Selective quantitative bioanalysis of proteins in biological fluids by on-line immunoaffinity chromatography–protein digestion–liquid chromatography–mass spectrometry

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Abstract

A quantitative method for the determination of proteins in complex biological matrices has been developed based on the selectivity of antibodies for sample purification followed by proteolytic digestion and quantitative mass spectrometry. An immunosorbent of polyclonal anti-bovine serum albumin (BSA) antibodies immobilized on CNBR agarose is used in the on-line mode for selective sample pretreatment. Next, the purified sample is trypsin digested to obtain protein specific peptide markers. Subsequent analysis of the peptide mixture using a desalination procedure and a separation step coupled, on-line to an ion-trap mass spectrometer, reveals that this method enables selective determination of proteins in biological matrices like diluted human plasma. This approach enhances substantially the selectivity compared to common quantitative analysis executed with immunoassays and colorimetry, fluorimetry or luminescence detection. Hyphenation of the immunoaffinity chromatography with on-line digestion and chromatography–mass spectrometry is performed and a completely on-line quantification of the model protein BSA in bovine and human urine was established. A detection limit of 170 nmol/l and a quantification limit of 280 nmol/l is obtained using 50 μl of either standard or spiked biological matrix. The model system allows fully automated absolute quantitative mass spectrometric analysis of intact proteins in biological matrices without time-consuming labeling procedures.
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1. Introduction

The quantitative bioanalysis of protein drugs in biological matrices is an important issue in early stage drug development. Different approaches for the absolute quantitative analysis of proteins in biological fluids have been reviewed by Hopfgartner and Varesio [1]. They emphasize that mass spectrometry (MS) has great potential for quantification of peptides and proteins in the off-line mode. Other groups used a stable isotope-incorporated peptide as internal standard for the absolute quantification of proteins and peptides [2,3]. Barnidge et al. [4] evaluated the use of tryptic cleavage sites incorporated

into an internal standard synthetic peptide. Storme et al. [5] performed absolute quantification of Cystatin C by analysis of tryptic peptide markers. Application of this approach is limited by laborious sample pre-treatment to remove biological matrices which has not been described in this work. Nelson et al. [6] used MALDI-MS for the quantification of proteins comprising an isotopically labeled internal standard. ESI-MS for the quantification of relatively small protein comprising a similar protein as internal standard derived from a different species that is mass shifted has been performed by Ji et al. [7]. Garbis et al. [8] amplify in their review the drawbacks of protein quantification procedures using MS. Generally, the procedures applied in the quantification of proteins are significantly different from those applied for the bioanalysis of small drug molecules. In fact, enzyme immunoassays are frequently used for the quantification of proteins in biological matrices [9,10], while combined liquid

chromatography tandem mass spectrometry (LC–MS/MS) and selected-reaction monitoring (SRM) is the method-of-choice for small molecules [11]. In enzyme immunoassays, which are considered to be selective, detection of protein drugs in, e.g., serum or plasma is usually performed using colorimetry, fluorescence or luminescence. However, these detection methods do not supply additional information on the target measured, and therefore strongly rely on the selectivity of the immunoassay. In this report, the development of an alternative approach for the quantitative bioanalysis of proteins is described. The fully automated method is based on an on-line coupling of immunoaffinity chromatography, proteolytic digestion, and LC–MS. In this way, absolute quantitation of the protein drug can be achieved with improved selectivity and certainty of identity.

Immunoaffinity chromatography (IAC) has frequently been used for selective sample purification, both off-line and on-line to the subsequent LC [12–15] separation. This approach was reviewed by Hennion and Pichon [16]. While in off-line enzyme immunoassays for protein drugs, often two or more antibody–antigen interactions are needed for capturing and detection [10], in IAC only one type of immobilized antibody is needed. The applicability of IAC as a selective sample-pretreatment method for protein drugs strongly depends on the ability to immobilize appropriate antibodies against the target protein drugs onto a chromatographic support and to pack IAC columns with sufficient efficiency. The IAC procedure comprises two separate steps: capturing of the analyte from the biological matrix and washing interferences away, and desorption of the captured analytes towards the next stage of analysis.

The on-line coupling of IAC to LC–MS was pioneered by Rule et al. [17,18] for the determination of small molecules, such as carbofuran in environmental samples. On-line IAC–LC and IAC–LC–MS are frequently performed via a short solid-phase extraction (SPE) trapping column to concentrate the eluate from the IAC column prior to injection into the LC. An eluent composition switch can be achieved in this stage as well, enabling the use of optimal eluent in both IAC and LC. The latter is especially important in LC–MS which poses stringent restrictions to the mobile-phase composition. In this case, SPE is used for the pre-concentration of peptides from the protein digest step (see below).

