Journal of Analytical Toxicology 2015;39:270–279
doi:10.1093/jat/bkv010 Advance Access publication February 21, 2015

Article

Evidence of Sulfur Mustard Exposure in Human Plasma by LC–ESI–MS-MS Detection of the Albumin-Derived Alkylated HETE–CP Dipeptide and Chromatographic Investigation of Its *Cis*/*Trans* Isomerism

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Sulfur mustard (SM) is a chemical warfare agent that causes painful blisters and chemically modifies endogenous biomacromolecules by alkylation to hydroxyethylthioethyl (HETE) adducts representing valuable long-term markers for post-exposure analysis. The albumin adduct formed in human plasma *in vitro* (HETE bound to the side chain of cysteine 34) was isolated and cleaved by current lots of pronase primarily generating the internal modified dipeptide (HETE–cysteine–proline, HETE–CP) instead of the formerly reported HETE–CPF tripeptide. The analyte was detected by liquid chromatography–electrospray ionization tandem-mass spectrometry (LC–ESI–MS-MS). In principle, HETE–CP undergoes a dynamic on-column equilibrium of *cis–trans* isomerism thus requiring separation at 50°C to obtain one narrow peak. Accordingly, we developed both a novel longer lasting but more sensitive microbore (1 mm i.d., flow 30 μL/min, cycle time 60 min, LOD 50 nM) and a faster, less sensitive narrowbore (2.1 mm i.d., 200 μL/min, cycle time 16 min, LOD 100 nM, both on Atlantis T3 material at 50°C) LC–ESI–MS-MS method suitable for verification analysis. The corresponding tri- and tetrapeptide, Q(HETE)–CPF were monitored simultaneously. HETE–CP peak areas were directly proportional to SM concentrations added to plasma *in vitro* (0.05–100 μM). Albumin adducts formed by deuterated SM (d8-SM) served as internal standard.

Introduction

Sulfur mustard (SM, bis(2-chloroethyl)sulfide, CAS no. 505-60-2) is a chemical warfare agent belonging to the class of vesicants. Exposed skin areas may develop erythema and painful blisters that are characterized by complicated and delayed wound healing (1).

Worldwide stockpiled SM is controlled by the Organisation for the Prohibition of Chemical Weapons (OPCW, Nobel Peace Prize laureate of 2013) and scheduled for destruction according to the Chemical Weapons Convention. Very recently, stocks of SM declared by the Syrian Arab Republic were destroyed by alkaline hydrolysis on the specially equipped US container ship MV Cape Ray (2). Nevertheless, SM that might be synthesized in smaller scale with moderate expertise still represents a threat for the military and civilian population especially in asymmetric or terroristic scenarios. Therefore, bioanalytical methods are demanded allowing evidence of exposure to SM. Due to the low stability and high reactivity of that poison, detection of the original compound or its hydrolysis products in biological specimens is a major challenge for verification analysis. Sample drawing even a few hours after exposure prevent successful detection of SM, whereas thiodiglycol (TDG, the most prominent product after hydrolysis) can be measured in urine for days. The most long-lived markers are covalent reaction products (adducts) to proteins (e.g., albumin or hemoglobin) and DNA (1). SM-adducts with diverse valine and histidine residues of hemoglobin were identified by Noort *et al.* (3) and corresponding peptide and amino acid derivatives were established as targets of sophisticated analytical procedures for verification (4). Additional analytical methods are based on non-enzymatic hydrolytic cleavage of the SM-derived moiety from any protein thus generating TDG for subsequent gas chromatography–mass spectrometric (GC–MS) analysis (5).

SM–albumin adducts have been shown to have a half-life *in vivo* of 3–4 weeks (6) thus providing a much longer time frame for verification post-exposure. SM is known to alkylate the only free, not disulfide-linked cysteine residue in human albumin producing an *S*-linked hydroxyethylthioethyl-derivative (HETE–albumin) (6). This chemical modification has been the target of diverse bioanalytical procedures (7–13) that are all based on the fundamental work of Noort *et al.* (6). According to this strategy albumin and its HETE adducts are extracted from plasma or serum by affinity chromatography, desalted on PD-10 columns and proteolytically cleaved by pronase. Pronase—a mixture of diverse endo- and exopeptidases of undefined composition (14)—was reported to generate an alkylated tripeptide, HETE–CPF, suitable for reliable analysis by liquid chromatography–electrospray ionization tandem-mass spectrometry (LC–ESI–MS-MS) (6). In reversed-phase (RP) chromatography this peptide derivative elutes as a narrow peak producing two major product ions after collision-induced-dissociation (CID) at m/z 105 and m/z 137 (6).

When trying to establish this procedure in our laboratory we failed to detect that HETE–CPF tripeptide. This unexpected result presumed to be due to variable enzyme activity of the different lots of pronase used. Instead of the tripeptide we identified the HETE–CP dipeptide nearly exclusively. Even though the occurrence of this proteolysis product has been mentioned before (6), no report has focused on that marker for verification analysis so far. This fact might primarily be due to the non-favorable broad elution profile of the dipeptide in RP-chromatography that hampers the most sensitive analysis.

However, based on the fact that many if not most current lots of commercially available pronase predominantly produce the dipeptide instead of the tripeptide, we present the development of a method targeting the HETE–CP molecule for verification analysis. Following this idea, we unraveled the scientific nature of its broad peak phenomenon, modified chromatographic

parameters to sharpen the peak and thus developed two novel methods for qualitative post-exposure analysis of SM using d8-SM incubated plasma as internal standard.

