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LC-Tandem MS Detection of Covalent Binding of Acetaminophen to Human Serum Albumin.

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LIST OF ABBREVIATIONS:

Cyt P450, cytochrome P450; APAP, acetaminophen; GSH, glutathione, NAc, N-acetyl-L-cysteine; Cys, cysteine; NAPQI, N-acetyl-p-benzoquinoneimine; CPF, Cysteine-Proline-Phenylalanine; HSA, human serum albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LC-Tandem MS, liquid chromatography-tandem mass spectrometry; ADRs, adverse drug reactions; IDRs, idiosyncratic drug reactions.

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ABSTRACT

Covalent binding of reactive electrophilic intermediates to proteins is considered to play an important role in the processes leading to adverse drug reactions (ADRs) and idiosyncratic drug reactions (IDRs). Consequently, both for the discovery and the development of new drugs, there is a great interest in sensitive methodologies that enable the detection of covalent binding of drugs and drug candidates *in vivo*. In this work, we present a strategy for the generation and analysis of drug adducts to human serum albumin. Our methodology is based on the isolation of albumin from blood, its digestion to peptides by pronase E and the sensitive detection of adducts to the characteristic Cysteine-Proline-Phenylalanine (CPF) tripeptide by LC-Tandem MS. We chose acetaminophen as a model compound because this drug is known to induce covalent binding to proteins when bioactivated by cytochrome P450s to its reactive N-acetyl-p-benzoquinoneimine metabolite. First, by microsomal incubations of acetaminophen in presence of CPF and/or intact albumin, *in vitro* reference adducts were generated in order to determine the mass spectrometric characteristics of the expected CPF adducts and to confirm their formation upon pronase E digestion of the alkylated protein. When applying this methodology to albumin isolated from blood of patients exposed to acetaminophen, we were indeed able to detect the corresponding CPF adducts. This strategy could therefore be seen as a potential bio-monitoring tool to detect *in vivo* reactive intermediates of drugs and drug candidates, e.g. in the preclinical and clinical development phase.

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Although much effort has been made in the development of predictive animal models useful for the early assessment of toxicity of drugs and drug candidates, the prediction of drug toxicity in humans stays difficult. While some adverse drug reactions (ADRs) can be predicted from preclinical safety studies, others are idiosyncratic in nature and only show up after the drug is already introduced on the market. These idiosyncratic types of drug reactions can lead to severe, in some cases fatal, toxicities in several organs, in particular the liver, skin and blood (Park et al., 2005; Smith and Schmid, 2006).

Even though the underlying mechanisms of most ADRs are as yet poorly understood, formation of reactive metabolites is considered to be a major trigger in the cascade of events leading to these adverse events (Williams et al., 2002). Drugs can be bioactivated both by phase I and by phase II enzymes to reactive electrophilic intermediates, which subsequently react with nucleophilic sites in macromolecules to form covalent adducts to proteins (Evans et al., 2004; Zhou et al., 2005). Covalent binding to proteins with subsequent inactivation of enzymes and/or disruption of cellular signaling processes are events that are thought to be related to the onset of ADRs (Zhou et al., 2005). By serving as haptens, drug-protein adducts may also trigger the autoimmune reactions which are often observed in case of idiosyncratic drug reactions (Utrecht, 1999; Park et al., 2000).

As reviewed by Caldwell and Yan (2006) and Zhou (2003), different methodologies are used for the detection of adducts resulting from formation of reactive intermediates. Briefly, these methods involve *in silico* screening of potentially toxic motives in molecules, the use of small nucleophilic trapping agents followed by mass spectrometric analysis of adducts formed *in vitro* and mechanism-based inhibition of Cytochrome P450. An estimation of the levels of total covalent binding to proteins, *in vitro* and/or *in vivo*, can eventually be obtained by using radiolabeled drugs in animal experiments. However, because extrapolation of animal data to

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evaluate potential risks in humans stays complicated (Olson et al., 2000; Smith and Schmid, 2006), there is still a need for sensitive and selective methods for the assessment of covalent binding to proteins *in vivo* in humans. In the present study, a recently developed LC-Tandem MS methodology will be applied to monitor covalent binding of acetaminophen to the blood protein albumin.

The concept of using blood protein adducts as biomarkers of human exposure to electrophilic compounds dates back to the 1970's and was originally applied for the *in vivo* monitoring of occupational exposures to reactive, potentially genotoxic, compounds (van Welie et al., 1992; Tornqvist et al., 2002). Consistently, adducts to human serum albumin (HSA) have been found in populations exposed to several environmental contaminants (Sherson et al., 1990; Wild et al., 1990; Omland et al., 1994; Rappaport et al., 2005).

The aim of the present study is to evaluate the applicability of a recently developed LC-Tandem MS methodology for the *in vivo* biomonitoring of reactive drug metabolites to the blood protein albumin. This methodology, which has been applied successfully for the biomonitoring of exposure of humans to chemical warfare agents (Noort et al., 1999; Noort et al., 2002), is based on the digestion of albumin by pronase E and subsequent selective detection of covalent adducts to the tripeptide Cysteine³⁴-Proline-Phenylalanine (CPF) by LC-Tandem MS. Cysteine³⁴ is the only free thiol-group in HSA and is capable of reacting with electrophiles. In this work, we evaluated the applicability of this methodology for the monitoring of reactive drug metabolites using acetaminophen (APAP) as model compound. At therapeutic doses, APAP is primarily metabolized by phase II enzymes to stable glucuronic acid and sulphate conjugates. A small proportion of the drug is bioactivated by Cytochrome P450s to a reactive N-acetyl-p-benzoquinone imine (NAPQI) intermediate that under normal conditions is detoxified by conjugation to glutathione (GSH) (Gibson et al., 1996; Zhou et al., 1996; Qiu et al., 1998). When

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taken in overdoses, the high levels of NAPQI produced will deplete the glutathione stores resulting in strongly increased covalent binding to liver proteins, oxidative stress and ultimately to severe hepatotoxicity (Bessems and Vermeulen, 2001).

We propose a general strategy which consists of the biosynthesis of reference adducts to CPF and to albumin in order to determine the mass spectrometric characteristics of the CPF-adducts and to determine whether pronase E treatment of alkylated albumin is able to generate the corresponding CPF-adducts (Figure 1). The analytical procedure was subsequently applied for the measurement of adducts in the blood of humans exposed to high doses of acetaminophen.

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MATERIAL AND METHODS

Materials.

Pronase E (protease from *Streptomyces griseus*, Type XIV, 3.4.24.31), GSH (reduced glutathione, 98%) and NAc (N-Acetyl-L-cysteine, 98%) were purchased from Sigma (Germany). NADPH-tetrasodium salt was obtained from AppliChem BioChemica (Germany). Amicon Ultra-4 (10 kDa molecular mass cutoff) centrifugal filters were purchased from Millipore (Millipore Corporation, Bedford, USA). The HiTrapTM Blue HP affinity columns (1 mL; pre-packed with blue Sepharose High Performance, with Cibacron Blue F3G-A as the ligand) and the PD-10 columns (containing 10 mL of Sephadex G 25 material) were obtained from Amersham Biosciences (Uppsala, Sweden). The Acrodisc LC PVDF filters (0.45 μ m, 25 mm) were obtained from Waters Corporation and the Strata-X columns (33 μ m Polymeric Sorbent) from Phenomenex. β -naphthoflavone-induced rat liver microsomes were prepared according to the standard protocol of our laboratory (Rooseboom et al., 2001). Control human blood was obtained from healthy volunteers and blood samples from patients overdosed with acetaminophen were kindly provided to us by Dr. D. Touw from the Apotheek Haagse Ziekenhuizen in The Hague, The Netherlands. Human plasma exposed to perdeuterated sulfur mustard was used as internal standard and was prepared as described previously (Noort et al., 2004). All other chemicals were of the highest grade and were obtained from standard providers.

Instrumentation.

LC-ES MS(/MS) analyses were conducted on a Q-TOFTM hybrid instrument (Micromass, Altrincham, UK) equipped with a standard Z-sprayTM ES interface (Micromass) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware

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for this system consisted of a precolumn splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a six-port valve (Valco, Schenkon, Switzerland) with a 50 μ L injection loop mounted and a PepMap C18 column (15 cm \times 1 mm i.d., 3- μ m particles; LC Packings). A gradient of eluents A (H₂O with 0.2 % formic acid) and B (acetonitrile with 0.2 % formic acid) was used to achieve separation, following: 100 % A (at time 0 min, 0.1 mL/min flow) to 100% A (at 5 min, 0.6 mL/min flow) to 30 % A and 70 % B (at 60 min, 0.6 mL/min flow). The flow delivered by the liquid chromatograph was split precolumn to allow a flow of approximately 40 μ L/min through the column and into the ES MS interface. The Q-TOF was operated at a cone voltage of 20-30 V, employing nitrogen as the nebulizer and desolvation gas (at a flow of 20 and 400 L/h, respectively). MS/MS product ion spectra were recorded using a collision energy between 20 and 30 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

Standard tripeptide assay.