Performing quantitative bioanalysis of proteins by LC–MS results in additional challenges. First of all it should be emphasized that the procedure developed aims at absolute protein quantitation, thereby excluding the use of the elegant isotope-coded affinity-tag labeling (ICAT) currently frequently applied for relative quantitation in proteomics studies [19,20]. Challenges in the absolute quantitation of protein drugs are related to the need to achieve an efficient LC analysis of proteins and problems due to the formation of an ion envelope of multiply-charged ions in electrospray ionization-MS (ESI-MS). Although quantitative LC–MS analysis of a 10 kDa peptide using an internal standard was described by Ji et al. [21], this procedure seems to show various limitations. The relative abundance of the peaks in the ion envelope may be a delicate function of experimental parameters, which may hamper reliable quantitation [22].

In order to avoid such problems, an on-line proteolytic digestion on an immobilized trypsin column has been applied in this study [23,24]. For the resulting peptide mixture, simpler and more efficient chromatographic procedures can be applied and the peptide mass spectra are significantly less complex than the protein mass spectra.

In order to enable the use of the on-line trypsin column, two issues have to be addressed. First, the pH of the solution used to elute the analytes from the IAC column must be modified to comply with the conditions required for the protein digestion [25]. Secondly, after the digestion which is performed in a phosphate buffer, an SPE trapping column has to be applied to concentrate the eluate from the trypsin column, to desalinate the sample, and to switch from a phosphate to a formic acid containing eluent which is compatible with LC–MS [26].

Finally, LC–MS is applied to separate and detect the peptides from the mixture. The peptide map acquired in this way can in principle be used to confirm the identity of the protein, e.g., via database searching using Mascot software (Matrix Science Inc.), and/or via additional MS–MS or MS^n experiments [27]. More importantly, the peak areas of selected peptide fragments can, after adequate validation, be applied to quantify the amount of protein initially introduced into the system.

This paper describes the development of this hyphenated method. Initially, various steps of the procedure were separately developed and optimized. Preliminary evaluation and validation was performed for a system comprising IAC, off-line protein digestion, and SPE–LC–MS analysis of the digested protein samples, enabling the overnight analysis of different batches of samples, running IAC, protein digestion, and SPE–LC–MS in parallel. However, especially the off-line digestion step is a very time-consuming procedure requiring some manual sample and reagent manipulation. Finally, hyphenation of IAC and SPE–LC–MS via the on-column trypsin digestion was achieved.

2. Experimental

2.1. Reagents

Sodium di-hydrogen phosphate and glycine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Disodium hydrogenphosphate and bovine serum albumin (purity 97%, HPCE) were obtained from Fluka (Buchs, Switzerland) and sodium chloride and formic acid came from Riedel-de-Haën (Seelze, Germany). Acetonitrile and hydrochloric acid (36–38%) were supplied by J.T. Baker (Deventer, The Netherlands). Water was produced by a Millipore (Bedford, MA, USA) Milli-Q unit. Sodium hydroxide was obtained from Merck (Darmstadt, Germany). Proteins were digested using sequencing-grade modified trypsin (Promega, Madison, WI). A 2 l stock of a 10-fold concentrated PBS buffer was made by dissolving 57.30 g of $Na_2HPO_4 \cdot 12H_2O$, 137.99 g of NaH_2PO_4, 175.30 g NaCl and 4.03 g KCl in 2 l of water. This stock was used to prepare the PBS by diluting 100 ml of the concentrate with 900 ml of water. The pH was brought to the required value with 8 M sodium hydroxide or hydrochoric acid.

2.2. Columns

The immunoaffinity column was in-house built (3.0 mm i.d. × 15 mm, peek material). Column frits were purchased from VICI AG international (Peek alloyed with Teflon, PAT), 1/16 in. thick × 0.188 in. diameter, porosity 5 μm). The column was filled with a slurry of immunosorbent (Polyclonal antibody against BSA from rabbit, immobilized on agarose, particle size 45–185 μm, Sigma, St. Louis, MO, USA). Before packing, the column was closed on one end with a PAT frit. The column was filled with the gel, and the bed was allowed to settle for 10 min. After this, the supernatant was carefully removed using a Pasteur pipette without disturbing the settled surface. Agarose gel slurry was added again. These steps were repeated several times until the column was completely filled with the affinity material. The excess of material was removed. The column was closed using a second PAT frit, and subsequently placed into an in-house built column holder. The column was flushed with a PBS solution of pH 7.4 for 20 min at a flow rate of 0.2 ml/min.

Some experiments were done using a cyanogen bromide (CNBr)–glycine ester agarose column, which was packed with a sorbent, produced by allowing CNBr agarose (particle size 45–185 μm, Sigma, St. Louis, MO, USA) to react with glycine, resulting in a CNBr–glycine ester. This column was packed and treated the same way as the affinity column (described above).

The SPE cartridge (C18, 2.0 mm i.d. × 4.0 mm) used for pre-concentration and desalination and the analytical column (Luna C18(2), 2.0 mm i.d. × 150 mm) were supplied by Phenomenex (Torrance, CA, USA). The trypsin column used for the on-line procedure was a StyrosZyme column (2.1 mm i.d. × 50 mm), purchased from OraChrom, Inc. (St. Woburn, MA, USA).