Experimental

Chemicals

Acetonitrile (ACN, gradient grade), water (HPLC grade) and isopropanol (iPrOH, GC grade) were purchased from Merck (Darmstadt, Germany). Formic acid (FA, ≥98% pro analysi) was obtained from Carl Roth (Karlsruhe, Germany). NH_4HCO_3 (Ultra grade, ≥99.5%) was from Fluka (Buchs, Switzerland). Human serum albumin (HSA) was delivered by Sigma-Aldrich (Steinheim, Germany). Both pronase and protease type XIV (pronase E) from *Streptomyces griseus* were provided by Roche (Mannheim, Germany; lot no. 70327222) and Sigma-Aldrich (lot no. SLBJ2160V), respectively. SM and 8-fold deuterated SM (d8-SM) were made available by the German Ministry of Defence and tested for integrity and purity in-house by nuclear magnetic resonance spectroscopy. Deuterated atropine (d3-atropine) was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Human ethylenediamine tetraacetic acid (EDTA) plasma was delivered by Dunn Labortechnik (Asbach, Germany).

Incubation of plasma and pure albumin with SM and d8-SM

Albumin solution (1,440 μL, 40 mg/mL) or EDTA–plasma (1,440 μL) was incubated with SM (60 μL in iPrOH) or d8-SM (60 μL in iPrOH) for 2 h at 37°C under gentle shaking. SM concentrations in plasma ranged from 0.05 to 100 μM. Samples were stored at −25°C until further processing if necessary.

Isolation of SM–albumin adducts from plasma

Albumin from plasma (100 μL) was extracted by the use of a *albumin removal columns* (ProteoExtract, Calbiochem, Merck, Darmstadt, Germany). Isolation based on affinity extraction was performed as published previously (15). The albumin-containing eluate was concentrated by ultrafiltration (UF, molecular weight cut-off, MWCO, 10 kDa, Vivaspin 500 centrifugal concentrator, Satorius Stedim, Göttingen, Germany) to 100 μL. The retentate was washed two times with NH_4HCO_3 buffer (600 μL, 50 mM) by UF (MWCO 10 kDa) and remaining retentate (100 μL) was diluted with the same buffer (100 μL).

Quantification of albumin and investigation of purity

Quantification of extracted albumin as well as testing for purity was performed as described earlier (15). In brief, quantification was carried out by a colorimetric modified Lowry assay in 96-well plate format according to the instructions of the manufacturer (BC-assay, Interchim, Montlucon, France) to calculate extraction recovery. Purity was checked by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining following common protocols (15).

Enzymatic cleavage of SM–albumin adducts with pronase

Pronase (100 μL, 10 mg/mL in 50 mM NH_4HCO_3) was added to isolated albumin after UF (200 μL, see above) allowing incubation for 2 h at 37°C under gentle shaking. Following UF (MWCO 10 kDa), the retentate (50 μL) was washed twice with 100 μL NH_4HCO_3 buffer as described above. Filtrates containing peptide cleavage products were combined (400 μL) and an aliquot was diluted 1:3 with 0.5% (v/v) FA for subsequent LC-ESI–MS-MS measurements.

LC–ESI–MS-MS analysis of SM–albumin adducts

Three different LC–ESI–MS-MS methods were developed differing in gradient applied and column dimensions: narrowbore (inner diameter, i.d., 2.1 mm) and microbore (i.d. 1 mm).

Narrowbore separations

All narrowbore separations were carried out on an UltiMate 3000 Standard LC System (Dionex, Sunnyvale, CA, USA) comprising of an UltiMate 3000 pump, autosampler and column compartment. The chromatographic system was on-line coupled to an API 4000 QTrap mass spectrometer (AB SCIEX, Darmstadt, Germany). Separations of 20 μL sample were carried out on an Atlantis T3 column (150 × 2.1 mm i.d., 3 μm, 100 A; Waters, Eschborn, Germany) connected with a precolumn (security guard cartridges, widepore C18 4 × 2 mm i.d.; Phenomenex, Aschaffenburg, Germany) at a flow of 200 μL/min. Solvent A (0.1% v/v FA) and solvent B (ACN/H_2O, 80:20 v/v, 0.1% v/v FA) served as mobile phase in gradient mode (see below). MS data analysis and control of the mass spectrometer were done with Analyst 1.6 software (AB SCIEX) and a Dionex chromatography MS link (version 2.12.0.3414). MS parameters were set as follows: curtain gas (CUR) 50 psi (3.45×10^5 Pa), ionization voltage (IS) 4,500 V, temperature 300°C, declustering potential (DP) 60 V (HETE adducts) and 86 V (d3-atropine), entrance potential (EP) 10 V, cell exit potential (CXP) 10 V, heater gas (GS1) and turbo ion spray gas (GS2) both 60 psi (4.14×10^5 Pa) and dwell time 90 ms.

MS–MS transitions were recorded from m/z 323.1 to m/z 105.0 (quantifying ion) and m/z 137.0 (qualifying ion) for HETE–CP (collision energy, CE 30 V); m/z 331.1 to m/z 145.0 (quantifying ion) and m/z 113.0 (qualifying ion) for d8-HETE–CP (CE 30 V); m/z 470.1 to m/z 105.0 and m/z 137.0 for HETE–CPF (CE 30 V); m/z 478.1 to m/z 113.0 and m/z 145.0 for d8-HETE–CPF (CE 30 V); m/z 598.2 to m/z 105.0 and m/z 137.0 for Q(HETE)–CPF (CE 30 V); m/z 606.2 to m/z 113.0 and m/z 145.0 for Q(d8-HETE)–CPF (CE 30 V) and m/z 293.2 to m/z 93.2 and m/z 127.1 for d3-atropine (CE 35 V).

Narrowbore separations for verification analysis

The method used for verification analysis worked at a fixed column temperature of 50°C with the following step-gradient: t(min)/B(%): 0/0; 1/0; 1.02/40; 4/40; 4.02/80; 8/80; 9/0; 15/0 with an initial 1 min equilibration period under starting conditions. For MS parameters see above.