Adducts to human serum albumin were analyzed using a slightly modified methodology that has been described previously (Noort et al., 2004). Briefly, human blood was first centrifuged at 3000g in order to separate plasma from erythrocytes. The obtained plasma (500 μ L) was then diluted with 2 mL of buffer A (50 mM KH₂PO₄ PH 7.00). In order to control the procedure, the samples were spiked with 50 μ L of an internal standard consisting of plasma isolated from human blood exposed to 100 μ M of perdeuteriated sulfur mustard gas. The pronase E digest of albumin alkylated with perdeuteriated sulfur mustard gas is known to produce the characteristic d₈-S-(2-hydroxyethylthioethyl)-Cys-Pro-Phe (d₈-HETE-CPF) adduct as has been described in Noort et al. (2004). The samples were then filtered with 0.45 μ m Acrodisc filters and the albumin was subsequently isolated from the filtrate using HiTrapTM Blue HP affinity columns. These columns were firstly conditioned with 10 mL of buffer A. The whole sample (2.55 mL) was then

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applied on the columns and washed with 10 mL of buffer A. Elution took place with 3 mL of buffer B (50 mM KH_2PO_4 with 1.5M KCl). The HiTrap columns were regenerated by washing with 10 mL of buffer A. PD-10 columns were used to desalt the obtained albumin fractions. After equilibration of the PD-10 columns with 25 mL of 50 mM NH_4HCO_3 , the samples were applied on the columns (3 mL) and eluted with 3 mL of the same bicarbonate buffer. The digestion procedure of the desalted albumin solution with pronase E was as follows: 100 μL of a freshly prepared pronase E solution (10 mg/mL stock solution in aqueous 50 mM NH_4HCO_3) was added to 750 μL of the albumin fraction (in 50 mM NH_4HCO_3). After 2 hours of incubation at 37 °C, the mixture was passed through molecular mass cutoff filters (10 kDa) under centrifugation at 2772 x g in order to remove the enzyme. Under these conditions, pronase E digestion of albumin adducts is completed (Noort et al, 1999). The filtrate was subsequently analyzed by LC-Tandem MS. If not analyzed immediately, all samples were stored at -20°C until analysis.

Synthesis and purification of NAPQI-CPF adducts.

The reactive metabolite of acetaminophen, N-acetyl-p-benzoquinoneimine (NAPQI), was synthesized according to a previously described method (Bessemers et al., 1996). Briefly, fresh silver oxide was prepared by adding a silver nitrate solution (170 mg in 10 mL H_2O) to a solution of potassium hydroxide (100 mg in 18 mL H_2O). The mixture was left for 15 minutes on ice in order to yield the highest amounts of the silver oxide precipitate. After filtration of the solution and three washing steps with acetone, the obtained silver oxide powder was added to a solution of acetaminophen (10 mg in 10 mL of chloroform). This reaction mixture was stirred at room temperature for 1 hour in order to obtain 10 mL of a yellowish NAPQI solution (in chloroform). A portion of this NAPQI solution (2 mL) was filtered, evaporated to dryness by rotaevaporation

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at room temperature and further taken-up in 10 μ L of acetonitrile. This concentrated NAPQI solution was then reacted with 100 μ L of synthetic Cysteine–Proline-Phenylalanine (CPF) tripeptide solution (stock concentration 2.5 mM in 50 mM NH_4HCO_3) in a total volume of 1.5 mL of a 30% acetonitrile solution (in bicarbonate buffer). After 2 hours of reaction at 37°C, the sample was measured by LC-Tandem MS. LC-Tandem MS analysis of the reaction mixture showed the presence of a product having the molecular mass of the expected NAPQI-CPF adduct (Figure 2). The product ion spectrum of this protonated molecule (m/z 515.18 [MH^+]) had characteristic fragments at m/z 497.18 ($\text{MH}^+ - \text{H}_2\text{O}$), m/z 350.11 (b_2), m/z 322.13 (a_2), m/z 263.16 (y_2''), m/z 225.07 (a_1), m/z 208.06 ($a_1 - \text{NH}_3$), m/z 166.06 (y_1''), m/z 152.07 ($\text{APAP} + \text{H}^+$) and m/z 120.07 (immonium ion of phenylalanine).

To further characterise the NAPQI-CPF product, and to enable quantification of NAPQI-CPF in patient samples, a large scale synthesis was performed. Acetaminophen (32 mg) was oxidised to NAPQI in 75 mL chloroform, as described above, and stirred vigorously for 2 hours at room temperature with 25 mg CPF in 30 mL 100 mM potassium phosphate buffer (pH 7.4). The resulting water phase was isolated and washed three times with a mixture of chloroform:isopropanol (3:1 vol) in order to remove excess NAPQI and lipophilic side products. The water phase was subsequently dried overnight by nitrogen stream. The residue was taken up in 1 mL of 10% acetonitril and applied to preparative HPLC to purify NAPQI-CPF-adducts.

The preparative HPLC consisted of a Luna C18 column (250x10 mm; 5 μ m particles) eluted at a flow-rate of 2 mL/min. A gradient of eluents A (1% acetonitril/0.2% formic acid/98.2% water) and B (99% acetonitril/0.2% formic acid/0.8% water) was used to achieve separation of analytes. The gradient used started at 15% B (0 min) and was followed by a linear increase to 25% B (50 min). Peaks absorbing at 254 nm were collected and screened for presence of NAPQI-CPF by LC-MS. Two peaks at 21.9 (minor, 5%) and 23.6 minutes (major, 95%) were shown to contain

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NAPQI-CPF. The fractions containing purified NAPQI-CPF were pooled, dried by nitrogen stream and taken up in 500 μ L deuterium oxide. The ^1H -NMR spectrum was recorded on a Bruker MSL 400 system operating at 376.43 Hz. The ^1H -NMR-spectrum of the major NAPQI-CPF-isomer was consistent with a NAPQI-CPF adduct, although protons could not be assigned individually due to the complexity and overlap of the ^1H -NMR-signals. Because aromatic protons of the phenylalanine-residue interfere with the signals of the acetaminophen-residue, the position of the thioether-bond could not be assigned. ^1H -NMR-spectrum: 6.8-7.6 ppm (aromatic protons; multiplet; 8H), 3.8-4.5 ppm (methine protons of phenylalanine, cysteine and proline; multiplet; 3H), 2.8-3.55 ppm (methylene protons of phenylalanine and cysteine; four methylene protons of proline; multiplet; 8H), 1.65-2.3 ppm (two methylene protons of proline and acetyl protons of acetaminophen; multiplet; 5H). The concentration of the minor NAPQI-CPF-isomer was too low to obtain a good ^1H -NMR-spectrum.

To calibrate the concentration of NAPQI-CPF-solution, difluoroacetic acid (final concentration 720 μ M) was added to the solution of NAPQI-CPF and analysed by ^1H -NMR. Difluoroacetic acid was selected as internal standard because its signals (triplet at 5.82 ppm, $^2J_{\text{FH}}$ 51 Hz) do not interfere with the signals of NAPQI-CPF. By integrating the signals of aromatic protons of NAPQI-CPF ([8H]) and that of difluoroacetic acid ([1H]), the concentration of the NAPQI-CPF-solution was estimated to be 650 μ M. This solution was used for the quantification of NAPQI-CPF-adducts formed by pronase E-treatment of patient samples.

Incubation of synthetic NAPQI in human plasma.

The synthetic NAPQI solution described above was used to expose human plasma to the reactive alkylating compound. In these experiments, 3 mL of the NAPQI solution (in chloroform) was evaporated to dryness by rotaevaporation at room temperature and further taken-up in 10 μ L

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of acetonitrile. The concentrated NAPQI solution was then reacted with 2 mL of human plasma for 2 hours at 37 °C. Subsequently, 500 µL of that incubation mixture was diluted with 2 mL of buffer A and spiked with 50 µL of the internal standard. The sample was consistently passed through the Acrodisc filter and the albumin was isolated on the HiTrap column as described above. The albumin fraction was then desalted on PD-10 columns and 750 µL of that filtrate were treated with pronase E for 2 hours at 37 °C. After a centrifugation step with the molecular mass cutoff filters, the filtrate was analyzed by LC-Tandem MS.

Microsomal incubations of acetaminophen in presence of synthetic CPF.

Microsomal incubations of acetaminophen and CPF had a final volume of 500 µL and were conducted at 37°C in a heated shaking water bath. The incubation occurred in a 100 mM KH₂PO₄ buffer PH 7.4 and the mixture was composed as follows (in final concentrations): 1 mM of APAP, β-naphthoflavone-induced rat liver microsomes (RLM, 2 mg protein/mL) and 1 mM of the synthetic Cysteine–Proline-Phenylalanine (CPF) tripeptide solution. After 5 minutes of pre-incubation, the co-factor NADP(H) (final concentration 2 mM) was added to the mixture and the samples were incubated for 1 hour at 37°C. After the incubation time, the samples were placed on ice and 200 µL of 2.00 M HClO₄ was added in order to stop the incubations and precipitate proteins. The samples were vortexed and kept on ice for 5 additional minutes. The tubes were subsequently centrifuged for 15 minutes at 4000 rpm and a 400 µL aliquot of the supernatant was neutralized by the addition of an equal volume of K₂HPO₄ 1.00 M. The samples were vortexed, centrifuged again for 15 minutes at 4000 rpm and the supernatant was analyzed by LC-Tandem MS. An incubation performed without APAP was processed in parallel and served as control.

