Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 859 (2007) 147–156
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Automated detection of covalent adducts to human serum albumin by immunoaffinity chromatography, on-line solution phase digestion and liquid chromatography–mass spectrometry

Johannes S. Hoos [a,*,1], Micaela C. Damsten [b,*,1], Jon S.B. de Vlieger [b], Jan N.M. Commandeur [b], Nico P.E. Vermeulen [b], Wilfried M.A. Niessen [a], Henk Lingeman [a], Hubertus Irth [a]

[a] *Vrije Universiteit Amsterdam, Faculty of Sciences, Section Analytical Chemistry & Applied Spectroscopy, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*
[b] *Vrije Universiteit Amsterdam, Faculty of Sciences, Section Molecular Toxicology, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands*

Received 25 May 2007; accepted 12 September 2007
Available online 19 September 2007

Abstract

A generic method for the detection of covalent adducts to the cysteine-34 residue of human serum albumin (HSA) has been developed, based on an on-line combination of immunoaffinity chromatography for selective sample pre-treatment, solution phase digestion, liquid chromatography and tandem mass spectrometry. Selective anti-HSA antibodies immobilized on agarose were used for sample pre-concentration and purification of albumin from the chemically produced alkylated HSA. After elution, HSA and HSA adducts are mixed with pronase and directed to a reaction capillary kept at a digestion temperature of 70 °C. The digestion products were trapped on-line on a C18 SPE cartridge. The peptides were separated on a reversed-phase column using a gradient of organic modifier and subsequently detected using tandem mass spectrometry. Modified albumin samples consisted of synthetically alkylated HSA by the reactive metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), and using the alkylating agent 1-chloro-2,4-dinitrobenzene (CDNB) as reference. The resulting mixture of alkylated versus non-modified albumin has been applied to the on-line system, and alkylation of HSA is revealed by the detection of the modified marker tetra-peptide glutamine–cysteine–proline–phenylalanine (QCPF) adducts NAPQI-QCPF and CDNB-QCPF. Detection of alkylated species was enabled by the use of data comparison algorithms to distinguish between unmodified and modified HSA samples. The in-solution digestion proved to be a useful tool for enabling fast (less than 2 min) and reproducible on-line digestion of HSA. A detection limit of 1.5 μmol/L of modified HSA could be obtained by applying 10 μL of NAPQI-HSA sample.

Keywords: Affinity; On-line; Pronase; Protein adduct formation; Digestion; Protein; Albumin; LC–MS; NAPQI

1. Introduction

Covalent binding of reactive electrophilic metabolites of drugs to proteins has been suggested to be involved in the onset of serious adverse drug reactions (ADRs) and idiosyncratic drug reactions (IDRs) in humans [1]. Therefore, methods that can detect covalent adducts to proteins are of great interest for risk assessment purposes, e.g. during the development of novel drugs and/or drug candidates [2,3]. Several methodologies exist for the quantitative and qualitative analysis of protein adducts [4]. Briefly, these methods first consist of the isolation of protein adducts with chromatographic and/or electrophoretic techniques (e.g. immunoaffinity chromatography, ion-exchange chromatography or HPLC), and the subsequent adduct analysis using immunological, radioactivity and/or mass spectrometry methodologies [5]. Although successful to some extent, these techniques usually remain labor-intensive and none of these answers the need for automated on-line isolation and detection of protein adducts.

Recently, we have described a concept where human serum albumin is used as an *in vivo* biomarker for bioactivation of

* Corresponding authors. Tel.: +31 20 5987536; fax: +31 20 5987543.
E-mail addresses: hoosj@hotmail.com (J.S. Hoos), Damsten@few.vu.nl (M.C. Damsten).
[1] Both authors contributed equally to this work.

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.09.015

drugs towards reactive intermediates and for covalent binding to proteins [6]. The methodology consists of the isolation of albumin from blood, its digestion with pronase and the LC-Tandem MS detection of alkylated and/or unalkylated peptides containing the free cysteine-34 residue of HSA. This site is the only free cysteine residue of albumin and has already been shown to be alkylated by several electrophilic compounds [7–12].

In the present paper, we demonstrate the full automation of the mass spectrometric detection of covalent adducts to the cysteine-34 residue of human serum albumin, integrating several sample pre-treatment and separation steps. First, immunoaffinity chromatography (IAC) was performed for selective purification of albumin from the alkylated HSA samples using specific antibodies against human serum albumin. The modified and non-alkylated albumin was retained and concentrated by immobilized antibodies while matrix components (i.e. remains from the alkylation reaction) can be flushed to waste. Purified albumin was subsequently eluted using a low pH buffer and further digested using pronase which is known to form small peptides containing the cystein-34 residue of HSA [6]. In this way, adducts to cysteine-34 could be monitored by LC–MS analysis on relatively small peptides. Pronase consists of a mixture of different enzymes, and cleaves in a relatively non-specific way compared to trypsin, chymotrypsin and pepsin. Recently, on-line digestion using immobilized enzyme reactors (IMERs) has been reported in various research areas [13–16] and trypsin is most frequently applied for proteolysis. Developments and applications of IMERs immobilized with trypsin have been reviewed by Massolini and Calleri [17]. Using IMERs is a very elegant way to perform on-line digestion; the reactors are relatively stable and re-usable when used under suitable conditions. Although the first application of a pronase immobilized IMER has been shown recently by Temporini et al. [18], their applicability is limited by the ability to immobilize the proteolytic enzyme without affecting its activity. Furthermore, the validation and characterization of IMERs prior to application in quantitative protein bioanalysis is a delicate task. In the present paper, we report the use of an on-line, pre-column solution phase digestion method that overcomes the need for immobilization. Recently, a comparable approach has been utilized in our group to perform in-flow digestion using pepsin for post-column digestion of protein mixtures [19]. We have integrated the solution phase digestion module in an automated methodology that allows the detection of NAPQI-QCPF and CDNB-QCPF, i.e. two peptide adducts formed after pronase digestions of HSA that previously was incubated with the chemically produced reactive metabolite of acetaminophen (NAPQI) and with the direct alkylating agent CDNB (1-chloro-2, 4-dinitrobenzene).

2. Experimental

2.1. Reagents

Sodium di-hydrogen phosphate, glycine hydrochloride, silver nitrate, human serum albumin (HSA), 1-chloro-2,4-dinitrobenzene, Tris base, Tris hydrochloride and pronase (protease type XIV from Streptomyces griseus, EC 3.4.24.31) were purchased from Sigma (Germany). Activity of the enzyme was 5.2 units per mg solid (units in amount of enzyme activity which will catalyse the transformation of 1 μmol of the substrate per minute under conditions given by the supplier). Disodium hydrogenphosphate was obtained from Fluka (Buchs, Switzerland) and sodium chloride and formic acid came from Riedel-de-Haën (Seelze, Germany). Acetonitrile, acetone, diethyl ether, chloroform, potassium chloride and hydrochloric acid (36–38%) were supplied by J.T. Baker (Deventer, The Netherlands). Water was purified by a Millipore (Bedford, MA, USA) Milli-Q unit. Sodium hydroxide was obtained from Merck (Darmstadt, Germany). 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 resulting PBS buffer consists of 10 mM sodium phosphate, 150 mM sodium chloride and 3.4 mM potassium chloride. The pH was set to the required value with 8 M sodium hydroxide or hydrochloric acid.