Narrowbore separations for characterization of temperature-dependent elution profile

For monitoring the temperature-dependent elution profile of the HETE–CP dipeptide the following gradient was used: t[min]/B[%]: 0/10; 13/50; 14/85; 17/85; 18/10; 20/10 with an initial

1 min equilibration period under starting conditions. For this study temperature of the column and solvent reservoir was adjusted to 1, 10, 15, 20, 23, 30, 40 and 50°C, respectively. MS parameters were as described above.

Microbore separations

Microbore separations (μLC–ESI–MS-MS) were developed for verification analysis. The μLC system consisted of a 1431 MicroPro pump (Eldex Laboratories, Napa, CA, USA), an Edurance autosampler and Mistral column oven (Spark Holland, Emmen, The Netherlands) and a Degasys Populaire degasser (Sunchrom, Friedrichsdorf, Germany) on-line coupled to the same API 4000 QTrap mass spectrometer as used for narrowbore chromatography. Pumps were controlled by MicroPro 1.0 software (SCPA, Weyhe-Leeste, Germany), and the autosampler by Endurance/Midas 3.10 (SCPA). Separations of 20 μL sample were performed on an Atlantis T3 column (150 × 1.0 mm i.d., 3 μm, 100 A, Waters) protected by a precolumn (security guard cartridges, widepore C18 4 × 2 mm i.d., Phenomenex) at a flow of 30 μL/min. The following gradient of solvent A (0.05% v/v FA) and solvent B (ACN/H_2O 80:20 v/v, 0.05% v/v FA) was applied: t(min)/B(%): 0/0; 38/40; 39/80; 43/80; 44/0; 45/0 including an initial 15 min equilibration period under starting conditions. Temperature of column (50°C) and solvent reservoir (40°C) was controlled. MS parameters were as follows: CUR 30 psi (2.07×10^5 Pa), IS 4,500 V, temperature 200°C, DP 60 V for the HETE adducts and 86 V for d3-atropine, EP 10 V, CXP 10 V, both GS1 and GS2 50 psi (3.45×10^5 Pa) and dwell time 90 ms. Transitions monitored and the corresponding parameters were the same as described for narrowbore separations (see above).

Characterization and comparison of narrowbore and microbore analysis

To elaborate well-suited approaches for verification of SM exposure both narrowbore and microbore methods were compared and characterized with respect to ion suppression, sensitivity, linear range, lower limit of detection (LOD) and stability of HETE–CP stored in the autosampler.

Linear range and LOD

For elaboration of linearity of HETE–CP peak area and estimation of the technical LOD nine standards were produced and analyzed by both methods. A human plasma sample incubated with 100 μM SM was prepared for LC–ESI–MS-MS analysis and aliquots were mixed with 0.5% v/v FA resulting in dilutions and SM concentration equivalents of 1:2 (50 μM), 1:10 (10 μM); 1:20 (5 μM); 1:100 (1 μM); 1:200 (500 nM); 1:1,000 (100 nM); 1:2,000 (50 nM) and 1:10,000 (10 nM).

Ion suppression and sensitivity

For investigation of ion suppression of HETE–CP caused by any component present in the samples after proteolysis of albuminspecified mixtures of solution A and solution B (see below) were analyzed. Solution A was produced as follows: human plasma was separately incubated with SM and d8-SM (both 100 μM) and prepared as described above. Final filtrates after pronase cleavage were mixed in equal volumes and diluted 1:3 with 0.5% (v/v) FA. Filtrates obtained from blank plasma were diluted accordingly yielding solution B. Solutions A and B were mixed in defined ratios ranging from 0% to 100% (v/v) solution A in steps of 10%. A similar set of samples was produced by using 0.5% v/v FA instead of solution B. All samples were measured with both narrowbore and microbore methods. Resulting peak areas of HETE–CP were plotted versus relative content of solution A prior to appropriate regression. Ion suppression in both methods was characterized by the ratios of the slopes resulting from solution B (m_B) and FA (m_{FA}) as m_B/m_{FA}.

Peak areas obtained for the two fragment ions of HETE–CP were used to calculate ion ratios of quantifying (m/z 105.0) to qualifying ion (m/z 137.0). In addition, peak area ratios of quantifying ions of HETE–CP and d8-HETE–CP were calculated for all samples.

Stability of HETE–CP in autosampler

Stability of HETE–CP in prepared plasma samples stored in the autosampler for 48 h at 15°C was investigated by consecutive μLC–ESI–MS-MS analyses in the presence of d3-atropine (2 ng/mL) as internal standard.

Proteolysis kinetics of pronase

Commercially available human albumin (40 mg/mL in physiological NaCl solution) was separately incubated with SM and d8-SM (both 100 μM) for 2 h at 37°C. Afterward, 150 μL of each reaction batch was mixed and ultrafiltrated for buffer exchange (50 mM NH_4HCO_3) and concentration to a final volume of 200 μL. This solution was transferred into a 1.5-mL Eppendorf tube and mixed with 250 μL buffer used for washing the UF device. Subsequently, 300 μL pronase solution (10 mg/mL in 50 mM NH_4HCO_3) was added to start proteolysis at 37°C under gentle shaking. Aliquots (50 μL) were taken at 0.3, 3, 5, 10, 20, 30, 45, 60, 90 and 120 min, precipitated by addition of 100 μL ACN, vortex mixed and centrifuged (5 min at 10,400g). A portion of the supernatant (100 μL) was evaporated to dryness under a gentle stream of nitrogen and redissolved in 60 μL 0.5% (v/v) FA prior to μLC-ESI–MS-MS analysis. This procedure was carried out in duplicate for both lots of pronase from Sigma-Aldrich (lot no. SLBJ2160 V) and Roche (lot no. 70327222). For quality control, ratios of quantifying ions of HETE–CP/d8-HETE–CP were calculated.