2.3. Apparatus

For some experiments, pre-concentration of the protein was done with an Amicon cell using membranes with a 10 kDa cut-off supplied by Millipore (Bedford, MA, USA).

IAC was performed with a Shimadzu LC2010HT (Chiyoda-ku, Tokyo, Japan) liquid chromatograph. A post-column make-up flow was delivered by a Knauer HPLC Pump K-500 (Berlin, Germany). Fraction collection was done using a Gilson 234 auto injector (Middleton, WI, USA) which was adapted to serve as a fraction collector in combination with a MUST multiport switch from Spark Holland (Emmen, The Netherlands). The multiport switch was triggered using the contact closure module of the Shimadzu LC2010HT system. Samples were incubated in an in-house built water bath containing a Lauda-temp thermostat (Königshofen, Germany).

The SPE–LC–MS measurement was done using an Agilent Technologies (Palo Alto, CA, USA) 1100 capillary HPLC system (containing degasser, binary pump, temperature-controlled auto sampler and two temperature controllable column switches) in combination with an Agilent 1100 VL-series MSD ion trap mass spectrometer. For re-equilibration purposes of the analytical column, a Gilson 302 (Middleton, WI, USA) HPLC pump was used in combination with an in-house built pulse-damper.

On-line pH measurements were performed by connecting the Shimadzu 2010HT system to a pH flow cell of a Pharmacia Äkta explorer (Uppsala, Sweden) HPLC system.

2.4. Immunoaffinity chromatography

A detailed scheme of the off-line IAC set-up can be found in Fig. 1A. The IAC column was operated by pump 1 at a flow rate of 0.2 ml/min unless specified otherwise. The column was conditioned using A: PBS solution of pH 7.4. Bovine serum albumin (BSA) from standard solutions and biological samples was trapped by injecting a volume of 50 μl (Injector 1). After 2 min, the column was flushed with 1 ml of B: 0.15 M sodium chloride solution to remove phosphates and matrix components to waste. After this, BSA was eluted from the column for 10 min with C: 0.15 M sodium chloride and 20 mM glycine hydrochloride, pH 2.7. A post column make-up flow of D: PBS solution at pH 10.8 was delivered by pump 2 at a flow rate of 0.2 ml/min. The MUST valve (valve 2) was switched to collect 0.64 ml of the eluate containing BSA. After elution, the column was re-equilibrated. The total run-time of this step was 19.5 min.

2.5. Off-line digestion procedure

The fraction collected was homogenized with a stirrer for a few seconds. Two hundred microliters of the fraction was digested by adding 5 μl of trypsin (concentration 0.1 mg/ml in PBS solution of pH 7.4) and incubation in a water bath at 37 °C. After 14 h, the reaction was stopped by adding 5 μl of formic acid. In most cases, this step was performed batch wise and overnight with three samples collected from IAC.

2.6. Desalination and separation

A detailed overview of the SPE–LC–MS system can be found in Fig. 1A. After digestion, the samples were kept at 4 °C in the Agilent autosampler (Injector 2) prior to analysis. Gradient elution was performed using two eluent solutions, E: acetonitrile/water (5/95, v/v) containing 0.2% formic acid and F: acetonitrile/water (95/5, v/v) containing 0.2% formic acid. Pump 4 was used at a flow rate of 0.2 ml/min. The SPE cartridge was conditioned for 12 min using solvent E: acetonitrile/water (5/95, v/v) containing 0.2% formic acid. One hundred microliters of the sample were injected onto the column and desalination was done by washing for 2 min with the same solvent. After this, the SPE cartridge was switched on-line with the analytical column by switching valve 4. The analytical column was equilibrated before with pump 5 at a flow of 0.2 ml/min for 12 min using solvent E. A solvent gradient with pump 4 was run from 0 to 30% solvent F in 22 min. After analysis, both columns were flushed with 95% of this solvent for 3 min and subsequently re-equilibrated using solvent E.