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Microsomal incubations of acetaminophen in presence of human plasma.

Microsomal incubations of acetaminophen in human plasma were performed similarly as described above. Briefly, 75 μL of acetaminophen (20 mM stock in H_2O) and 150 μL of RLM (10 mg of protein/mL stock) were added to 500 μL of human plasma. After 5 minutes of pre-incubation at 37°C, 150 μL of NADP(H) (10 mM stock in 100 mM K-P buffer PH 7.4) was added to the incubation mixture. The mixture was incubated for 1 hour at 37°C. After the incubation time, 2 mL of buffer A and 50 μL of the internal standard were added to the samples. The albumin isolation, pronase E digestion and peptide filtration steps were performed similar to the procedure described for the exposure of human plasma to synthetic NAPQI. The samples were subsequently analyzed by LC-Tandem MS. An incubation performed without substrate served as control.

Analysis of human serum samples.

Consistently, 500 μL of human serum of patients exposed to high levels of acetaminophen was diluted with 2 mL of buffer A and the samples were spiked with 50 μL of the internal standard. The rest of the procedure was similar to that described earlier. Blood from healthy volunteers, not being exposed to either APAP or NAC, was taken as control and processed in parallel.

Initial measurements of the patient's blood samples revealed a significant background signal interfering with the expected NAPQI-CPF adducts. Therefore, to increase the sensitivity in the albumin adduct detection, the protocol was slightly adapted by including a solid phase extraction (SPE) step in the procedure. The human serum samples were analyzed with the modified protocol as follows. After isolation and desalting of albumin as described above, 300 μL of a fresh pronase E solution (10 mg/mL) were added to approximately 2 mL of the albumin solution. After 2 hours

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of incubation at 37°C, the samples were centrifuged at 2772 x g to eliminate the excess of enzyme. The freshly digested samples were acidified with TFA (final concentration 0.1 % TFA) and Strata X columns were used for the solid phase extraction procedure. The columns were first activated with 10 mL of methanol and then equilibrated with 10 mL of 100 % H₂O with 0.1% TFA. The samples were applied on the column and subsequently eluted with 2 mL fractions of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% acetonitrile solutions containing 0.1 % TFA. These different fractions were collected, freeze-dried, taken-up in 120 µL of 100 % H₂O with 0.2 % formic acid and analyzed by LC-Tandem MS. The NAPQI-CPF adducts were eluted by the 30% acetonitril/0.1% TFA fraction.

To quantify NAPQI-CPF-adducts formed by pronase E-treatment of albumin, the albumin-fractions isolated from the plasma samples were also spiked with 65 nmol of purified synthetical NAPQI-CPF (major-isomer), prior to the treatment with pronase E. By measuring the increase in the ratio of the peak area of NAPQI-CPF to that of the internal standard (d₈-HETE-CPF), the amount of NAPQI-CPF in the non-spiked albumin-fraction was estimated.

In order to analyze small molecular weight adducts, the eluates obtained from the application of the serum samples on the HiTrap columns were collected, filtered with molecular mass cutoff filters (10 kDa) under centrifugation at 2772 x g and analyzed by LC-Tandem MS.

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RESULTS

Incubation of synthetic NAPQI with human plasma.

In order to assess if NAPQI is also reactive towards the free cysteine³⁴ residue of HSA and if the pronase E digest of alkylated albumin is leading to the formation of NAPQI-CPF, human plasma was incubated *in vitro* with freshly synthesized NAPQI. Full MS analysis was used to screen the fragments obtained after fragmentation of the selected molecular ion with an m/z of 515.2. The reconstructed ion chromatogram of the summed ions corresponding to specific fragments of the synthetic adduct revealed the presence of two NAPQI-CPF adducts in the reaction mixture (Peak 1 and Peak 2 in Figure 3-A). Their product ion spectra were comparable to that of our reference adduct. Only slight differences in the fragmentation pattern of the two peaks were observed suggesting the formation of two regioisomeric NAPQI-CPF adducts.

Incubations of acetaminophen with rat liver microsomes.

Next to the chemical synthesis of the albumin and CPF-adducts of NAPQI, it was also investigated whether *in vitro* incubations of APAP in presence of albumin or CPF produced the same adducts. We therefore used β NF-induced rat liver microsomes (RLM) in order to bioactivate APAP to NAPQI in a biological system. By performing incubations using synthetic CPF as trapping agent for NAPQI, two NAPQI-CPF adducts were found by LC-Tandem MS analysis (Figure 3-B).

When APAP was incubated with rat liver microsomes in presence of human plasma, two NAPQI-CPF adduct isomers were observed after pronase E digestion of the albumin fraction. The adducts had the same characteristic differences in their product ion spectra as observed in the products formed by synthetic NAPQI (Figure 3C).

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Analysis of human serum samples.

The developed methodology was applied to analyze blood of patients exposed to high levels of acetaminophen. Three patients were selected with the characteristics presented in Table 1. Patient TOX 444 suffered from severe hepatotoxicity as indicated by increased plasma aspartate transaminase (AST) and alanine aminotransferase (ALT) levels. The two other patients (TOX 438 and TOX 440) had normal plasma transaminase levels and were therefore considered to be in a non-hepatotoxic condition. Initially, the serum samples of these patients were analyzed with the standard tripeptide assay as described in Noort et al. (2004). However, by this assay no NAPQI-CPF adducts were observed in the sera of patients TOX 438 and TOX 440 due to background interference, whereas two weak NAPQI-CPF signals were found in the serum of patient TOX 444 (data not shown). By using the solid phase extraction step in the procedure, it was found that the NAPQI-CPF peaks eluted in the 30% acetonitrile/0.1 % TFA] fraction. The signal-to-noise ratio of the NAPQI-CPF signals was significantly increased using this clean-up step. Two NAPQI-CPF adducts were observed in the sera of patient TOX 444 and signals corresponding to the major NAPQI-CPF adduct were now also observed in the sera of patients TOX 438 and TOX 440. Based on the peak areas of the summed ion current chromatograms, the level of the major NAPQI-CPF adduct was about 10-fold higher in serum of TOX 444, when compared to that in serum of TOX 438 and 440. The product ion spectra of these adducts were identical to those obtained in our previous *in vitro* experiments. No NAPQI-CPF adducts were present in the control subjects (Figure 5).

By spiking the isolated albumin fractions of patient TOX 444 with 65 pmol of synthetic NAPQI-CPF, prior to pronase E treatment, the amount of NAPQI-CPF in the pronase E-digest was quantified. By comparing the peak areas of NAPQI-CPF in samples of patient TOX 444 with and without spiking with synthetic NAPQI-CPF, the amount of NAPQI-CPF in the

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unspiked sample was estimated to be 35 ± 15 pmol/mL serum. Based on the 10-fold lower peak areas in patient samples TOX 438 and 440, Figure 5, the levels of NAPQI-CPF in these serum samples are estimated to be approximately 3-4 pmol/mL serum.

The eluates obtained during the albumin isolation step were also screened for the presence of other NAPQI-derived adducts. No GSH conjugates were observed in the human plasma samples. However, very high amounts of NAPQI-cysteine (NAPQI-Cys) and NAPQI-N-acetyl cysteine (NAPQI-NAc) adducts were found in the sera of these patients (Figure 6). Their specific fragmentation pattern confirmed the identity of these adducts of which the characteristics are presented in Table 2.

Interestingly, another high signal was found in the pronase E digest of the isolated albumin fraction of patient TOX 444 suggesting that another albumin adduct is formed *in vivo*. The ion chromatogram of the protonated molecule (m/z 655.4 $[\text{MH}^+]$) is depicted in Figure 7-A. The product ion spectrum of this ion showed characteristic signals at m/z 637.4 ($\text{MH}^+ - \text{H}_2\text{O}$), m/z 527.3 (y_3''), m/z 490.3 (b_3), m/z 393.2 (b_2), m/z 375.2 ($b_2 - \text{H}_2\text{O}$), m/z 347.2 ($a_2 - \text{H}_2\text{O}$), m/z 263.2 (y_2''), m/z 230.1 ($b_2 - \text{NAc}$), m/z 162.1 $[\text{NAc-H}]^+$ and m/z 101.1 (a_1). This fragmentation pattern is consistent with the Glutamine-Cysteine³⁴-Proline-Phenylalanine (QCPF) tetrapeptide from which the Cysteine³⁴ residue formed a mixed disulfide with N-acetyl cysteine (Figure 7-B). This adduct was also found in the serum of patient TOX 438 but was absent in patient TOX 440. As the formation of a tetrapeptide instead of the CPF tripeptide was surprising, we checked the enzymatic activity of pronase E by analyzing the internal standard that was systematically added to the serum samples. The expected d₈-HETE-CPF tripeptide adduct was consistently found in the samples thereby confirming a correct activity of the protease (data not shown). The human samples were also screened for the possible formation of NAPQI-QCPF adducts but no signals corresponding to these adducts were observed suggesting a complete digestion towards NAPQI-

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CPF adducts. Eventually, the levels of the different adducts analyzed for each patient were found to be constant in time.