Dependence of SM concentrations added to plasma on HETE–CP formation

For characterization of dependence of the added SM amount on albumin adduct formation human plasma was incubated with SM concentrations of 0.05, 0.1, 0.5, 1, 2, 5, 10, 20, 50 and 100 μM in triplicate. For relative quantitative evaluation an aliquot of these plasma samples (90 μL) was mixed with human plasma (10 μL) exposed to 100 μM d8-SM before. The d8-SM derived adduct served as internal standard. Following sample preparation the HETE–CP dipeptide and its d8-HETE–CP analog were measured by both narrowbore and microbore methods. In addition, prepared samples (240 μL) derived from incubations with 0.05–20 μM SM were evaporated to dryness, redissolved in 60 μL 0.5% v/v FA and analyzed again.

SM concentrations were plotted versus absolute peak areas of HETE–CP and versus peak area ratios of HETE–CP/d8-HETE–CP.

Results

Isolation, quantification and purity of albumin from plasma

As described earlier (15) albumin extraction resulted in a yield of 3.6 mg protein from 100 μL human plasma indicating a recovery of 90% (assuming 40 mg albumin/mL plasma). SDS-PAGE of extracted protein from human plasma yielded only one band with an apparent molecular weight of 68 kDa (data not shown).

Cleavage products of albumin adducts generated by pronase

Initial LC–ESI–MS-MS analyses of prepared plasma samples and pure albumin both incubated with SM before were carried out according to Noort *et al.* (6). But only smallest peaks were found for the expected HETE–CPF tripeptide when monitoring selective transitions of m/z 470.1 to m/z 105 and m/z 137 as well as for the Q(HETE)–CPF tetrapeptide recorded by transitions from m/z 598.2 to the same fragments. In contrast, we detected the dipeptide HETE–CP (precursor at m/z 323.1) almost exclusively with high intensities by monitoring transitions to the common product ions mentioned above. Results were the same for two different lots of pronase currently available from Roche and Sigma-Aldrich. Under standard chromatographic conditions using a common RP C18 stationary phase (Acclaim PepMap 100 C18, 150 × 1.0 mm i.d., 3 μm, 100 A; ThermoFisher, Schwerte, Germany) the HETE–CP adduct eluted as a broad peak with a peak width at the base of 4.5 min (Figure 1A). Mass spectrometric CID of this protonated adduct yielded major fragments at m/z 105 and m/z 137. Figure 2A shows the MS–MS spectrum extracted from a μLC–ESI–MS-MS run. Corresponding signals obtained from d8-HETE–CP (m/z 331.1) were found at m/z 113 and m/z 145 (Figure 2B).

Temperature-dependent narrowbore separations

Figure 3 illustrates the temperature-dependent elution profile of the alkylated dipeptide HETE–CP analyzed by narrowbore LC–ESI–MS-MS. At 1°C two baseline-separated narrow peaks were found at retention times (t_R) of 5.8 and 7.9 min (Figure 3A). Successive increase of temperature caused shifting of the peaks toward each other thereby producing a broad overlapping and continuously growing elution zone between both signals. At the highest temperature tested (50°C) only one single narrow peak was observed (t_R 3.9 min, Figure 3H). The total peak areas of each run considering all peaks detected were constant with a relative standard deviation (RSD) of 3.2% over all temperatures tested. The ratio of quantifying and qualifying ion of HETE–CP was found to be constant at 1.8 ± 6% for all peaks detected independent of their elution profile.

Characterization and comparison of narrowbore and microbore analysis

Both methods designed for verification purposes allowed detection of HETE–CP in prepared plasma samples as a narrow peak at