2.7. On-line digestion procedure

A detailed schematic view of the on-line procedure can be found in Fig. 1B. The starting position of the valves was as
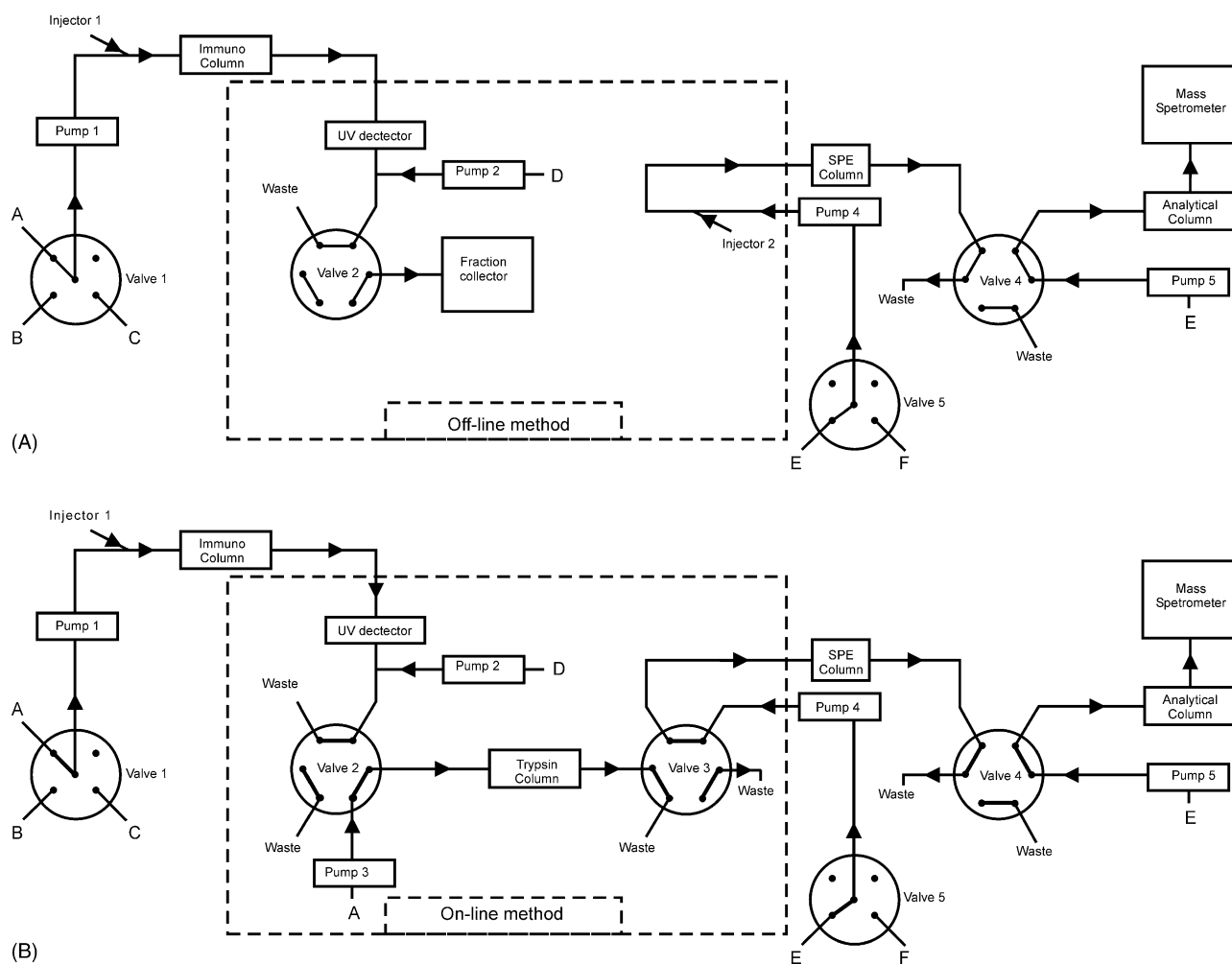
Fig. 1. Schematic overview of the off-line procedure (A) and the on-line procedure (B).

depicted in the Figure. The IAC column and the trypsin column were conditioned using A: PBS solution of pH 7.4, and the SPE cartridge and the analytical column were conditioned using solvent E: acetonitrile/water (5/95, v/v) containing 0.2% formic acid, all at a flow rate of 0.2 ml/min. Fifty microliters of sample or standard were injected onto the IAC column and the column was washed with solution A: PBS solution of pH 7.4 for 2 min, subsequently the column was washed with 1 ml of B: 0.15 M sodium chloride solution. After this, valves 2 and 3 were switched and albumin was eluted using C: 0.15 M sodium chloride and 20 mM glycine hydrochloride (pH 2.7) at 0.05 ml/min. Pump 2 was also operated at a flow of 0.05 ml/min, delivering solution D: a PBS solution of pH 10.8. The overall flow rate over the trypsin column, thermostated in a column oven at 37 °C, thus was 0.1 ml/min. After 15 min, the SPE column containing peptides from the trypsin column was desalinated for 5 min by switching back valves 2 and 3 and by using gradient pump 4 with solvent E: acetonitrile/water (5/95, v/v) containing 0.2% formic acid. Subsequently, valve 4 was switched and a gradient was run at a flow rate of 0.2 ml/min to 30% solvent F: acetonitrile/water (95/5, v/v) containing 0.2% formic acid in 22 min. After analysis, the SPE and analytical columns were flushed with 95% of this solvent for 3 min. The procedure results in a total run

time of 55 min including reconditioning of all columns. However, because the IAC analysis can already be started during the re-equilibration of the SPE cartridge the total analysis run time in a series of samples is 47 min per sample.