2.2. Synthesis of albumin adducts

A general scheme of the synthesis of the reactive intermediate of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), and of the formation of NAPQI-HSA adducts is depicted in Fig. 1a. For the formation of NAPQI, fresh silver oxide was synthesized as follows [20]. While stirring, 50 mL of a 2 M sodium hydroxide solution was added to 12.5 mL of a 0.4 M silver nitrate solution. The reaction mixture was kept on ice and stirred for 30 min. Isolation of silver oxide was performed by filtration over a Büchner funnel and the product was subsequently washed with 60 mL water, 60 mL acetone and 60 mL diethyl ether. The freshly prepared silver oxide was added to 10 mL of a 6.6 mM acetaminophen solution in chloroform and purged with nitrogen. The glass vial was sealed and the solution was stirred for 1.5 h at room temperature. After filtration of the mixture, 4 mL of the yellowish NAPQI solution was added drop wise under stirring to 5 mL of HSA (2.5 mg/mL) in PBS (pH 7.4). This mixture was stirred for 1 h at room temperature. The layers were allowed to settle for 10 min and the water layer containing NAPQI-HSA was stored at −80 °C until further analysis.

As NAPQI can also react with other nucleophilic residues of HSA (s.a. lysines and histidines), the degree of alkylation of the free cys-34 residue of NAPQI-HSA was assessed. Two HSA syntheses were performed in parallel as described above; one HSA solution (2.5 mg/mL) was reacted with 4 mL of NAPQI (in chloroform) and one HSA solution (2.5 mg/mL) with 4 mL of chloroform. After 1 h of reaction, water layers were isolated and free thiols were determined using a modification of Ellman's method [21]. Ellman reagent consisted in 10 mM DTNB in buffer 7.0 (100 mM NaH_2PO_4 containing 0.2 mM EDTA, adjusted to pH 7). Briefly, HSA samples were prepared in PBS (137 mM NaCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4 and 1 mM EDTA, adjusted to pH 7.4) by mixing 1000, 800, 600, 400, 200 and 0 μL of the HSA syntheses with PBS to a final volume of 1000 μL. Then, 200 μL of a strong buffer (100 mM boric acid

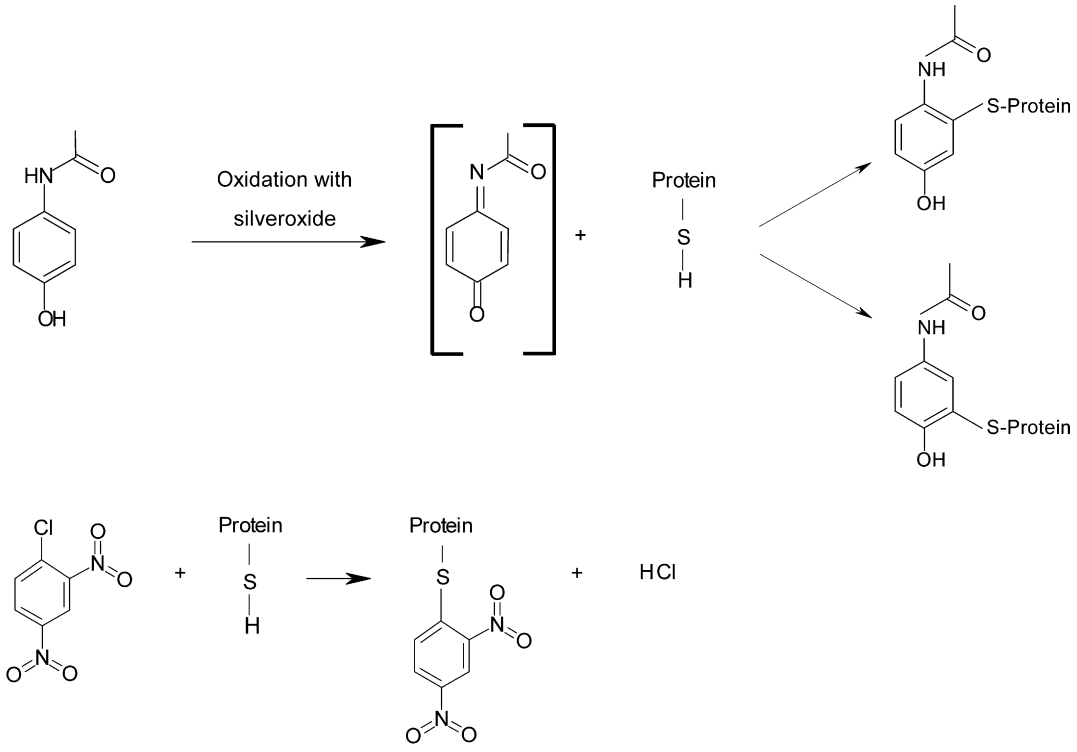

Fig. 1. (a) Synthesis of NAPQI-HSA protein adduct. From left to right: acetaminophen is oxidized by silver oxide to the reactive NAPQI intermediate (between brackets). NAPQI can subsequently react with the free cysteine-34 residue of HSA to form two possible NAPQI-HSA adduct isomers. (b) Synthesis of CDNB-HSA protein adduct. 1-Chloro-2,4-dinitrobenzene (CDNB) reacts with the free cysteine-34 residue of HSA to form the CDNB-HSA adduct.

and 0.2 mM EDTA, adjusted to pH 8.2 with NaOH) and 20 μL of Ellman reagent were added to each HSA sample. The mixtures were immediately vortexed, incubated for 15 min at room temperature and the absorbance was read at 412 nm (A_s) on a Pharmacia Biotech Ultrospec 2000 UV/Vis spectrophotometer. Control incubations consisted of incubations performed without HSA sample (A_p) and/or without Ellman reagent (A_{er}). The amounts of free thiols in the samples were determined according to Eq. (1) [21]:

$$\text{Mol SH} = \frac{0.00122L \times (A_s - A_p - A_{er})}{\Delta\varepsilon_{413} \times 1\,\text{cm}} \quad (1)$$

with

$$\Delta\varepsilon_{412} = 14150\,\text{M}^{-1}\,\text{cm}^{-1}$$

All measurements were performed in triplicate.