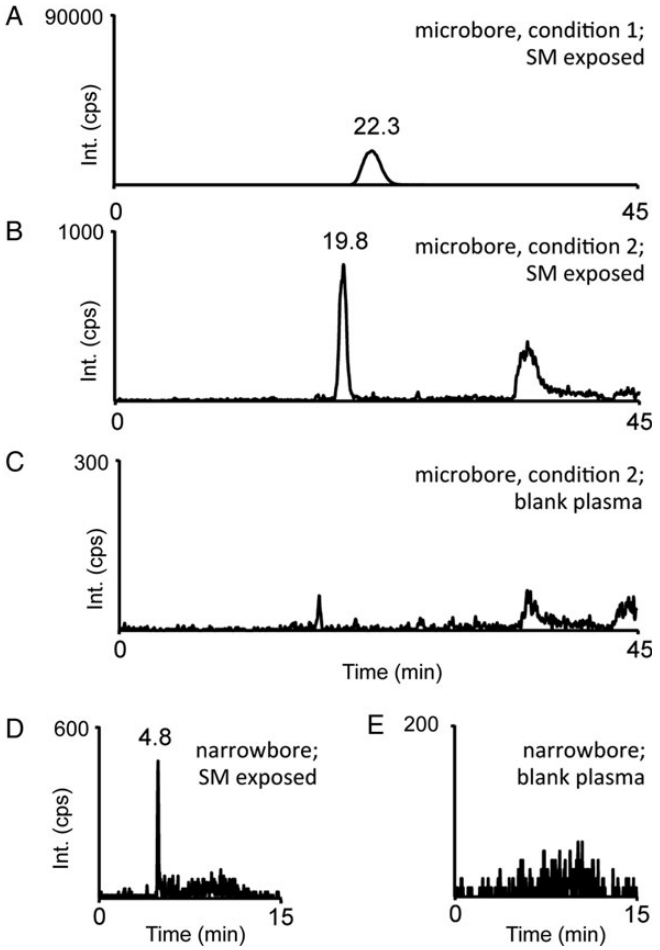

Figure 1. LC–ESI–MS-MS detection of HETE–CP in albumin proteolysis mixtures. HETE–CP was detected in multiple reaction mode monitoring transitions from m/z 323.1 to m/z 105.0 (quantifying ion) and to m/z 137.0 (qualifying ion). For reasons of clarity only traces of the quantifying ion are shown. (A) Conventional microbore RP chromatography (condition 1: Acclaim PepMap 100 C18, 150 × 1.0 mm i.d., 3 μm, 100 A) at 30°C (20 μL/min). Sample derived from human plasma incubated with 100 μM SM. HETE–CP peak width at the base: 4.5 min. (B and C) Optimized microbore method for verification (condition 2: Atlantis T3, 150 × 1.0 mm i.d., 3 μm, 100 A) at 50°C (30 μL/min), cycle time 60 min. Sample derived from (B) human plasma incubated with 100 nM SM and (C) blank plasma. HETE–CP peak width at the base: 1.5 min. (D and E) Narrowbore method for verification (Atlantis T3, 150 × 2.1 mm i.d., 3 μm, 100 A) at 50°C (200 μL/min), cycle time 16 min. Sample derived from (D) human plasma incubated with 100 nM SM and (E) blank plasma. HETE–CP peak width at the base: 0.4 min.

50°C. The retention time of HETE–CP in the narrowbore method was 4.8 min with a cycle time (time between two injections) of 16 min (Figure 1D) whereas t_R in the microbore method was 19.8 min (HETE–CP) and 19.6 min (d8-HETE–CP) with a 60-min cycle time (Figure 1B). Peak width at the base was 0.4 min in the narrowbore and 1.5 min in the microbore method.

Linear range and technical LOD

Plotting peak areas of HETE–CP (in counts, cts) versus the relative SM concentration equivalent (in μM) allowed excellent linear regression in the range from 50 nM to 50 μM for the microbore method (3 orders of magnitude, $y = 30{,}558x + 2{,}223$,

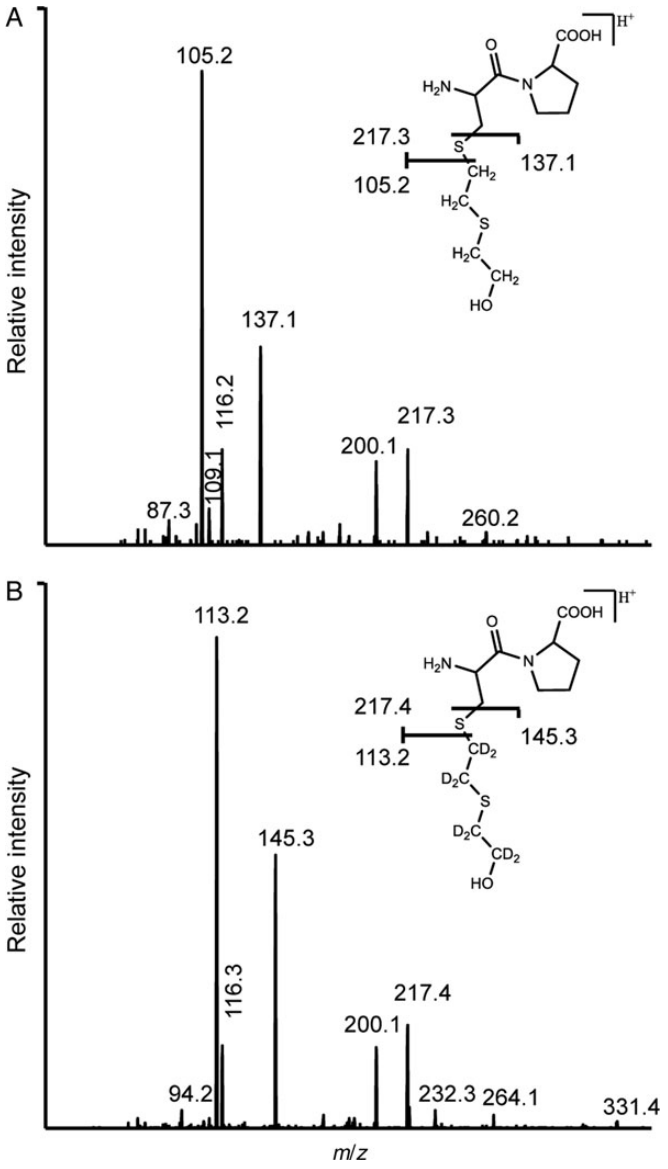

Figure 2. MS-MS spectra of alkylated dipeptide from human albumin. (A) HETE–CP and (B) d8-HETE–CP. Spectra were extracted from a LC–ESI–MS-MS run of alkylated albumin cleaved by pronase in product ion scan mode of (A) m/z 323.1 and (B) m/z 331.1. Sites of fragmentation by CID are marked.

$r^2 = 0.9996$) and from 100 nM to 10 μM for narrowbore analysis (2 orders of magnitude, $y = 6{,}579x + 75$, $r^2 = 0.9996$). Respective higher concentration up to 100 μM clearly demonstrated a SM dose-dependent increase of the peak areas but this effect was smaller than expected from linear regression. Accordingly, technical LODs of 50 nM (S/N = 4) were found for the microbore and 100 nM (S/N = 4) for the narrowbore method.