2.8. Mass spectrometry

Positive ESI-LC–MS was performed in full-spectrum acquisition mode in the range of m/z 400–1500. The spray voltage was set at 4.5 kV with a drying temperature of 350 °C, nitrogen drying gas flow at 8 l/min and nebulizer pressure at 40 psi. The maximum accumulation time was 100 ms and the ICC target was set to 30,000, three spectra were averaged. Data processing was performed using LC/MSD trap software version 5.2, build 382.

2.9. Quantification procedure

For quantification purposes, a standard series of protein concentration was analyzed in triplicate. From these standards, a dilution series were made, resulting in a calibration curve with a concentration between 15 μmol/l and 300 nmol/l. Fifty microliters of the spiked biological matrices or samples were injected

and reconstructed extracted ion chromatograms were generated for relevant *m/z* values and the peak was integrated. Unless mentioned otherwise, BSA tryptic fragment T 78 (LVVSTQ-TALA) at *m/z* 1002.6 was used for analysis. The counts of the integrated peak were analyzed by linear regression by using chemometric software (Origin (Northampton, MA, USA) version 6.1052).

3. Results and discussion

Initially, the analytical system was developed as three separate units, i.e., the immunoaffinity chromatography part, the protein digestion part, and the SPE–LC–MS part. Each unit was developed while keeping in mind the need for final on-line coupling, which is for instance reflected by the choice of solutions and time schedules. The quantitative bioanalysis with the system consisting of the three separate steps was first tested and validated before the three steps were hyphenated into one system, which was tested and validated again. Given the complexity of the complete system, development and optimization of the separate steps are described in detail as well.

Although the system was developed for the bioanalysis of protein drugs, the present study describes the bioanalysis of bovine serum albumin (BSA) as a model protein. The relatively low costs of this target molecule as well as of the immunoaffinity material makes BSA highly suitable for method development and initial validation.

3.1. Immunoaffinity chromatography

The capacity of the column prepared for the analysis of the model protein BSA was determined by a binding-capacity curve (Fig. 2). For this, the elution peak area from the UV chromatogram (Fig. 3) was plotted against the amount of BSA injected. The resulting binding-capacity curve shows a linear part and a plateau with a significantly lower slope. The capacity can be easily estimated by calculating the amount of BSA eluted within the linear part. The capacity was determined to be 1215 nmol corresponding to 81 μg. In the IAC-UV chro-
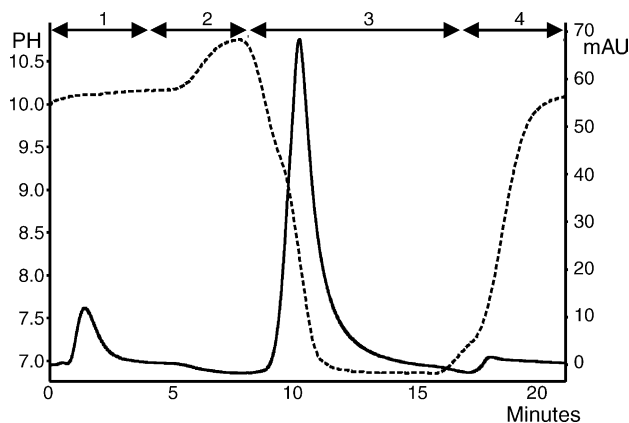
Fig. 3. Solid line: monitoring of the UV (280 nm) of the IAC procedure. Forty micrograms injection of BSA in 40 μl PBS (pH 7.4). (1.3 min: elution of impurities and/or BSA aggregates, 10.4 min: elution of BSA). The dashed line represents the pH generated by the mixture of make-up solution (PBS, pH 10.8) with (1) binding and washing of sample using PBS (pH 7.4), (2) washing with 0.15 M NaCl, (3) elution of BSA from the column using 20 mM glycine hydrochloride with 0.15 M NaCl and (4) re-equilibration of IAC column with PBS (pH 7.4).

matogram (280 nm, Fig. 3), initial elution occurs shortly after injection of the BSA sample, while a major peak is observed at the expected time in the chromatogram. Since a 597 pmol injection of BSA in 40 μl PBS solution of pH 7.4 is within the range of the column capacity, the influence of the concentration of the sample in IAC was studied. For this, a 20 μl sample containing 30 μmol/l BSA was injected and the area of the eluted peak was compared to a 100 μl injection containing 6 μmol/l BSA. The elution peaks were found to have similar peak areas with a relative standard deviation lower than 2%. This indicates that the sorption reaction at the immunoaffinity material is sufficiently fast under the conditions chosen. Therefore, the initial elution peak is most likely due to impurities and/or presence of BSA aggregates. The latter possibility was studied by collecting this peak, and re-concentrating the fraction 10-fold in an Amicon cell using a membrane with a cut-off of 10 kDa. From the supernatant, 200 μl were digested for five hours at 37 °C with 5 μl trypsin solution (0.1 mg/ml in PBS solution of pH 7.4). The reaction was stopped by addition of 5 μl formic acid, and the resulting mixture was injected on the SPE–LC–MS system. Peptides from BSA were found in low abundance, which were not present in blank samples. A possible explanation for this phenomenon is the presence of BSA aggregates that are not recognized by the immobilized antibodies. However, further studies are necessary to elucidate the structure of the unretained compound.