Table 3 summarizes the different adducts identified in the human serum samples and gives a semi-quantitative overview of their relative concentrations.

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DISCUSSION

Predicting the potential of drugs and drug candidates to lead to adverse drug reactions in humans via electrophilic reactive intermediates is still a difficult and speculative task. While the current way to assess this issue is usually to combine *in vitro* data on reactive intermediate formation with protein covalent binding studies *in vivo* in animals with radiolabeled drugs, extrapolation of these data to assess potential risks for humans remains complicated (Caldwell and Yan, 2006; Smith and Schmid, 2006). In this study, we evaluated the use of albumin adducts as biomarkers of bioactivation of drugs to reactive metabolites *in vivo* in humans. An overall strategy for the generation and analysis of those adducts is proposed. The methodology used is based on LC-Tandem MS analysis of covalent adducts to free cysteine³⁴ residue of HSA by detecting an alkylated Cysteine-Proline-Phenylalanine (CPF) tripeptide obtained after pronase E digestion of the protein. Acetaminophen (APAP) was chosen as model compound to study this concept (Bessems and Vermeulen, 2001; James et al., 2003).

We have shown that microsomal incubations were able to generate the same albumin adducts as those obtained with synthetic NAPQI (Figure 3), indicating that the biosynthesis of reference adducts is possible. This biosynthetic approach therefore allows the development of sensitive and selective analytical methods for the detection of CPF adducts without the requirement for chemical synthesis of reference adducts. The NAPQI-CPF adducts observed had slightly different product ion spectra and consequently suggested the formation of two isomers. Based on literature, we assume that the major peak is most likely the 3'-NAPQI-S-CPF adduct, because several studies have demonstrated that conjugation of thiols to NAPQI predominantly takes place at the 3'-position of NAPQI (Hoffmann et al., 1985; Axworthy et al., 1988; Pumford et al., 1997; Chen et al., 1999). As the minor NAPQI-CPF-adduct, two different products can be considered: a

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thioether ipso-adduct and a 2'-NAPQI-S-CPF-adduct. By Chen et al. (1999) it was demonstrated that the ipso-adduct was formed at slightly higher levels than the 2'-isomer, after reaction NAPQI with GSH at pH 6. However, the ipso-adduct was shown to be highly unstable at lower pH, with a half live of 0.5 minutes at pH 4. Because of the lengthy procedure, involving albumun isolation and pronase E-digestion, and the fact that the solid phase extraction was performed at acid pH (~pH 2), it seems unlikely the minor NAPQI-CPF-adduct will corresponds to the ipso-adduct. We therefore propose that the minor NAPQI-CPF-adduct corresponds to the 2'-regioisomer.

When applying the developed methodology to blood samples from patients exposed to high doses of acetaminophen, the major NAPQI-CPF isomer was observed in all patients (Figure 5). The patient with severe hepatotoxicity, TOX 444, showed an approximately ten-fold higher level of NAPQI-CPF-adduct when compared to the patients without hepatotoxicity. The minor NAPQI-CPF isomer could only be observed in blood sample of patient TOX 444. To our best knowledge, this is the first time that two NAPQI-HSA adduct regioisomers have been observed *in vivo* in humans. The levels of NAPQI-CPF adduct present in the serum of patient 444 was estimated to be about 35 pmol/mL of serum. It has been reported previously, that significantly higher levels of NAPQI-protein adducts can be found in serum in patients showing severe hepatotoxicity in comparison with serum of patients without or with low hepatotoxicity (Muldrew et al., 2002; James et al., 2006). However, because a different methodology was used in these studies, measuring total NAPQI-cysteine adducts after complete hydrolysis of whole serum, the levels of our NAPQI-CPF-adducts were not compared quantitatively to the adduct levels reported previously.

Besides the NAPQI-CPF adducts, very high levels of NAPQI-Cys and NAPQI-NAc adducts were found in the sera of all patients. These adducts probably originate either from the direct trapping of NAPQI by the antidote N-acetyl-cysteine (NAc) and/or from the catabolism of

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corresponding GSH adducts (Commandeur et al., 1995; Bessems and Vermeulen, 2001). However, the fact that patient 440, who did not receive NAc, forms equal amounts of NAPQI-NAc suggests that the major amount might be derived from GSH adduct catabolism. The formation of the QCPF-NAc adduct can be explained by mixed disulfide formation between the cysteine thiol of HSA and NAc, as has been shown *in vivo* in the plasma of rats treated with NAc (Harada et al., 2002). As NAPQI is a strong oxidizing agent (Gibson et al., 1996) and as the formation of mixed disulfides are known indicators of oxidation (Eaton, 2006), the QCPF-NAc adduct could therefore be considered as a marker of oxidative stress. However, because human blood samples from patients exposed to NAc and not to APAP are not available, the precise impact of NAPQI on QCPF-NAc adduct formation could not be directly assessed.

From the present results it is clear that, although the doses of acetaminophen were extremely high, the levels of NAPQI-HSA adducts in the human samples were unexpectedly low. One explanation might be the extensive mixed disulfide formation due to the oxidative properties of NAPQI that might have prevented the formation of NAPQI-CPF adducts by blocking the free cysteine³⁴-thiol group of HSA. The fact that lower amounts of NAPQI-CPF adducts were observed in the blood of patient TOX 438, who ingested similar amounts of APAP than TOX 440 but was treated with NAc, might support this hypothesis. Another explanation is that alkylation is taking place to pre-albumin in the liver and that the NAPQI-CPF adducts found in plasma result from the leakage of albumin adducts into the blood stream in cases of overt liver damage. This is consistent with the observation that much higher levels of NAPQI-CPF adducts were found in patient TOX 444, that showed high leakage of transaminases, in comparison to the adduct levels detected in patients TOX 438 and 440. Figure 8 summarizes the proposed interaction between NAPQI and the various trapping reactions involved.

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Previous methods that have been used in order to investigate covalent binding of APAP to proteins *in vivo* include several immunoassays (Roberts et al., 1987; Bartolone et al., 1988; Matthews et al., 1996; James et al., 2001) and the use of radiolabeled acetaminophen (Axworthy et al., 1988; Zhou et al., 1996; Qiu et al., 1998). Still, these methods often lack the necessary sensitivity when concerning human serum samples (Zhou, 2003). More recently, a HPLC-electrochemical detection (ECD) methodology was developed by Muldrew et al. (2002) in order to quantify covalent protein binding of acetaminophen. The methodology involves the dialysis of serum protein samples, subsequent digestion with protease and the measurement of the NAPQI-Cys adducts formed. Whereas the sensitivity of the HPLC-ECD method was increased compared to the previously mentioned technologies, it remains a long and labor-intensive procedure. Comparatively, our LC-Tandem MS methodology is quicker, sensitive, particularly selective and can provide precise structural information on the adducts formed to HSA as proven by the detection of two regioisomeric NAPQI adducts. This method also proved valuable to quantify the amounts of NAPQI-CPF adducts measured in the human serum samples. However, when synthesis of the reference drug-CPF adduct is not possible, this methodology can already as such be applied for the retrospective and/or relative comparison of drug-albumin adduct levels between individuals. Eventually, the possibility of scanning for neutral losses corresponding to “non-drug related” fragments of drug-CPF adducts (e.g. the y_2 ’ fragment corresponding to the PF part of the drug-CPF adduct) would allow the detection of novel, yet uncharacterized, drug-protein adducts.

In summary, we propose a generic strategy to assess the potential of drugs to be bioactivated to reactive electrophilic metabolites and to covalently bind to proteins *in vivo* (Figure 1). The strategy consists firstly in the biosynthesis of reference drug-albumin adducts for method development purposes. This biosynthetic approach is of particular importance since the synthesis

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of reactive metabolites of drugs is generally not feasible. Secondly, the developed analytical methodology is applied in the “*in vivo* approach” where serum samples of patients exposed to the drug are analyzed for the presence of similar albumin adducts. Smaller adducts, such as N-acetyl cysteine and cysteine adducts, can be measured as well hereby further confirming the bioactivation potential of the drug *in vivo*. While many adverse drug reactions seem not to be directly related to the concentration of the parent drug, it is likely that the onset of ADRs is related to the levels of reactive metabolites formed *in vivo*. Consequently, information on blood levels of “drug-protein adducts” will give an indication of the potential of a drug to be bioactivated to reactive electrophilic metabolites *in vivo*, and consequently on potential ADRs. As human serum albumin has a half-life of approximately 20 days, these drug-HSA adducts might accumulate in time. This could be of interest since IDRs usually have a delayed onset of occurrence and seem to take place essentially in patients taking a drug in relatively high doses, for a longer period of time. In this perspective, this strategy might also be seen as an *in vivo* dosimetry methodology to assess levels of covalent binding to proteins (Yang et al., 2006). Consequently, this technology could constitute a potential bio-monitoring tool that could improve the risk assessment of ADRs and IDRs of novel drugs and/or drug candidates.