It was found that both HSA (Abs = 0.0562 [HSA] (in mg/mL)) and NAPQI-HSA samples (Abs = 0.0041 × [HSA] (in mg/mL)) gave a linear response in the dilution series described above (data not shown). Standard deviations were consistently below 1.5% absorbance units. From Eq. (1), the free thiol concentration measured in the HSA sample was determined to be 0.32 ± 0.002 mol SH/mol HSA, which is in agreement with previous data from literature [22]. The degree of alkylation of the cys-34 residues of NAPQI-HSA was determined with the ratio of the slopes of HSA versus NAPQI-HSA. Consequently, it was found that NAPQI-HSA contained approximately 7.3% of free thiols compared to the HSA sample, which was set to 100% as reference. This suggests that more than 90% of the cys-34 residues of HSA have been alkylated by NAPQI and that the NAPQI-HSA synthesis can be considered as complete.

The CDNB-HSA adduct was produced by reacting HSA with the direct alkylating agent 1-chloro-2, 4-dinitrobenzene (CDNB). The reaction scheme is shown in Fig. 1b. Briefly, a total 80 μL of a 100 mM CDNB solution in methanol was added drop wise under continuous stirring to 1 mL of a 5 mg/mL solution of HSA in 100 mM Tris buffer (pH 8.0). The mixture was allowed to react for 4 h at 40 °C. The obtained yellowish solution was subsequently stored at −80 °C until further analysis.

2.3. Columns

The immunoaffinity chromatography column was produced in-house (1.0 mm × 15 mm I.D., peek material). Column frits were purchased from VICI AG international (peek alloyed with Teflon, PAT, 1/32″ thick × 1/16″ diameter, porosity 5 μm). Immunoaffinity material was used from the HSA removal kit purchased from Vivascience (Sartorius, Goettingen, Germany). This kit includes agarose slurry containing immobilized antibody fractions for specific removal of albumin from biological samples. The material (particle size, 45–185 μm) was used for on-line sample pre-treatment in this research. The column was filled with the anti-HSA agarose slurry using an in-house built packing device, which allows packing under low-pressure conditions. This packing device consisted of a column holder and a connector fitting with a common luer tip connection. Before packing, the column was closed on one end with a PAT frit. The column was packed with the slurry using a 1 mL syringe and

the excess of material was carefully 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 PBS (pH 7.4) for 20 min at a flow rate of 0.040 mL/min.

The SPE cartridge (C18, 5 μm particles, 2.0 mm × 4.0 mm I.D.) used for pre-concentration and desalination and the analytical column (Luna C18(2), 5 μm particles, 2.0 mm × 150 mm I.D.) were both supplied by Phenomenex (Torrance, CA, USA).

2.4. Apparatus

IAC was performed with a binary pump (Agilent, Amstelveen, The Netherlands) in combination with a MUST multiport switch from Spark Holland (Emmen, The Netherlands) which was triggered using a contact closure of the same Agilent binary pump. Elution was done using a Gilson 302 (Middleton, WI, USA) pump and enzyme solution was delivered by a Knauer (Berlin, Germany) pump. Injection was done by a temperature controllable autoinjector from Agilent which was kept at 8 °C. Continuous flow digestion was performed by using an in-house built tee-piece infusing both elution solution and pronase solution into a reaction capillary (PEEK, polyetheretherketone) with an I.D. of 0.50 mm and an inner volume of 140 μL and was supplied by Bester BV (Amstelveen, The Netherlands). The reaction capillary was kept inside a column oven to control the digestion reaction temperature. Analysis of the reaction mixture was performed by switching the reaction capillary in-line with a SPE cartridge which was equilibrated by a second Agilent 1100 (Amstelveen, The Netherlands) micro-flow pump. This pump also performed separation of the digest products including a gradient over an analytical column, which was switched in-line with the SPE column after desalination. Both the latter switching events were operated by two temperature controlled column switches from Agilent. All Agilent apparatus were controlled by Chemstation version 10.02, and analysis was done by an Agilent 1100 MSD VL-series ion-trap. Positive ESI LC–MS was performed in full-spectrum acquisition mode in the range of *m/z* 200–1000. For identification purposes, tandem MS or MS^n experiments were performed using the smart-frag option. 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, two spectra were averaged. Data processing was performed using LC/MSD trap software version 5.2, build 382 or ACD/SpecManager software version 9.15. Extracted ion chromatograms were generated for relevant *m/z* values and the peaks were integrated.

2.5. System set-up

2.5.1. Off-line digestion procedure

The pronase digestion of NAPQI-HSA was first evaluated by performing off-line batch experiments. The digestion products were analyzed and characterized by MS^2 experiments on the SPE-LC–MS system. This was done by addition of 10 μL of a pronase solution (1 mg/mL in PBS, pH 7.4) to a sample of 1000 μL containing 100 μg/mL of NAPQI adducted albumin in PBS, pH 7.4. The mixture was immediately incubated at 37 °C in a water bath and incubated for 8 h. After this, 20 μL of the reacted sample was injected onto a SPE-LC–MS system consisting of a gradient pump, a conditioning pump and a switching valve. The sample was injected onto the SPE cartridge and desalinated for 5 min using the equilibration solution (99.3% water, 0.5% acetonitrile and 0.2% of formic acid). The analytical C18 column was switched in-line by switching the valve. A gradient was run from 0% of the equilibration solution up to 40% of the elution solution (99.3% acetonitrile, 0.5% water and 0.2% formic acid) within 20 min. After this, the column was flushed by applying 80% of this elution solution for 2 min. After the sample valve was switched back, both columns were reconditioned for the next run. The analytical column was reconditioned by a Gilson 302 pump. The flow rate of both pumps was set to 0.2 mL/min, and total run-time including the reconditioning step was 35 min. Full scan mass spectrometric analysis was done using an *m/z* range between 100 and 1000 unless mentioned otherwise. MS^2 and MS^3 experiments were performed with the isolation window set to 4.0 and product ions were scanned in a range between *m/z* values 100 and 750.

2.6. Immunoaffinity chromatography procedure

The capacity of the IAC column was estimated by a method which has been previously published [23]. A 1 mg/mL solution of HSA was made in PBS (pH 7.4) solution. Different amounts of protein were applied by injecting an increasing volume of this solution onto the column using an Agilent 1100 autoinjector. The IAC was operated at a flow rate of 0.040 mL/min using PBS (pH 7.4). After 5 min, the column was flushed with 0.400 mL of 0.15 M NaCl to flush matrix components to waste. The elution of the protein was performed for 10 min using 10 mM glycine-HCl containing 150 mM NaCl (pH 2.7). The effluent was directed towards an Agilent 1100 series UV/VIS spectrometer and detection was done by using UV detection at 280 nm. The elution peak was integrated and the data were evaluated as reported in previous work.