The specificity of the interaction of BSA with the polyclonal antibodies in the column was verified by comparing UV chromatograms obtained in the IAC procedure with either the anti-BSA agarose column or a CNBr–glycine ester agarose column. No measurable non-specific interaction with the Agarose material was found (data not shown).

For fraction collection of the BSA elution, the repeatability of the elution time and the peak area are important parameters. This was tested by a 12-fold injection of 40 μg BSA. Elution time shift was lower than 0.1% and deviation of the peak area was
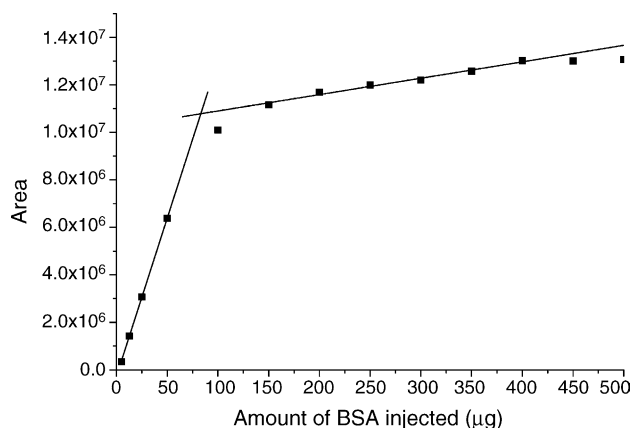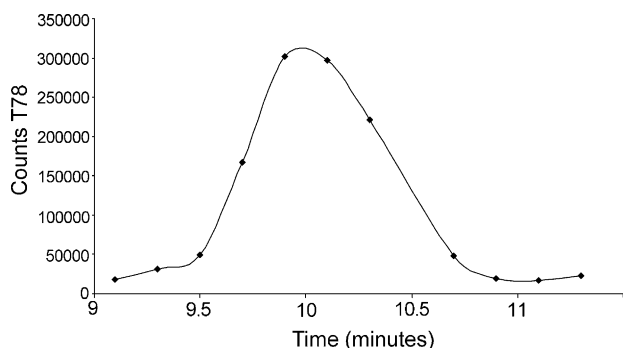

Fig. 2. Binding-capacity curve of an anti-BSA column measured by the amount of BSA injected as a function of the elution peak area using UV detection at 280 nm.

Fig. 4. Elution profile of BSA; area of peptide T78 (LVVSTQTALA) found at *m/z* 1002.5 from SPE–LC–MS chromatogram is illustrated.

lower than 2.5%. The affinity material did not show significant loss of capacity after 400 cycle runs.

3.2. Off-line digestion of IAC fraction

For these studies, the UV-cell was removed and samples were collected directly. The fractionation time was optimized by collecting small fractions from the IAC elution procedure. Forty microliters samples (corresponding to 0.2 min) were collected in 88 μl PBS solution of pH 7.4, 2 μl trypsin (0.1 mg/ml in PBS solution of pH 7.4) were added and the samples were incubated overnight (14 h) on a 96-well microplate. The digestion reaction was stopped by adding 5 μl of formic acid. Fifty microliters of this sample was injected onto the SPE–LC–MS system. From the resulting elution profile, acquired by monitoring the tryptic BSA fragments T78 (LVVSTQTALA) (Fig. 4), T34 (AEFVEVTK), T18 (YLYEIAR) and T72 (QTALVELLK), it was decided to collect the fraction eluting between 9.2 and 10.8 min.

The pH is a rather critical parameter for proteolytic reactions, with pH 6–9 being acceptable [25]. Therefore, the pH of the make-up flow was optimized by performing IAC with on-line measurement of the pH using a pH flow-cell. The pH shown in the chromatogram (see Fig. 3) is a complex function of the different solutions used during the IAC procedure and post-column mixed in a 1:1 ratio with the make-up buffer consisting of PBS solution at pH 10.8. In the first step (1), the sample was injected in PBS solution of pH 7.4. In the second step (2), the sample is thoroughly washed with 0.15 M sodium chloride solution. In step 3, BSA is eluted from the IAC column using elution buffer, consisting of 0.15 M sodium chloride and 20 mM glycine

hydrochloride, pH 2.7. After this, the column is re-equilibrated for the next run (4) using PBS solution of pH 7.4.