Ion suppression and sensitivity

The narrowbore method yielded linear relationships between HETE–CP peak areas (in cts) and the relative content of solution A (dimensionless) containing the SM and d8-SM dipeptide adducts. Regression for mixtures with FA ($0.198x + 0.496$, $r^2 = 0.98$) and solution B containing the plasma derived matrix ($0.135x + 0.289$, $r^2 = 0.99$) resulted in a slope ratio m_B/m_{FA} of 0.68 characterizing an ion suppression of 32% (corresponding to 68% remaining intensity). Linear relations were also obtained in microbore analysis for mixtures with FA ($1.142x + 0.077$, $r^2 = 0.99$) as well as solution B ($0.940x + 1.646$, $r^2 = 0.99$) yielding in a slope ratio m_B/m_{FA} of 0.82 indicating an improved ion suppression of 18% indicating 82% remaining intensity.

Accordingly, slope ratios (m_μ/m_n) obtained with the microbore (m_μ) and narrowbore method (m_n) for mixtures with FA ($m_\mu/m_n = 5.8$) and solution B ($m_\mu/m_n = 7.0$) revealed that the microbore method was about six to seven times more sensitive depending on matrix composition.

The peak area ratios of quantifying ions of HETE–CP/d8-HETE–CP was calculated to be 2.4 ± 4% in microbore and 2.2 ± 4% in narrowbore analysis. The peak area ratio of the quantifying and qualifying ion of HETE–CP was found to be constant over the entire range of content A in both methods thus indicating reproducible fragmentation conditions (1.85 ± 7%).

Stability of HETE–CP in autosampler

Peak areas of HETE–CP measured during the 48 h storage at 15°C in the autosampler did not show any trend and remained constant with a RSD of 9%. The same effect was observed when plotting the peak area ratios of HETE–CP to d3-atropine (RSD 6%) that is known to be stable under these conditions (16, 17). Furthermore, retention time did also not change.

Proteolysis kinetics of pronase

Production of HETE–CP (t_R, 19.8 min) as well as of HETE–CPF (t_R, 29.9 min) and Q(HETE)-CPF (t_R, 30.1 min) cleaved from HETE–albumin by pronase (Sigma-Aldrich) are illustrated in Figure 4. The corresponding concentration–time profile obtained with the other lot of pronase (Roche) was absolutely identical (data not shown). Furthermore, data obtained for albumin alkylated with d8-SM did not show any differences in the concentration–time profile. The following ion ratios were calculated for quality control: quantifying/qualifying ion of HETE–CP (1.86 ± 3%) indicating the absence of interferences and quantifying ions of HETE–CP/d8-HETE–CP (1.8 ± 3%) indicating albumin degradation without isotope-label distinction.

Dependence of SM concentrations added to plasma on HETE–CP formation

The relation between SM concentrations used for human plasma incubations (0.05–100 μM) and measured peak area ratios (HETE–CP/d8-HETE–CP) was linear over 3 orders of magnitude independent of the method used. Figure 5 illustrates similar regression results for the narrowbore (curve 3, slope 0.150/μM) and the microbore analysis (curve 2, slope 0.169/μM).

Corresponding plots of absolute HETE–CP peak areas differed more obviously. In narrowbore analysis a slope of 3595/μM ($r^2 = 0.97$) was found (Figure 5, curve 4) in contrast to 37,312/μM ($r^2 = 0.994$) obtained by the microbore method (Figure 5, curve 1). Concentrating the samples (by factor 4) by means of evaporation to dryness and subsequent redissolvation yielded higher slopes indicating a 4-fold increase of sensitivity (data not shown).

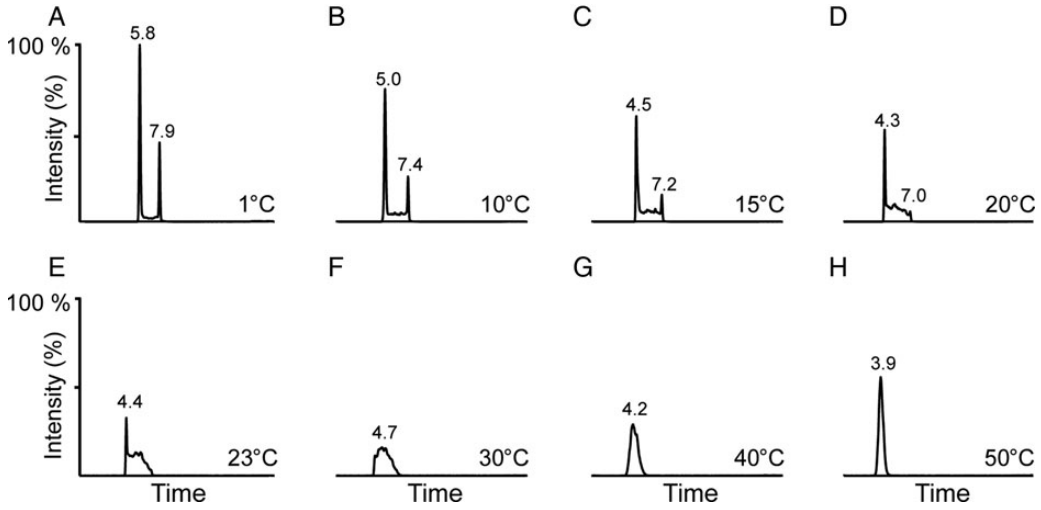

Figure 3. Temperature-dependent elution profile of HETE–CP. HETE–CP produced by human albumin proteolysis with pronase was chromatographed on Atlantis T3 (150 × 2.1 mm i.d., 3 μm, 100 A) with a flow of 200 μL/min at given temperatures and monitored in multiple reaction mode following transitions from m/z 323.1 to m/z 105.0 and m/z 137.0. For reasons of clarity only the traces of m/z 105 are shown. Peak profiles document the dynamic on-column equilibrium of *cis*- and *trans*-configuration at the imide bond between cysteine and its C-terminally bound proline in HETE–CP. At 1°C velocity of on-column conversion was minimized allowing separation of both isomers. At 50°C fastest conversion occurred yielding only one narrow peak suitable for sensitive detection. Total peak areas of each run were identical (RSD 3.2%) and peak area ratios of both product ions (m/z 105 and 137) were constant (1.8 ± 6%). Therefore, identification and assignment of isomers were not possible based on chromatographic and MS data.