2.7. On-line system

A detailed scheme of the entire on-line set-up is depicted in Fig. 2, and a table with the detailed chromatographic conditions of the entire analytical process can be found in Table 1. The IAC column was operated by pump 1 (P1) at a flow rate

Table 1
Table with the detailed chromatographic conditions of the entire analytical process

Time (min)	IAC action	SPE action	LCMS action
0–5	Sample application		
5–15	Flushing		
15–25	Elution		
18–23	Elution	Capturing	
23–26		Flushing	
26–42		Gradient	Gradient
42–44		Flushing	Flushing

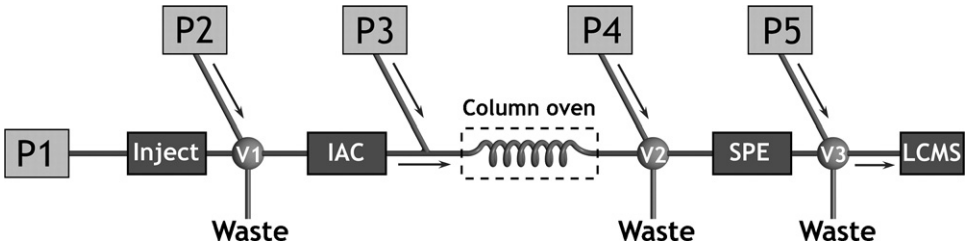

Fig. 2. Scheme of on-line set-up used for this research. Protein is injected onto immunoaffinity chromatography (IAC) column by pump 1 (P1). Pump 2 (P2) elutes the protein by switching valve 1 (V1). Pump 3 (P3) adds protease in solution. The digested product is captured on a solid phase extraction (SPE) cartridge by switching valve 2 (V2). Pump 4 (P4) desalinates the trapped peptides and elutes the peptides over the liquid chromatography–mass spectrometry (LC–MS) system by switching valve 3 (V3). Pump 5 (P5) equilibrates the LC–MS system.

of 0.040 mL/min. This column was conditioned using PBS (pH 7.4). Albumin samples were introduced by the injector and the sample was delivered by pump 1 (P1) using PBS (pH 7.4). After 5 min, the column was flushed with 0.400 mL of a 0.15 M NaCl solution to remove matrix components to waste. After this, the protein was eluted from the column by switching valve 1 (V1) to pump 2 (P2) for 10 min with a solution of 10 mM glycine-HCl containing 150 mM of NaCl (pH 2.7). The protein fraction eluting between 17 and 21 min was delivered to the SPE cartridge via the reaction capillary. A UV chromatogram of the IAC procedure can be found in Fig. 3. In the reaction capillary, the eluate from the IAC column was allowed to react with pronase dissolved in a PBS (pH 10.8) solution, delivered by pump 3 (P3, Fig. 2) at a flow rate of 0.040 mL/min. Compatibility of the elution conditions using low pH with the on-line solution phase digestion was achieved by neutralizing the elution fraction by mixing the effluents from the immunoaffinity chromatography column continuously with pronase solution in PBS at a pH of 10.8. The resulting pH will be between pH 6 and 8, which is compatible with the pH optimum for the pronase activity. The digestion temperature was controlled by using the temperature controlled column switch, which also delivered the digested eluate from the IAC column to the SPE column by switching valve 2 (V2). When the protein was eluted, valve 1 (V1) was switched back and the IAC column was equilibrated for analysis of the next sample. When the digested protein effluent was delivered to the SPE cartridge via the reaction capillary, valve 2 (V2) was switched back and solvent delivered by gradient pump 4 (P4) desalinated the captured effluent for 3 min using a solvent consisting of 99.3% water, 0.5% acetonitrile and 0.2% of formic acid

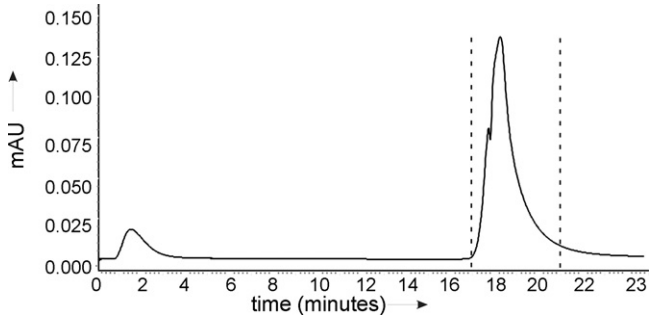

Fig. 3. UV (280 nm) monitoring of the immunoaffinity chromatography procedure. The elution peak (between dashed lines) is directed towards the on-line solution phase digestion procedure.

at a flow rate of 0.2 mL/min. After desalination, valve 3 (V3) was switched and a gradient was run to 50% within 16 min using a second solution consisting of 99.3% acetonitrile, 0.5% water and 0.2% formic acid to separate the digest products from the originally captured protein. The columns were washed with 80% of the elution solution for 2 min, and subsequently the analytical column was re-equilibrated by pump 5 (P5) after switching back valve 3 (V3) using 99.3% water, 0.5% acetonitrile and 0.2% formic acid. The total run-time was 44 min. During separation of the digest products and re-equilibration of the SPE cartridge, the next sample could already be applied onto the immunoaffinity column. Hereby, the overall run-time could be reduced to 25 min, in principle enabling a sample throughput of almost 60 samples per day.

2.8. On-line digestion procedure optimization

For the evaluation of the continuous flow digestion procedure, protein was injected into the previously described system whereas the concentration of pronase in the delivered solution (P3, Fig. 2) and the temperature of the reaction capillary were varied. Both the concentration of the protease as well as the thermal denaturation of albumin are important factors in protein digestion, as has been studied by Pico [24]. The reaction time was kept constant at 1.75 min.

2.9. Chromatogram evaluation

Software assisted data comparison of chromatograms was done in the ACD/Specmanager software package as follows: the Agilent chemstation file (*.d) file was exported to netCDF file format (*.cdf). As a result, the mass spectral data are automatically converted to centroided mass spectra. The chromatograms were imported to the software and the chromatograms were processed using the COMPARE algorithm. Data processing accuracy was set to 0.1, other settings were set to default. The program picks the differences between the chromatograms and highlights these in reconstructed extracted ion chromatograms.