3.3. SPE–LC–MS of digest products

The repeatability of the SPE–LC–MS procedure was checked by 20 repetitive injections of a mixture of the tryptic peptides from BSA. Reconstructed extracted ion chromatograms were made and peaks from peptide T78 at *m/z* 1002.6 were integrated. From this, a Shewart [28,29] control chart was calculated to monitor the SPE–LC–MS procedure, including the chromatographic repeatability, MS-source contamination and sample stability at given conditions. A test according to Neumann [28,30] revealed that there is no significant trend in the peak areas measured, suggesting a random distribution of errors. The same sample was injected as a control sample in triplicate prior and after analysis of a series of samples and standards. Measurements were considered to be valid if not more than one measurement in twenty was out of order, i.e., outside 2 s borders in the Shewart control chart. Two LC–MS chromatograms of BSA digests are given in Fig. 5. Repeatability of the areas of peptide peaks from reconstructed extracted ion chromatograms was found to be 95%.

3.4. Combining IAC with SPE–LC–MS via off-line digestion

The repeatability of the entire off-line procedure was found to be better than 85% (*n* = 10) by treating 300 pmol of BSA using IAC, off-line digestion and subsequent SPE–LC–MS. Linearity of the BSA analysis was estimated to be between 0.1 and 15 μmol/l for a 50 μl sample injection onto the IAC column. Total processing time of a single sample including IAC, off-line digestion (incubation time was kept constant at 14 h) and SPE–LC–MS procedure was approximately 16 h. When performing quantitative analytical measurements including calibration curve and sample measurements, continuous manual handling was needed because the reaction time of the trypsin with BSA had to be kept constant. In principle, an overall sample throughput of 15 samples per day could be achieved.

In order to perform a preliminary test of the selectivity of the method, BSA was spiked in untreated, undiluted human urine and the samples were analyzed. Accuracies of the analysis of spiked matrices were calculated from a calibration curve consisting of ten data points, triple measurements were done for
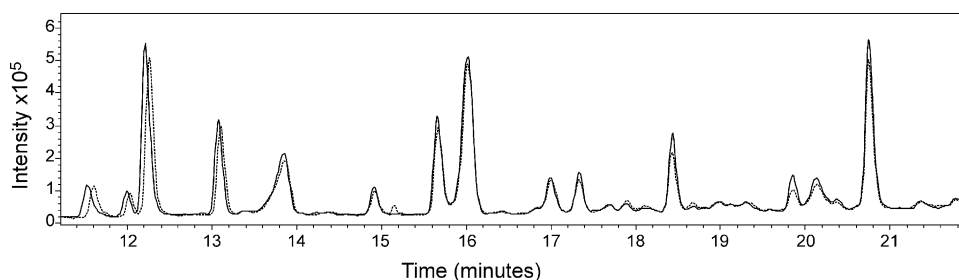

Fig. 5. LC–MS separation of digest products from BSA. Solid trace: TIC (total ion chromatogram) from bovine urine sample spiked with 2.2 μmol/l BSA, dashed trace: TIC from PBS (pH 7.4) containing 2.0 μmol/l BSA.

each point. Accuracies of 95% were found for a urine sample spiked with 10 μmol/l, 82% for a sample spiked with 7 μmol/l and 84% for a sample spiked with 3 μmol/l, respectively.

Alternatively, the possibility to apply this system to plasma samples has been shown by spiking human plasma with BSA. Human serum albumin (HSA) showed cross-reactivity with the immobilized antibodies. Nevertheless, this cross-reactivity is not a point of concern since BSA and HSA differ significantly in amino acid sequence. No tryptic fragments isobaric with the BSA T78 fragment were found in an HSA digest. In this case, SPE–LC–MS analysis of the peptides allowed us to distinguish between tryptic peptides from HSA and BSA. Prior to injection, samples were 50% diluted with a PBS solution of pH 7.4. Accuracy of a spiked sample was 98% for a 50 μl sample injection containing 12 μmol/l BSA.

3.5. Hyphenation of the methods

The off-line procedure as described above is very time-consuming and labor-intensive. It also requires an accurate time schedule for constant incubation times of the samples. It is therefore attractive to combine the three separate steps (IAC, digestion and SPE–LC–MS) into one single hyphenated system. This enables the unattended overnight analysis of series of samples. The key issue for hyphenation is having the proteolytic digestion procedure on-line. For on-line digestion, cartridges are available containing immobilized trypsin on a chromatographic support. In this study, an immobilized TPCK trypsin on a simulated monolithic bed cartridge was used. The continuous-flow digestion mode was chosen instead of a stop-flow procedure because of three reasons. Firstly, the monolithic material presents large mesopores, facilitating fast transfer of macromolecules through the monolith [23], thus enabling high exposure of the protein to the immobilized trypsin. Secondly, Wang and Regnier [31] reported no observable difference in proteolyic efficiency on such a column between the constant flow and stopped flow mode. Finally, there is no need to synchronize the interruption of the flow through the column with the elution time of the samples that are inside the trypsin column [24]. The temperature was found to be a crucial factor for the digestion efficiency. Therefore, a column oven was used to keep the temperature of eluents and the trypsin column constantly at 37 °C. The IAC procedure was performed using conditions previously optimized for the off-line approach (see Section 2), except for the flow rate of the eluent which was lowered to 50 μl/min gaining a total eluent flow rate over the trypsin column of 0.1 ml/min after addition of the make-up flow. By reducing the linear flow rate through the trypsin column, a somewhat higher exposure of the BSA to the immobilized trypsin on the trypsin column was achieved. A concentration series of BSA in PBS solution of pH 7.4 were injected onto the on-line system for a calibration curve. Linearity was shown within a range of 172 nmol/l and 15 μmol/l injecting 50 μl sample volumes. A sample throughput of 30 samples per day was achieved in this way compared to 15 samples per day using the off-line method. The on-line method reduced the risk of sample degradation or contamination due to manual sample handling, and enables unattended overnight operation.