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LEGENDS FOR FIGURES

Figure 1. Scheme of the strategy proposed for the generation and detection of drug-adducts to human serum albumin.

Figure 2. Electrospray product ion spectrum of the NAPQI-CPF adduct. The reference NAPQI-CPF adduct was obtained from the reaction of synthetic NAPQI with synthetic Cysteine-Proline-Phenylalanine tripeptide (CPF). The electrospray product ion spectrum of the adduct (MH^+ 515.18) was obtained with a collision energy of 24 eV.

Figure 3. Analysis of NAPQI-CPF adducts (MH^+ 515.2) generated in different *in vitro* systems. Ion chromatograms of summed ions: m/z 152.1, 208.1, 225.1, 322.1, 350.2 in (A) a pronase digest of alkylated HSA isolated from human plasma exposed to synthetic NAPQI; (B) an APAP microsomal incubation performed with synthetic CPF; (C) a pronase digest of alkylated HSA isolated from an APAP microsomal incubation performed in human plasma.

Figure 4. Electrospray product ion spectra of two NAPQI-CPF adduct regioisomers (MH^+ 515.2) with proposed structures: (A) NAPQI-CPF adduct corresponding to Peak 1 in Figure 3; (B) NAPQI-CPF adduct corresponding to Peak 2 in Figure 3.

Figure 5. Liquid chromatography-tandem mass spectrometry analysis of NAPQI-CPF adducts in human serum samples (MH^+ 515.2). Ion chromatograms of summed ions: m/z 152.1, 208.1, 225.1, 322.2, 323.2, 351.2 in (A) the control subject; (B) patient TOX 438; (C) patient TOX 440

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and (D) patient TOX 444. The analysis of the 23, 4 and 25 hours time point samples of patients TOX 438, 440 and 444 respectively are represented here.

Figure 6. Analysis of NAPQI-NAc and NAPQI-Cys adducts in the human serum samples. Liquid chromatography-tandem mass spectrometry analysis of (A) the NAPQI-Cys adduct (MH^+ 271.1; ion chromatogram of summed ions: m/z 140.0, 182.1) and (B) the NAPQI-NAc adduct (MH^+ 313.1; ion chromatogram of summed ions: m/z 208.1, 271.1) with proposed structures, analyzed in the 4 hours time point sample of patient TOX 440.

Figure 7. Analysis of the QCPF-NAc adduct in the human serum samples. Liquid chromatography-tandem mass spectrometry analysis of the QCPF-NAc adduct (MH^+ 655.4) in a pronase digest of alkylated HSA isolated from the 25 hours time point sample of patient TOX 444: (A) ion chromatogram of m/z 655.4 and (B) its product ion spectra with proposed structure.

Figure 8. General scheme proposing the different trapping reactions involved in the formation of the adducts detected in the human serum samples.

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TABLES

Table 1. Characteristics of the patients overdosed with acetaminophen.

The table includes the ingested dose of APAP; the time after APAP intake; the blood concentration of APAP in time; whether the patient was treated with the N-acetyl cysteine antidote (NAC); and the levels of hepatic enzymes ALT and AST in time.

Patient	APAP dose (g)	Time after intake (hours)	[APAP] (mg/l)	NAC treatment	ALT (U/l)	AST (U/l)
TOX 438	12	5	130	Yes	11	12
		23	4.5		n.d.	n.d.
TOX 440	10	2	150	No	16	30
		4	92		n.d.	n.d.
TOX 444	40	16	12	Yes	373	3150
		25	< 1		229	2282
		31	< 1		194	1683

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Table 2. Characterization of the NAPQI-Cys and NAPQI-NAc adducts.

Characteristics of the electrospray product ion spectra of the NAPQI-Cys and of the NAPQI-NAc adducts detected in the human serum samples. Product ion spectra's were obtained with a collision energy of 15 eV.

Adduct	Retention time (min)	Parent ion	Fragments
NAPQI-Cys	20.12	m/z 271.1 [MH ⁺]	m/z 254.1 [MH ⁺ -NH ₃]; m/z 225.1 [F2]; m/z 208.1 [F2-NH ₃]; m/z 182.1 [F1]; m/z 140.0 [F1-acetyl]
NAPQI-NAc	26.02	m/z 313.1 [MH ⁺]	m/z 271.1 [F1]; m/z 253.1 [F1-H ₂ O]; m/z 225.1 [F1-HCOOH]; m/z 208.1 [m/z 225.1-NH ₃]; m/z 182.1 [F2]; m/z 166.1 [m/z 208.1-acetyl]; m/z 162 [F3]; m/z 140.1 [F2-acetyl]

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Table 3. Overview of the adducts detected in the human serum samples^a.

Patient #	APAP dose (g)	NAC treatment	NAPQI-CPF	NAPQI-NAc	NAPQI-Cys	QCPF-NAc
TOX 444	40	Yes	++	++++	++++	+++
TOX 440	10	No	+	++++	++++	-
TOX 438	12	Yes	+	++++	++++	+++
Blank	0	No	-	-	-	-

a. Levels of analytes are ranked as: very abundant (++++); abundant (+++); average (++); present (+); absent (-). Because levels are based on peak areas of summed ion chromatograms of each analyte, only levels from the same column should be compared.