3. Results and discussion

The analytical system for the on-line detection of covalent adducts to human serum albumin comprises several pre-column steps, including immunoaffinity purification, solution

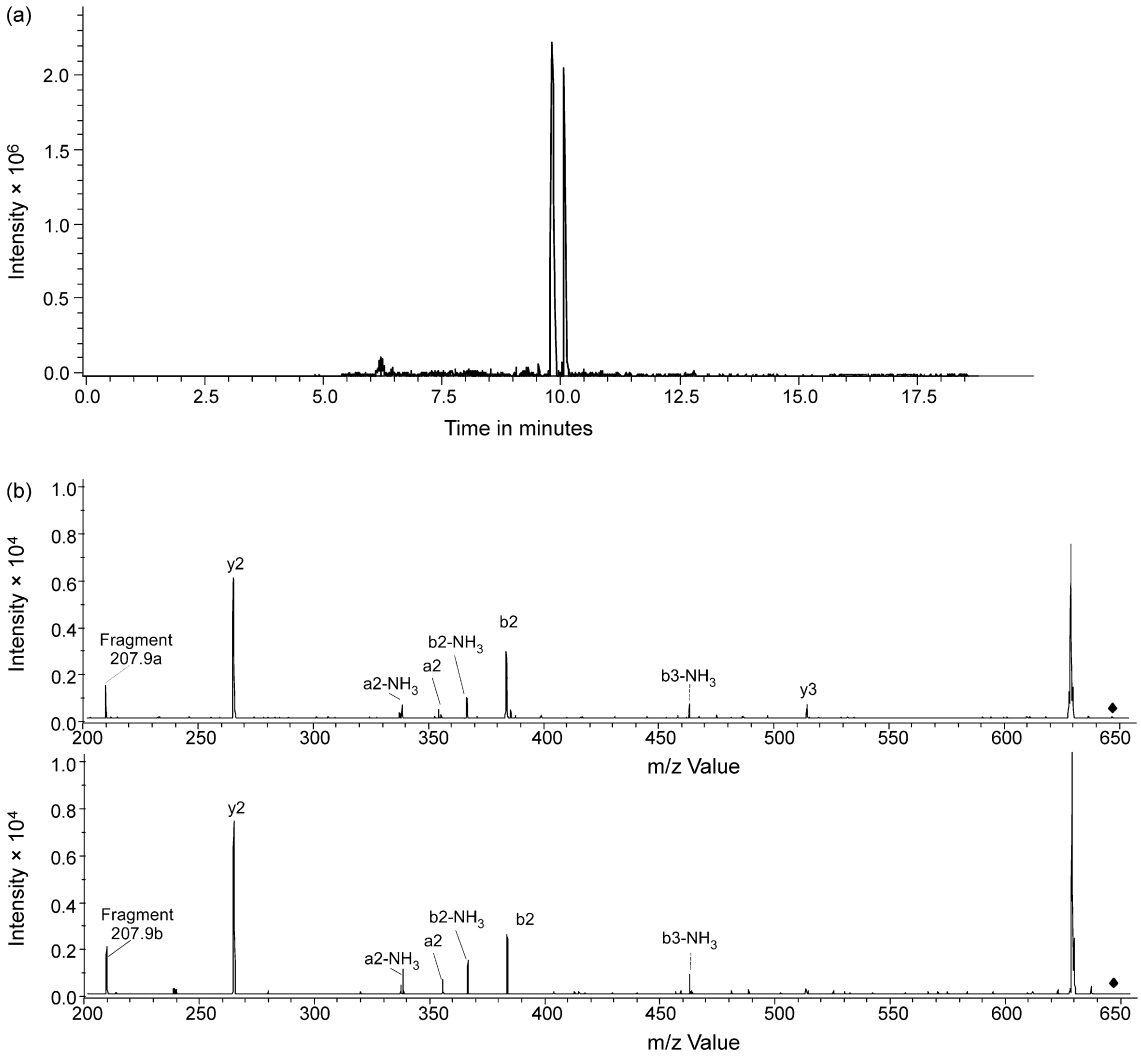

Fig. 4. (a) Reconstructed extracted ion chromatogram of NAPQI-QCPF (*m/z* 643.3) in a pronase digest of NAPQI-HSA (upper). (b) Tandem MS product ion scan of *m/z* 643.3 (upper) of the first peak and the product ion scan of the second peak (lower).

phase on-line digestion, and C18 solid phase extraction with the subsequent determination of peptide adducts using liquid chromatography–mass spectrometry. During method development main attention was paid to achieve compatibility of the optimum conditions of the different steps.

3.1. Identification of NAPQI-peptide adducts

The pronase digestion of NAPQI-HSA was first studied by performing in-batch off-line digestion experiments. In principle, adduct formation by NAPQI results in a mass shift of 149.1 Da. Since NAPQI is known to be reactive towards the cysteine-34 residue of HSA, the full scan MS chromatogram was screened for peptides containing that cysteine residue with an *m/z* value shifted by 149.1 Da. A series of peptides containing this cysteine-34 residue has already been published [9]. The analyzed NAPQI-HSA and unmodified HSA digest sample traces were compared using ACD/SpecManager. Two peaks of *m/z* 643.3 corresponding to the *m/z* of NAPQI-glutamine–cysteine–proline–phenylalanine (NAPQI-QCPF) were found in the NAPQI-HSA sample and were not present in the unmodified HSA sample. Fig. 4a shows an extracted ion chromatogram from a NAPQI-HSA digest at *m/z* 643.3. MS^2 experiments were subsequently performed on the ion at *m/z* 643.3 and the identity of the NAPQI-QCPF adducts could be confirmed (Fig. 4b). Both compounds show almost identical product ion mass spectra in MS^2 and MS^3 experiments. This suggests the formation of two NAPQI-QCPF isomers. The two adduct isomers may result from two different reactions of NAPQI with the cystein-34 group in HSA, either through electrophilic addition of thiolate to the carbonyl-carbon to yield a thio-hemi-ketal intermediate or by nucleophilic addition to the imine-carbon via an ipso intermediate [25]. Both structures may fragment to a product ion with *m/z* 208 forming the isomeric structures (1,4-benzothiazine and 1,4-benzooxathiine derivatives). Since it is not possible to distinguish between these isomeric structures in MS^2 mode, further attempts were done to assign the resulting spectra. MS^3 fragmentation of both ions with *m/z* 208 yield fragment ions with *m/z* 166, due to the loss of $H_2C{=}C{=}O$ from the acetyl group. Although the results of the

Table 2
Relative intensities of reconstructed extracted daughter ion peak areas of HSA and NAPQI-HSA peptides originating from samples consisting in modified HSA, of a 1:1 mixture of modified and unmodified HSA and of a control sample of unmodified HSA

Sample type	Area *m/z* 748.4 DEFKPL	Area *m/z* 643.3 NAPQI-QCPF
Modified HSA	1510048	122595
Modified + non-modified HSA 1:1	1409927	56220
Non-modified HSA	1301998	Not detectable

MS^2 and MS^3 experiments support the theory of isomer formation, further experiments especially including NMR have to be performed to confirm this phenomenon.