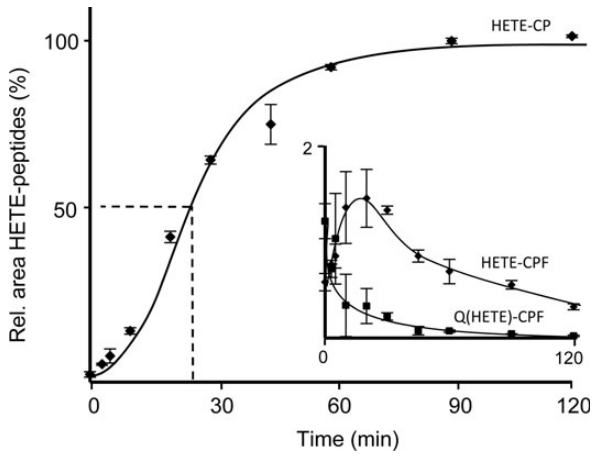

Figure 4. Kinetics of HETE–CP production by albumin cleavage with pronase. Human albumin incubated with SM (100 μM) was mixed with pronase (Sigma-Aldrich, lot no. SLBJ2160 V) at 37°C, aliquots were taken at distinct time points, precipitated with ACN to stop proteolysis and analyzed by microbore LC–ESI–MS-MS monitoring HETE–CP as well as HETE–CPF and Q(HETE)–CPF by their transitions to common product ions (m/z 105 and 137). Data points are mean and SD of duplicate incubations and duplicate LC–ESI–MS-MS analysis of each sample. HETE–CP was produced nearly exclusively reaching maximum concentration after ~60 min with a period of half-change of 25 min (dashed line) indicating stability against further proteolysis. Results were the same for pronase purchased from Roche (lot no. 70327222). Identical data were also obtained for production of d8-HETE–CP with both enzyme lots indicating no isotopic discrimination for proteolysis.

According to the standard protocol without concentrating, the LOD for the HETE–CP adduct corresponded to SM concentrations of 100 and 50 nM in plasma (S/N = 4) for the narrowbore and microbore method, respectively.

Discussion

Human albumin (SwissProt no. P02768) is known to be alkylated by SM at the only free non-disulfide-bridged cysteine residue (Cys^{34}, numbering does not include the signal peptide) (6). The resulting HETE-adduct thus serves as a marker for SM exposure (6–13). The original method suitable for verification analysis was presented by Noort *et al.* analyzing the albumin-derived HETE–CPF tripeptide by LC–ESI–MS-MS (6). According to an improved protocol of this procedure albumin was first extracted from plasma by HiTrapBlue affinity followed by desalting on PD-10 columns and final concentration by UF (9, 12). Intending to proceed with our good experience in using albumin removal columns (15) we decided to apply this tool for albumin isolation followed by UF thereby economizing one manual preparation step.

Isolation, quantification and purity of albumin from plasma

According to our earlier results (15), SDS-PAGE analysis revealed purified albumin extractable from human plasma with high selectivity and satisfying recoveries (90%). This extraction procedure thus appears as a valuable alternative to the use of HiTrapBlue and PD-10 columns (9). Nevertheless, it is much more laborious and time consuming than the 96-well plate method recently presented by Andacht *et al.* (13) but in contrast does not require laboratory equipment like extraction chambers or special centrifuges.

LC–ESI–MS-MS analysis of SM–albumin adducts

Based on earlier reports (6, 9) we set up a positive ESI–MS-MS protocol for simultaneous detection of the CP dipeptide, CPF tripeptide and QCPF tetrapeptide all alkylated at the cysteine side chain either by SM or d8-SM. LC–ESI–MS-MS-based techniques are the method of choice for peptides (18) as well as adducts with diverse poisons (19). Transitions to their major common product ions at m/z 105.0 and m/z 137.0 for SM adducts and