METHOD DEVELOPMENT

LC-MS/MS Methodology

Sample Preparation

APPLICATION

"*in vivo*" Samples

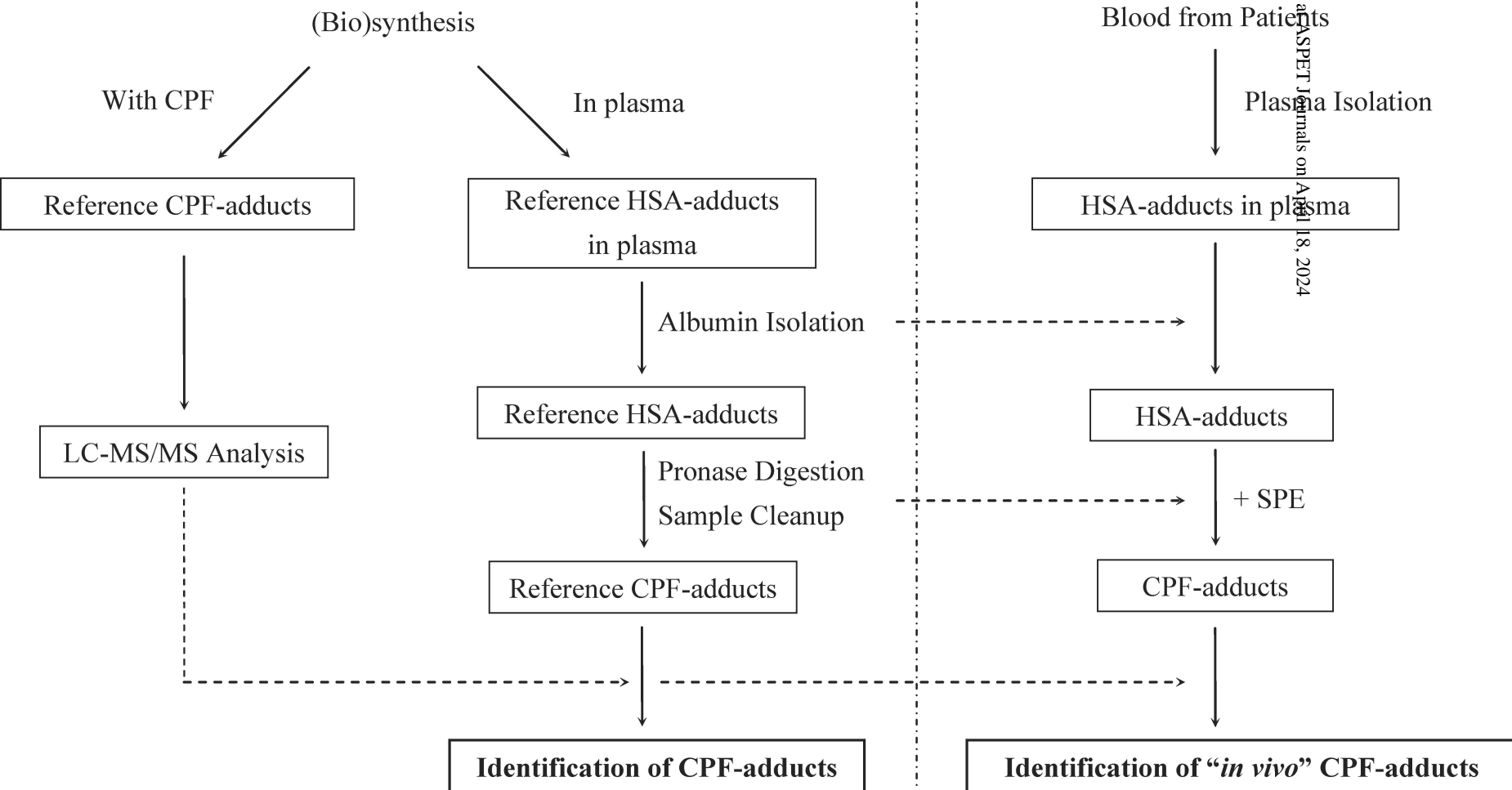


Figure 1

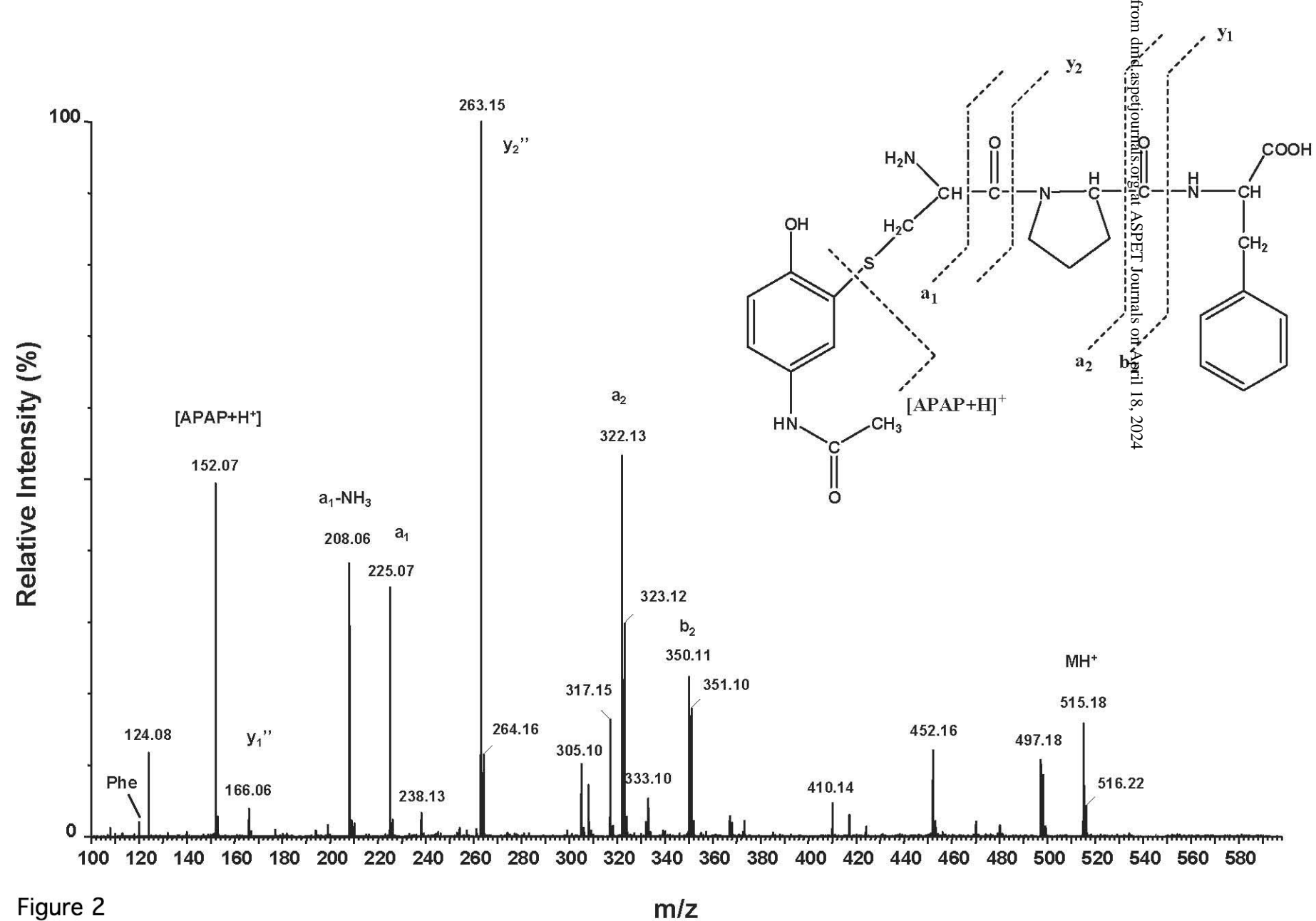