3.2. Immunoaffinity chromatography

An UV chromatogram of the IAC procedure can be found in Fig. 3. The effluent from the IAC column was continuously delivered via the in-solution digestion reaction capillary. There was no need to pre-select the protein fraction from the IAC procedure for digestion since the effluent (both matrix components and purified protein) is continuously mixed with fresh enzyme. The eluting albumin fraction from the IAC column (between dashed lines) has been heart-cut towards the SPE cartridge via the reaction capillary by switching valve 2 (V2), Fig. 2) for subsequent separation and analysis. The capacity of the IAC column is an important issue for the sample purification step. By means of a binding capacity curve [23], it was found that the capacity (390 pmol or 26 μg HSA) of the IAC column (inner volume of 12 μL) is in accordance with the specifications given by the supplier (2 mg/mL). Although a fixed sample volume with increasing sample concentrations would be analytically more appropriate, the use of a varying sample volume has not been of concern in our previous project [23]. Therefore, this approach has been used for the estimation of column capacity. By this experiment, the application in an on-line format could be realized. If a higher column capacity is needed, other column dimensions might be considered, e.g. by increasing the I.D. from 1 to 3 mm, enhancing the total capacity about 10-fold for both modified and unmodified HSA.

To assure that matrix components can be successfully removed at chosen conditions, a solution of myoglobin has been injected and analyzed (data not shown). For this, 50 μL of a solution of PBS (pH 7.4) containing 1 mg/mL myoglobin has been injected onto the anti-HSA immunoaffinity column. Apart from the breakthrough of myoglobin from the anti-HSA immunoaffinity column, the elution profile did not differ from a blank (PBS, pH 7.4) injection. This indicates that proteins, which are not supposed to bind to the material, are successfully removed during the flushing process using 0.15 M NaCl.

Another important aspect is the immunoaffinity of the immobilized antibodies towards modified proteins. In order to prove that modified HSA binds to the immobilized anti-HSA antibodies, the following experiments were performed: first, a number of native (unmodified) peptides from HSA were identified using the complete on-line system (IAC, solution phase digestion, LC and MS). The mass spectrometer was set to full scan mode. 30 μL of a blank (PBS, pH7.4) and HSA sample (0.1 mg/mL in PBS, pH 7.4) were injected and the results were compared. Since pronase generates significant background signals, comparison was performed with help of the ACD/SpecManager as described in Section 2. Using ACD/SpecManager, we were able to search for specific differences between blank samples (i.e. without HSA) with a rather high background and samples containing low abundant HSA within this background. The *m/z* signals with the highest intensity (those which were not present in the blank chromatogram) were selected for further MS/MS experiments. From the most intense *m/z* values, two peptides could be easily identified as native HSA peptides, i.e. VLIAF and DEFKPL. No further attempts were done to identify other peptides. The peptide DEFKPL (*m/z* 748.4) is present in modified as well as in the unmodified HSA and will therefore be used as marker for human serum albumin. The MS was subsequently set to the SRM mode and continuous MS/MS was done on precursors 643.3 and 748.4, both with an isolation width of 4.0. After the analysis, an extracted ion chromatogram of the identified product ion traces of the modified peptide and the unmodified peptide were generated. 10 μL of a 1 mg/mL solution of modified HSA was injected on the system. After a sample of NAPQI modified HSA was analyzed, the sample was spiked in a 1:1 ratio with unmodified HSA (1 mg/mL in PBS; pH 7.4). From this mixture also 10 μL were injected and the same product ion traces were summed for a reconstructed chromatogram. The peaks of both experiments were integrated and the results are shown in Table 2. The first line represents only the modified protein and the second line the modified and unmodified protein mixed in a 1:1 ratio. The same sample volume was injected. The area of the unmodified peptide marker is consistent with the area of the previously analyzed sample and the control sample since the same amount of peptide marker can be produced whereas the amount of modified peptide was reduced by 50% as half of the amount was injected compared to the first experiment. In the unmodified HSA sample, no NAPQI modified peptide could be found. From these results, it can be concluded that NAPQI-HSA was not displaced from the immunoaffinity column by native HSA, since the area of the native HSA marker peptide remained the same whereas the peak area of the modified peptide was reduced by approximately 50%. This indicates that it is possible to extract both the modified and the unmodified HSA using the anti-HSA immunoaffinity support. Although this experiment indicates sufficient affinity for both protein species, a dilution series will have to be considered for biological samples containing an excess of non-modified albumin. This is important to assure full recovery of (very) low levels of modified albumin.

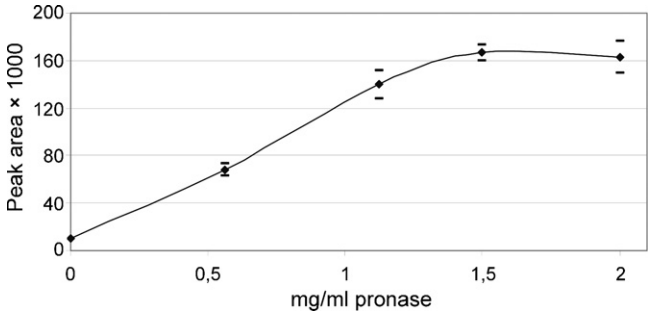

Fig. 5. Influence of the pronase concentration on the NAPQI-QCPF peptide formation (peak areas of reconstructed extracted ion chromatograms of *m/z* 643.3) during the in-solution digestion procedure.

3.3. Continuous flow digestion

In previous work, Damsten et al. [6] described a method to analyze NAPQI adducts to albumin in human serum samples. Briefly, albumin from serum samples of patients exposed to high levels of acetaminophen was isolated and digested with pronase. The resulting mixture was analyzed by LC–MS/MS after removal of the enzyme. In the present study, albumin was isolated by immunoaffinity chromatography including an elution step using a low pH buffer. As previously published [23], it was found that the elution solution used (10 mM glycine-HCl and 0.15 M NaCl, pH 2.7) could be neutralized by mixing with PBS (pH 10.8) in a 1:1 ratio. Pronase was dissolved in this neutralizing buffer and mixed with the effluent from the immunoaffinity chromatography using the same flow rate. In this way, the neutralizing solution was applied and the protease could also be delivered using a single pump (P2, Fig. 2). A schematic overview of this on-line system can be found in Fig. 2. In the reaction capillary, the enzyme interacts with the eluted protein to form marker peptide(s) for NAPQI adducted albumin. The influence of the enzyme concentration was evaluated by varying the concentration of pronase between 0.1 and 2.0 mg/mL. Analysis was done using the completely on-line system. Extracted ion chromatograms of the MS^2 product ions of NAPQI-QCPF were constructed, the results being shown in Fig. 5. The maximum amount of product ions was found at a concentration of 1.5 mg/mL of pronase. This concentration has been used for further analysis. It has to be kept in mind that the reaction concentration of pronase in the reaction capillary differs from the concentration in the buffered solution which was deliverd by pump 3 (P3, Fig. 2). Since the effluent from the IAC column is mixed with the pronase solution in a 1:1 ratio, a dilution of 50% has to be taken into account. This means that a maximum amount of NAPQI-QCPF has been found at a pronase concentration in the reaction capillary of 0.8 mg/mL at given conditions.

After this, the temperature of the reaction capillary (which was kept until this time at 37 °C) was varied between 8 and 80 °C. The reaction was monitored in a similar way as described for the enzyme concentration optimization, the results being depicted in Fig. 6. A maximum product formation was found at 70 °C, probably caused by both a higher activity of pronase and the thermal denaturation of albumin [24]. Further optimization studies were performed at a reaction capillary temperature of 70 °C.

