrich proteins from complex biological matrices with protocols that are as simple and straightforward as those of affinity-based methods. These methods take the specific physical-chemical characteristics of a protein of interest into account, such as the presence of transition metal-chelating sites, which can be targeted through immobilized metal affinity chromatography, or the presence of positively or negatively charged regions, which can be targeted by ion exchange resins. The resulting protocols enable the highly reproducible enrichment of low abundant proteins from serum, as demonstrated by the development and validation of a quantitative LC-MS method for sRAGE based on a strong cation exchange enrichment procedure. Our work furthermore emphasizes that protein concentrations must be seen in the context of the methodology that was used to measure them due to the complexity of the proteome and, as a consequence, the possibility that different methods may enrich a different subset of proteoforms. We showed further that ion exchange-based enrichment represents a relevant option for enriching other proteins of interest. However, for a rationally designed ion exchange-based enrichment procedure it is essential not to rely on the average, calculated isolectric point of a protein but to study its charge distribution along the three-dimensional orientation of the molecule.

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Appendix A. Supplementary data

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References