The accuracies of BSA spiked in untreated, undiluted human urine samples were calculated from the calibration curve which resulted in an equation of $y = 1556x + 2280$, an R^2 of 0.9655 and statistical F-test indicates that a calibration of the first order is significant ($N = 18$, $P = 95\%$). Accuracies in spiked, untreated and undiluted human urine were 93% for a 50 μl sample containing 4 μmol/l, and 95% for a 50 μl sample containing 15 μmol/l BSA in 50 μl. These accuracies indicate that it is possible to perform a reliable quantitative analysis of a protein in a complex biological matrix. The detection limit of the method is 170 nmol/l with a quantification limit of 280 nmol/l.

3.6. Recovery

The overall recovery of the on-line system as described in Section 2 was evaluated by comparing an off-line digested sample with on-line treated samples using T78 at *m/z* 1002.6 as test fragment. For this, a 1.5 μmol/l BSA solution was made in PBS solution of pH 7.4. Trypsin was added in a 1:20 mass-to-mass ratio and a sample was incubated in the Agilent autosampler which was thermostated at 37 °C. After preparing this sample, the off-line digestion reaction was continuously monitored by permanent injection of 50 μl onto the SPE–LC–MS (Fig. 1A) system. Conditions were the same as described in Section 2. The relative area of the peak from peptide T78 was found to increase until 15 h of incubation. From this moment, the area of this peak did not change significantly which indicates a completed digestion of the protein.

This peak area was then compared to the peak area resulting from a 50 μl duplicate injection of the 1.5 μmol/l BSA solution on the fully on-line system (Fig. 1B) with conditions given in Section 2. The relative peak areas of the reconstructed extracted ion chromatogram of peptide T78 from the off-line digested sample and the on-line measured sample were compared.

Despite of the complexity of this hyphenated system, a recovery of 4.0% of the tryptic fragment was found. This rather low recovery can entirely be attributed to the on-line digest process, since recoveries for immunoaffinity extraction and on-line SPE of the peptide fragments were 90 and 95%, respectively. It has to be further investigated if there are possibilities to improve this recovery, e.g., by increasing the temperature of the on-line digest and the use of different proteolytic enzymes. The reproducibility of the on-line system was found to be better than 15%, which was studied by 20 μl duplicate injections of five separately prepared samples containing 15 μmol/l BSA dissolved in PBS solution of pH 7.4.

3.7. Application to bovine urine samples

Four different bovine urine samples were spiked with different amounts of BSA and subsequently analyzed. The concentrations in spiked samples were calculated from the calibration curve and the accuracy was calculated. A comparison of a resulting total ion current of a spiked urine sample compared to BSA dissolved in PBS solution of pH 7.4 can be found in Fig. 5. The average accuracy was 104% with a relative standard deviation

of 3% between the four urine samples spiked with 2.2 µmol/l BSA, and 95% with a relative standard deviation of 6% for the urine samples spiked with 1.5 µmol/l BSA. Finally, the four different bovine urine samples were analyzed for the presence of BSA. No BSA was found, suggesting that the concentration of BSA is lower than 170 nmol/l, which may be in accordance with a study of Nakajima et al. [32], who showed a concentration of 307 nmol/l in bovine urine. However, in this off-line procedure the sample volume was 50 ml and the relative standard deviation was ca. 50%.

4. Conclusions

A selective on-line quantitative procedure for the analysis of proteins in biological samples is described where immunoaffinity chromatography using immobilized antibodies is combined with mass spectrometric detection using a proteolytic digestion. Accuracies of spiked untreated and undiluted human and bovine urine were good. The possibility to use the system for analysis of BSA in both urine and plasma samples was demonstrated. Despite of the relatively low recoveries, the major advantages of this on-line system are speed and minimized sample handling and tagging procedures, still providing detection limits of about 200 nmol/l.

Establishment of the on-line system significantly simplified the sample pre-treatment procedure. It greatly reduces the risk of sample degradation and/or contamination resulting from manual transport of samples between the various steps. Finally a significant improvement of analysis time in comparison with the off-line approaches has been obtained.

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