Since it can be expected that both the peptides from the protein and the protease itself are trapped on the C18 SPE column, we were interested to assess whether on-column digestion significantly contributes to the digestion of the HSA adducts. In order to study the contribution of on-column digestion, the temperature of the reaction capillary was kept constant at 8 °C in order to minimize the in-solution formation of products in the reaction capillary, while the SPE column temperature was raised to 70 °C to enhance possible on-column digestion. No significant traces of NAPQI-QCPF could be found using these settings and it can therefore be concluded that on-column digestion does not contribute in a significant way to the digestion of HSA adducts.

An estimation of the detection limit for NAPQI-QCPF was obtained by injection of a sample containing 0.1 mg/mL NAPQI-HSA into the complete on-line system using the optimized parameters. When 10 μL of a 1.5 μmol/L NAPQI-HSA solution were injected on the system, a signal-to-noise ratio was between 3 and 4 was obtained, resulting in an absolute detection limit of 15 pmol NAPQI adducted albumin via the cystein-34 functionality. It should be kept in mind that the modification of the cystein-34 residue by NAPQI is not a specific one, and other amino acid residues might be alkylated in a similar way. However, specific attention was paid to modifications of the cystein-34 residue in this work because this site has often been suggested as an attractive biomarker of exposure to electrophiles [8,26].

3.4. CDNB adducted HSA

Next to the detection NAPQI adducts to the cysteine-34 residue of human serum albumin, we also investigated the possibility to detect other albumin adducts. As 1-chloro-2, 4-dinitrobenzene (CDNB) is known as an electrophilic and thiol-reactive compound, it was chosen for the synthesis of a different albumin adduct. The synthesized CDNB-HSA adduct

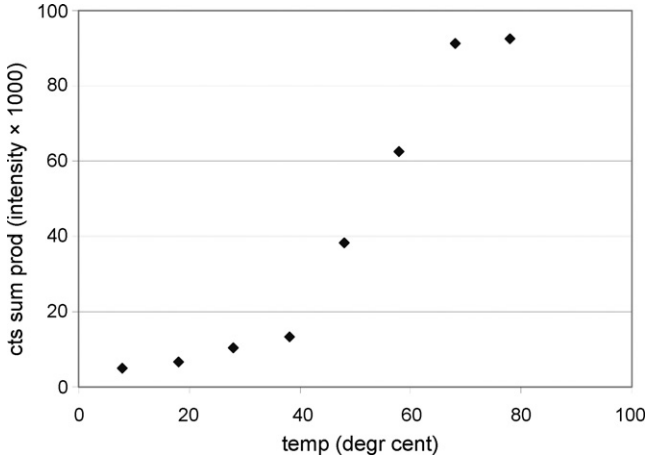

Fig. 6. Influence of the solution phase digestion capillary temperature on the NAPQI-QCPF peptide formation (peak areas of reconstructed extracted ion chromatograms of *m/z* 643.3).

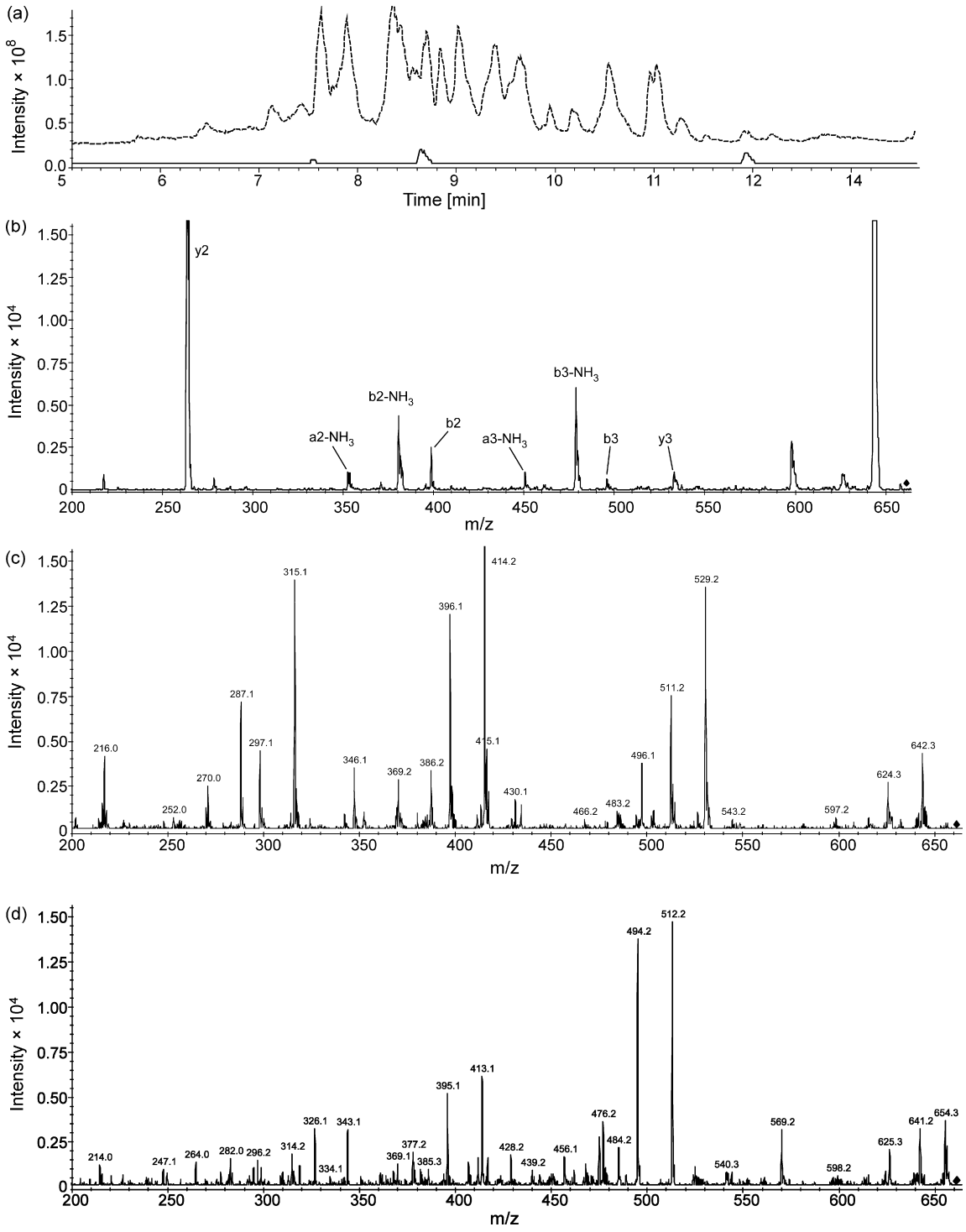

Fig. 7. (a) The total ion current chromatogram of a pronase digest of CDNB adducted HSA shown by the dashed trace; the reconstructed extracted ion chromatogram of *m/z* 660.3 is shown by the solid trace (blow-up 3-fold). (b) Tandem MS product ion scan of *m/z* 660.3 (blow-up 6-fold) at retention time 11.9 min. (c) Tandem MS product of *m/z* 660.3 at retention time 8.7 min. (d) Tandem MS product of *m/z* 660.3 at retention time 7.6 min.