Figure 2

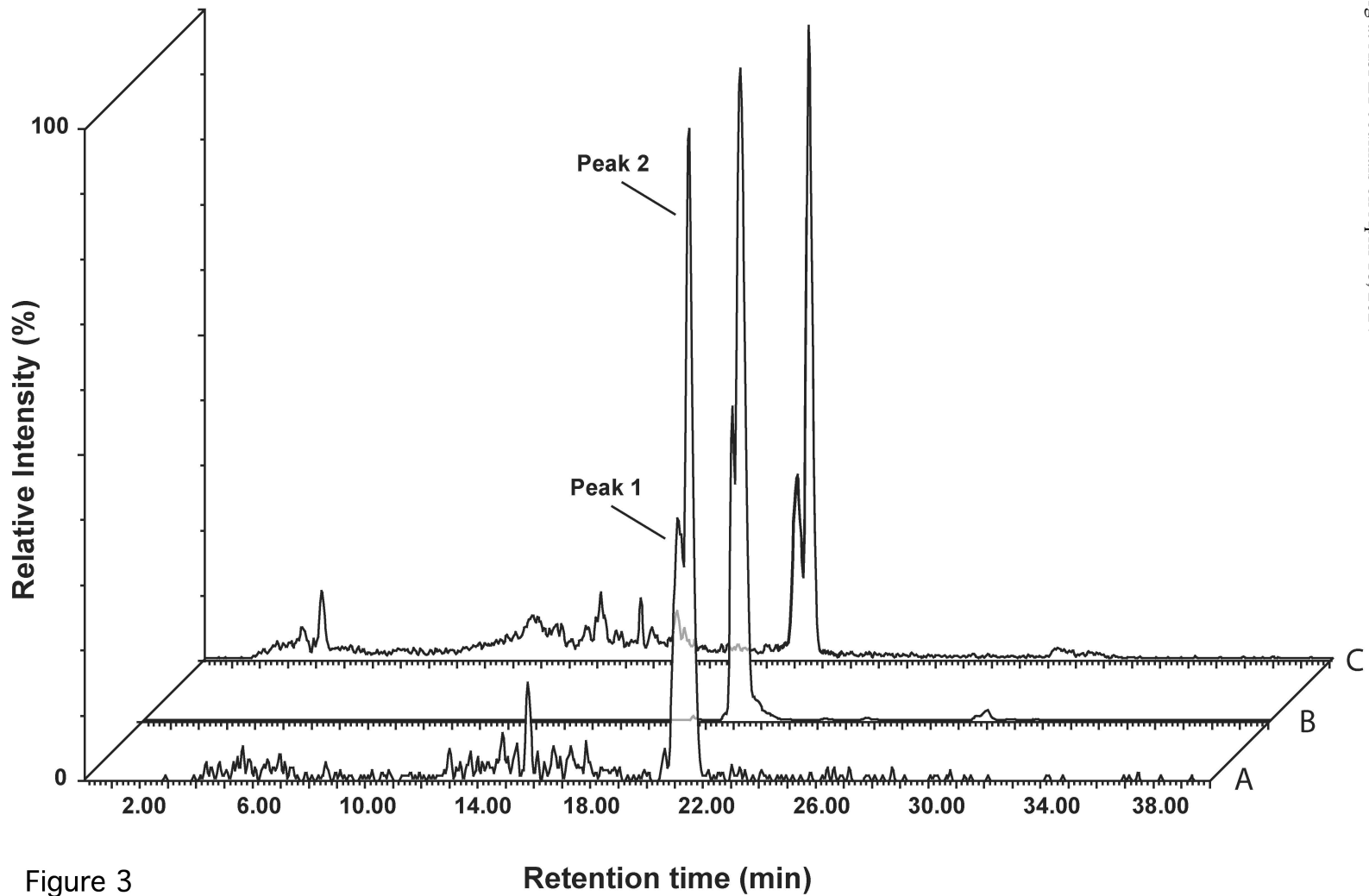


Figure 3

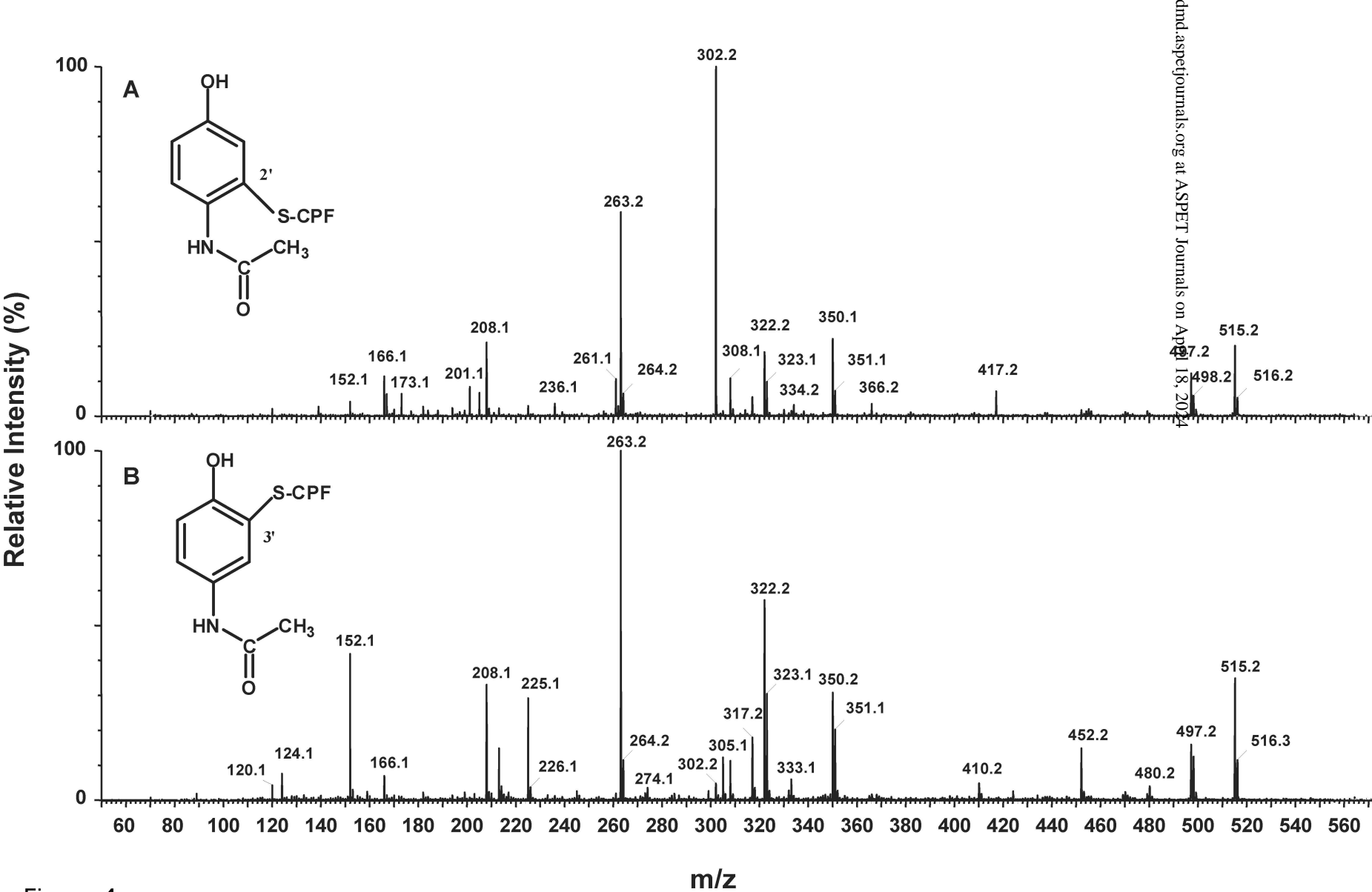


Figure 4

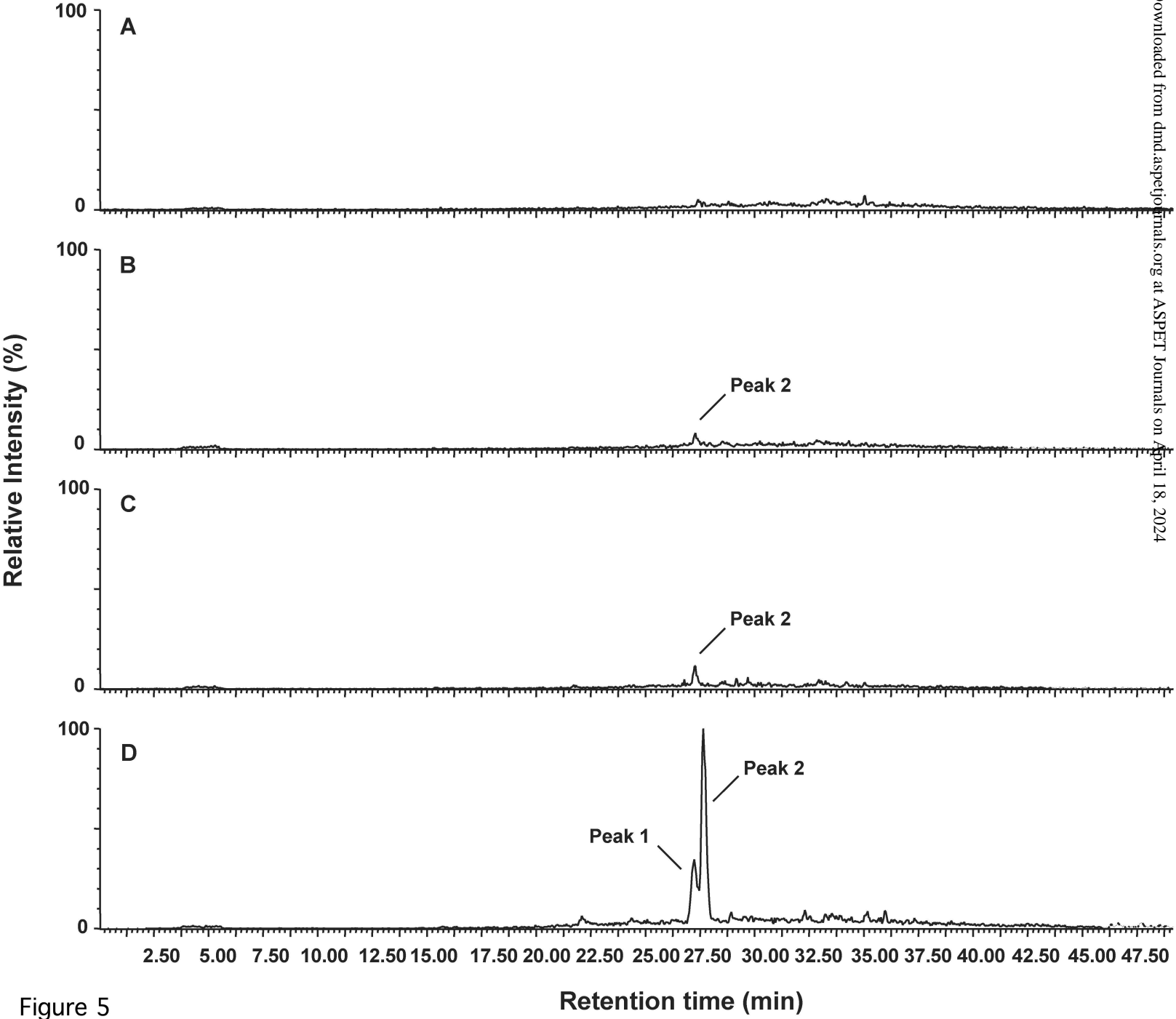
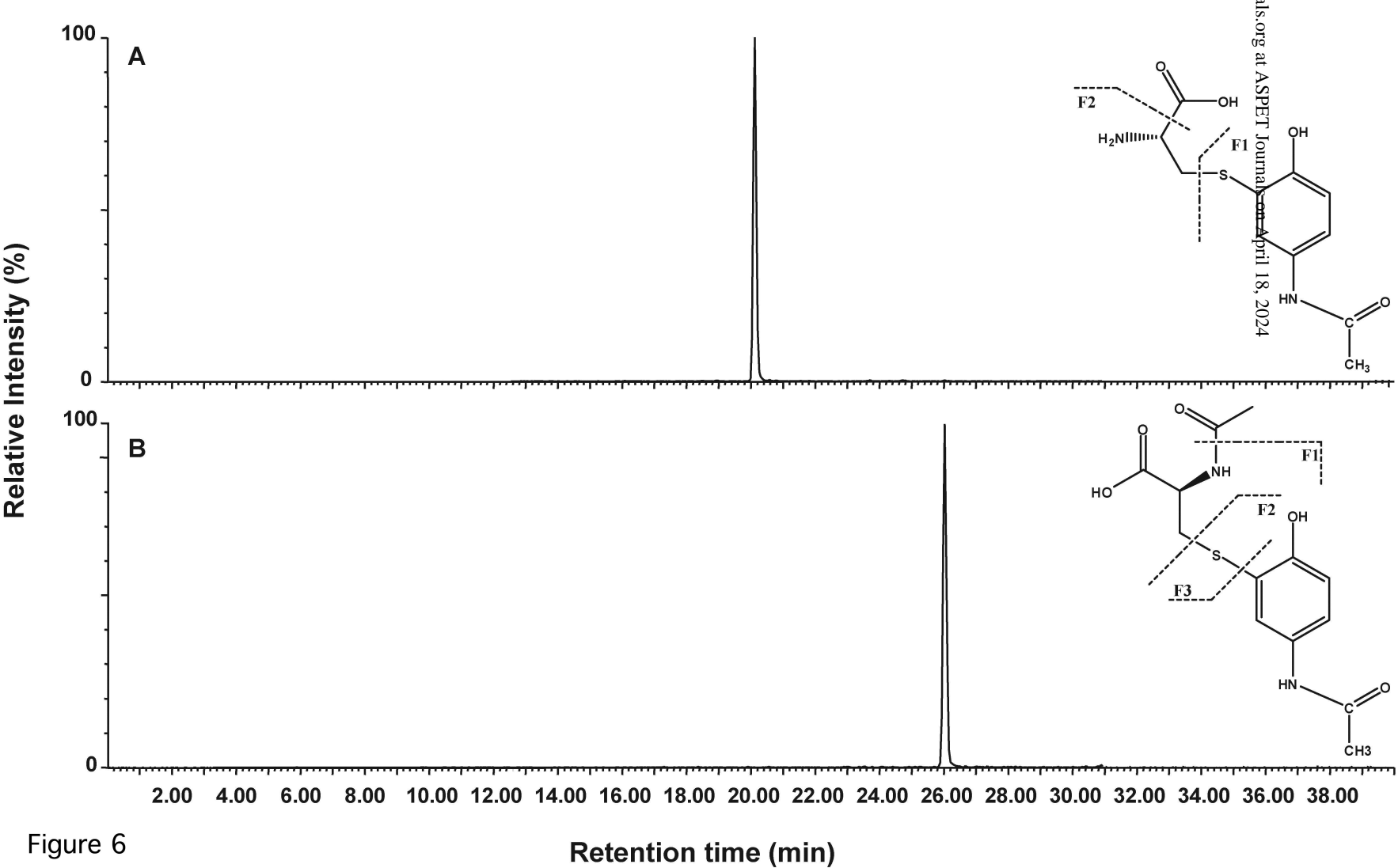