was used to evaluate the general applicability of the system. The albumin was reacted with CDNB and the resulting mixture was injected onto the on-line analysis system. The reaction with CDNB results in a mass shift 166.0 Dalton to the peptide QCPF resulting in an expected *m/z* ratio of 660.3. In Fig. 7a, three peaks can be observed in the extracted ion trace with *m/z* 660.3. Next, a tandem MS experiment was performed in an attempt to identify these peaks (Fig. 7b). The last peak was identified as the CDNB-QCPF peptide adduct. The product ion spectrum of this peak is presented in Fig. 7b. The two other peaks (Fig. 7c and d) were not identified in this work, since the product ions differ significantly from the fragments of the CDNB-QCPF peptide adduct.

4. Conclusions

A fully automated, on-line approach for the detection of covalent adducts to the cysteine-34 residue of human serum albumin has been developed. Human serum albumin adduct formation with the reactive metabolite of acetaminophen (NAPQI) could be detected by the analysis of the corresponding NAPQI-QCPF adduct by mass spectrometry. The applicability of the approach was confirmed by additional analysis of CDNB adducted human serum albumin. This approach might therefore constitute a useful tool for the screening of covalent adducts to HSA in human samples without requiring time-consuming manual sample handling. This work also confirmed that the cysteine-34 residue of HSA serves as a highly reactive site for covalent binding of reactive electrophilic compounds. This can be attributed to the fact that cysteine-34 is the only freely available thiol-group present in intact albumin. An absolute amount of 15 pmol NAPQI-HSA could be analyzed when injecting 10 μL of a 1.5 μmol/L NAPQI adducted human serum albumin solution onto the on-line system. Immunoaffinity chromatography has been applied as a selective sample purification step and it could be shown that the presence of native (non-adducted) albumin did not hinder the specific interaction with the immobilized antibodies. Also, continuous solution phase digestion has been presented as a new approach to perform on-line digestion, thereby excluding the need for immobilization of enzymes like as usually done with IMERs. Data analysis was supported by ACD/SpecManager and although a rather high background signal was observed, the identity of several peptides could be confirmed by mass spectrometric experiments.

Acknowledgements

This research was supported by Roche Diagnostics GmbH in Penzberg (Germany). We acknowledge Agilent (Amstelveen, The Netherlands) for providing technical parts and equipment service.

References

[1] S. Zhou, E. Chan, W. Duan, M. Huang, Y.Z. Chen, Drug Metab. Rev. 37 (2005) 41.
[2] D.C. Evans, A.P. Watt, D.A. Nicoll-Griffith, T.A. Baillie, Chem. Res. Toxicol. 17 (2004) 3.
[3] X.X. Yang, Z.P. Hu, S.Y. Chan, S.F. Zhou, Clin. Chim. Acta 365 (2006) 9.
[4] M. Tornqvist, C. Fred, J. Haglund, H. Helleberg, B. Paulsson, P. Rydberg, J. Chromatogr. B 778 (2002) 279.
[5] S. Zhou, J. Chromatogr. B 797 (2003) 63.
[6] M. Damsten, J.N. Commandeur, A. Fidder, A.G. Hulst, D. Touw, D. Noort, N.P. Vermeulen, Drug Metab. Dispos. 35 (2007) 1408.
[7] K.J. Hoffmann, A.J. Streeter, D.B. Axworthy, T.A. Baillie, Chem. Biol. Interact. 53 (1985) 155.
[8] S. Ito, T. Kato, K. Fujita, Biochem. Pharmacol. 37 (1988) 1707.
[9] D. Noort, A.G. Hulst, L.P. de Jong, H.P. Benschop, Chem. Res. Toxicol. 12 (1999) 715.
[10] D. Noort, A.G. Hulst, R. Jansen, Arch. Toxicol. 76 (2002) 83.
[11] D. Noort, A. Fidder, A.G. Hulst, A.R. Woolfitt, D. Ash, J.R. Barr, J. Anal. Toxicol. 28 (2004) 333.
[12] A.J. Streeter, D.C. Dahlin, S.D. Nelson, T.A. Baillie, Chem. Biol. Interact. 48 (1984) 349.
[13] C.L. Cardoso, V.V. Lima, A. Zottis, G. Oliva, A.D. Andricopulo, I.W. Wainer, R. Moaddel, Q.B. Cass, J. Chromatogr. A 1120 (2006) 151.
[14] L. Korecka, Z. Bilkova, M. Holeapek, J. Kralovsky, M. Benes, J. Lenfeld, N. Minc, R. Cecal, J.L. Viovy, M. Przybylski, J. Chromatogr. B 808 (2004) 15.
[15] J. Krenkova, F. Foret, Electrophoresis 25 (2004) 3550.
[16] N. Markoglou, R. Hsuesh, I.W. Wainer, J. Chromatogr. B 804 (2004) 295.
[17] G. Massolini, E. Calleri, J. Sep. Sci. 28 (2005) 7.
[18] C. Temporini, E. Perani, E. Calleri, L. Dolcini, D. Lubda, G. Caccialanza, G. Massolini, Anal. Chem. 79 (2007) 355.
[19] B. Bruyneel, J.S. Hoos, M.T. Smoluch, H. Lingeman, W.M. Niessen, H. Irth, Anal. Chem. 79 (2007) 1591.
[20] I.A. Blair, A.R. Boobis, D.S. Davies, T.M. Cresp, Tetrahedron Lett. 21 (1980) 4947.
[21] C.K. Riener, G. Kada, H.J. Gruber, Anal. Bioanal. Chem. 373 (2002) 266.
[22] R. Hurst, Y. Bao, S. Ridley, G. Williamson, Biochem. J. 338 (1999) 723.
[23] J.S. Hoos, H. Sudergat, J.P. Hoelck, M. Stahl, J.S.B. de Vlieger, W.M.A. Niessen, H. Lingeman, H. Irth, J. Chromatogr. B 830 (2006) 262.
[24] G.A. Pico, Int. J. Biol. Macromol. 20 (1997) 63.
[25] W. Chen, J.P. Shockcor, R. Tonge, A. Hunter, C. Gartner, S.D. Nelson, Biochemistry 38 (1999) 8159.
[26] D. Noort, A. Fidder, A.G. Hulst, Arch. Toxicol. 77 (2003) 543.