Figure 5



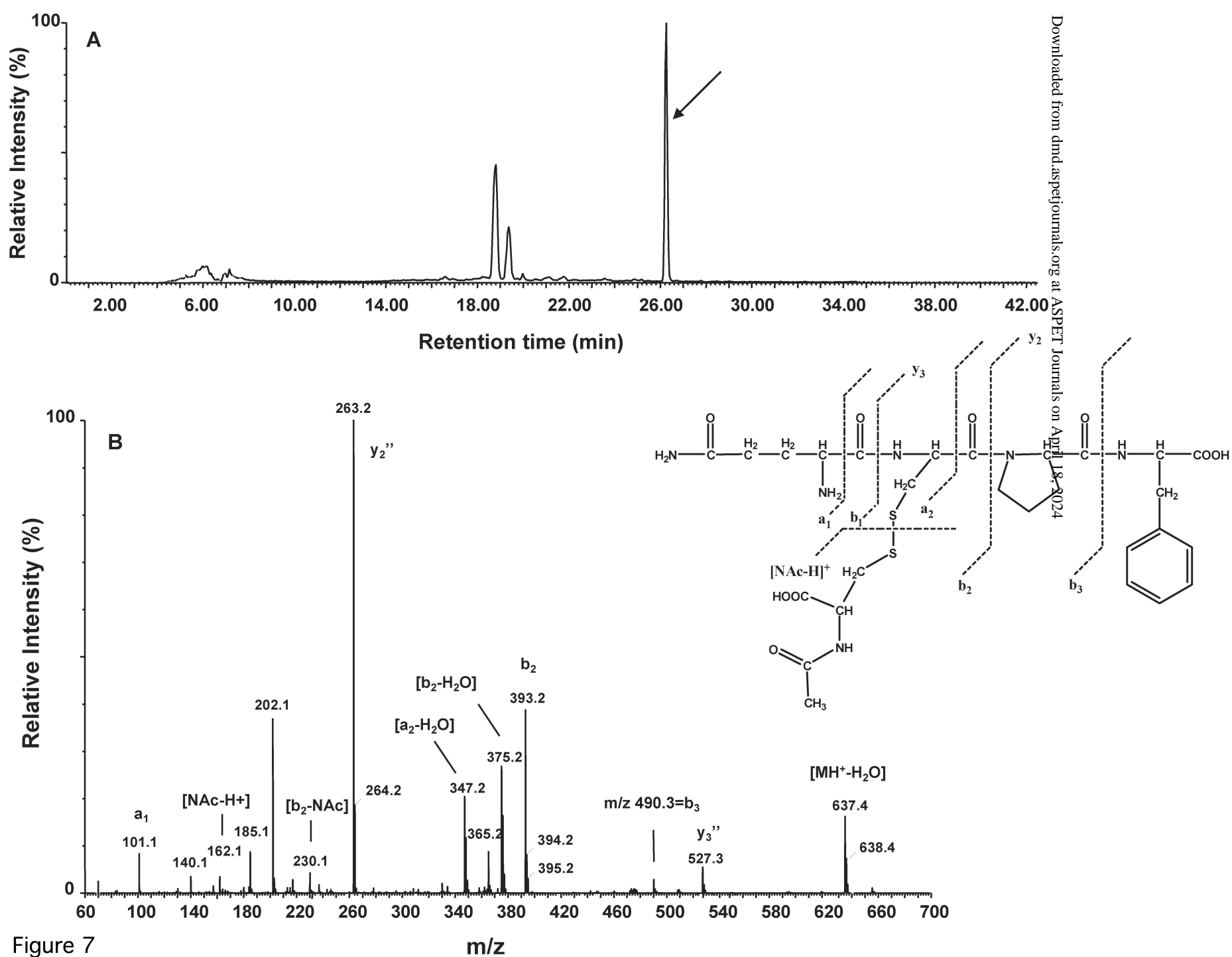


Figure 7

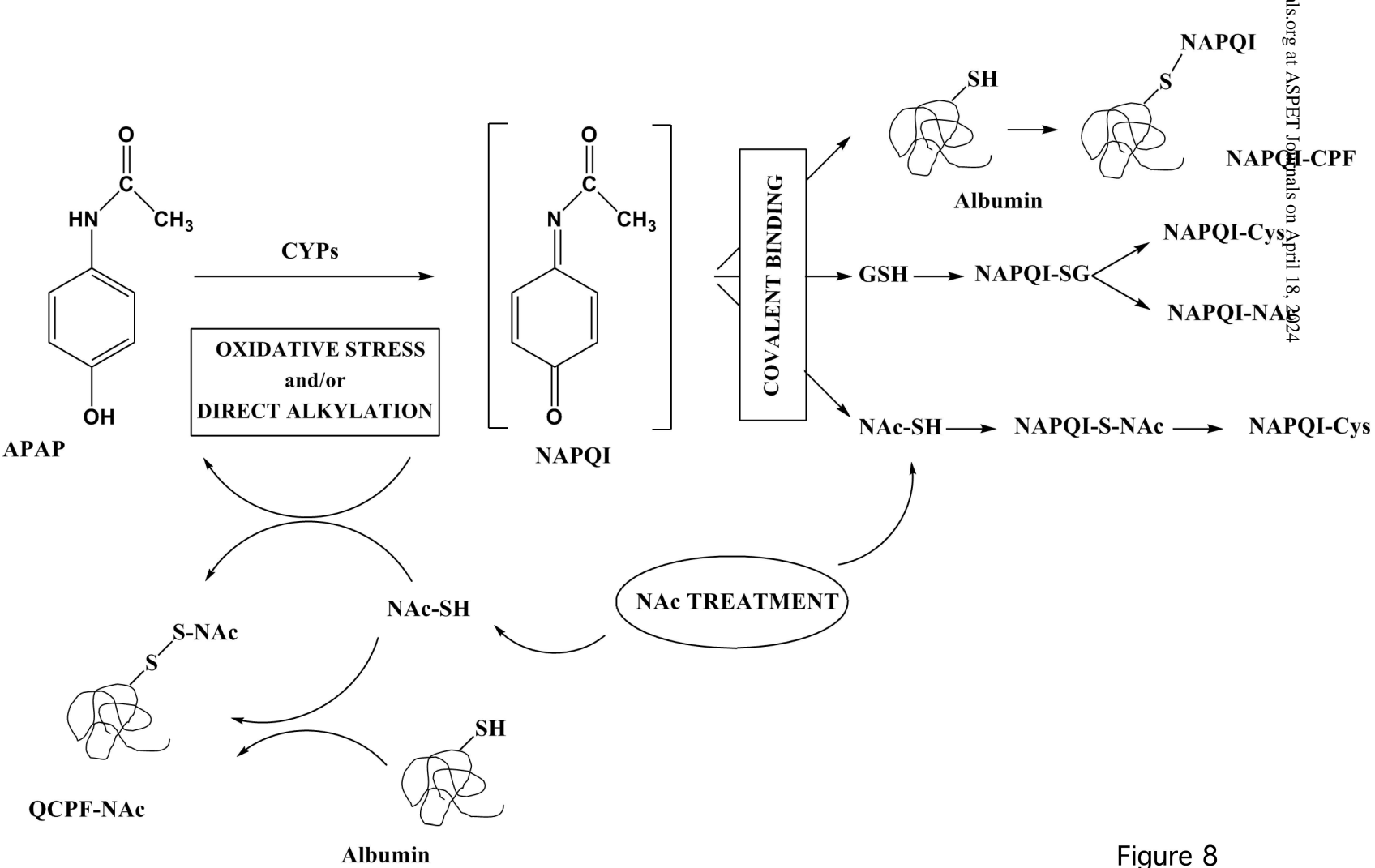


Figure 8