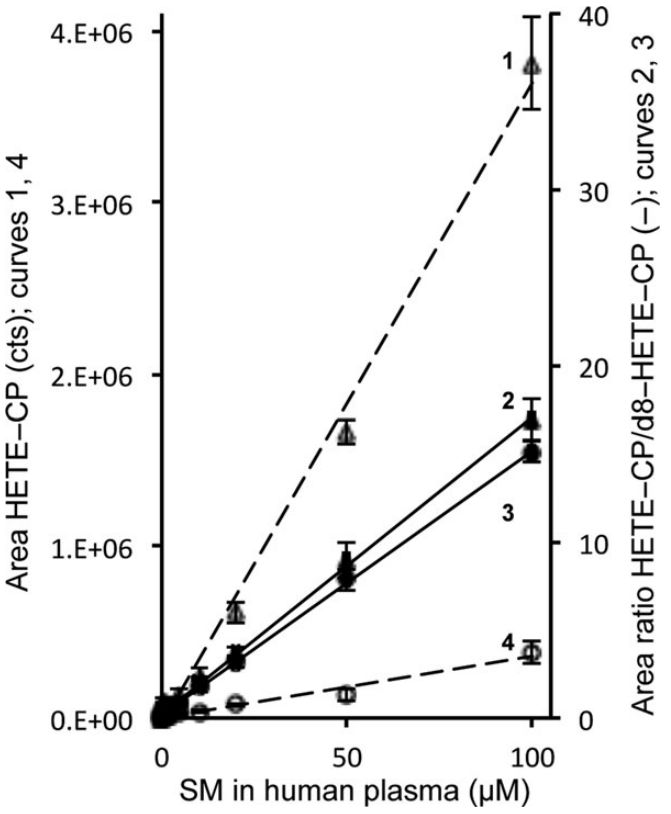

Figure 5. HETE–CP formation in dependence of the SM concentration in human plasma. Human plasma was incubated in triplicate with different concentrations of SM (0.05–100 μM) and prepared by the standard protocol after addition of 10% (v/v) human plasma incubated with 100 μM d8-SM before as internal standard (IS). Analyses were performed by the microbore (curves 1 and 2) and narrowbore LC–ESI–MS-MS methods (curves 3 and 4). Absolute peak areas of HETE–CP (transition to quantifying ion at m/z 105) were plotted against the SM concentrations and revealed that the microbore method (curve 1) was about 10 times more sensitive than the narrowbore method (curve 4). Plotting the peak area ratios of analyte to IS both curves were quite similar (curve 2: microbore, and curve 3: narrowbore) and documented that HETE–CP formation was directly proportional to added SM concentrations. LODs were found at 50 nM (microbore) and 100 nM (narrowbore) SM in human EDTA plasma. Data points are the mean and SD of triplicate incubations and duplicate LC–ESI–MS-MS analysis of each sample.

at m/z 113.0 and m/z 145.0 for d8-SM adducts were monitored. These major fragments correspond to the cleaved HETE-chain (m/z 105.0 and m/z 113.0) and the same ion prolonged by the sulfur atom of the cysteine side chain (m/z 137.0 and m/z 145.0; Figure 2). Applying this method to prepared albumin samples incubated with SM, only traces of the alkylated tri- and tetrapeptide were found whereas major peaks were obtained for the dipeptide products. This was attributed to a different specificity and reactivity of pronase. Pronase which is an undefined mixture of endo- and exopeptidases (14) is usually used for complete breakdown of peptides and proteins down to single amino acids. In accordance, Andacht *et al.* (13) also reported that current lots of pronase from three different vendors did not produce the HETE–CPF tripeptide to the same level as older lots used by Noort *et al.* did (6, 9). Therefore, we accounted for these diversifications and started development of alternative methods targeting the adducted dipeptide by making use of currently commercially available enzyme lots. Method development and optimization were performed with samples containing prepared alkylated human albumin due to the lack of commercially available HETE–CP reference.

In accordance to results mentioned by Noort *et al.* (6) the HETE–CP dipeptide eluted as a broad peak when separated on a conventional RP C18 stationary phase at typical 30°C thus being unfavorable for quantification (Figure 1A). Therefore, we initially tried to make the dipeptide elute as a narrower peak by using Atlantis T3 material as stationary phase that in our group has been proven to be highly suitable for small quite polar molecules (16, 17, 20). The first column used was of narrowbore dimension (150 × 2.1 mm i.d.) working with a 200 μL/min gradient flow promising shorter run times. Even though gradients were found yielding narrow peaks at 30°C we permanently observed a peak splitting effect showing two maxima independent on the retention time. This phenomenon is often interpreted as an effect of impurities, column deterioration, apparatus malfunction or ion suppression (21). However, it might also indicate dynamic equilibrium reactions during separation as demonstrated, e.g., by John and Schlegel for the anomers equilibrium of thromboxane B2 (22, 23). As velocity of such reactions depends on the temperature we varied this parameter for chromatography of the HETE–CP samples.

Temperature-dependent narrowbore separations

As illustrated in Figure 3 a strongly temperature-dependent elution profile of the alkylated dipeptide was found. Two baseline-separated peaks at 1°C were transferred into one peak at 50°C, thereby indicating a dynamic equilibrium of two components. This phenomenon was due to a dynamic *cis–trans* isomerization at the imide bond between cysteine and its C-terminally bound proline residue thus representing a mixture of two isomers with different chromatographic properties. Such equilibria were also observed for some other proline dipeptides (X-Pro) that could also be separated chromatographically under reduced temperature (21, 24, 25). Simple heating of column and solvent reservoir to 50°C increased the reaction velocity for the dynamic *cis–trans* conversion of HETE–CP and thus makes it much faster than the separation process. Accordingly, different chromatographic properties resulted in a single narrow peak anymore. As tandem-mass spectrometry did not allow differentiation of both isomers we could not identify which of the peaks was the *cis*- or *trans*-isomer. Nevertheless, this is the first report presenting elucidation of the reason for the broad peak profile of HETE–CP that presumably prevented its use for verification analysis so far. Therefore, we carried out chromatography at 50°C for further method development and present both a narrowbore and a microbore procedure suitable for verification analysis.

Characterization and comparison of narrowbore and microbore analysis

Even though the developed LC–ESI–MS-MS methods were not intended for quantitative analysis of HETE–CP, diverse assay characteristics essential for a reliable method were elaborated including linearity of peak area and injected amount, ion suppression and LOD. As no purified and quantified HETE–CP reference compound was commercially available any quantitative interpretation is related to the concentration of SM in plasma applied for

adduct formation. The same reference parameter was also used in earlier studies dealing with SM adduct detection as reported by, e.g., Noort *et al.* (9, 12) and Andacht *et al.* (13).

Linear range and technical LOD

According to the principle of quantitative interpretation mentioned above, a broad linear range for SM concentration-dependent HETE–CP peak areas was found (2 orders of magnitude for the narrowbore and 3 orders for the microbore method). Due to the fact that HETE–CP was not available as pure reference compound we had to use a prepared plasma sample (100 μM SM) containing the alkylated dipeptide analyte in the presence of a huge amount of additional albumin-derived proteolysis products. Diluting this sample to adjust smaller analyte concentration, matrix components were also diluted and could thus provoke a variable extent of ion suppression. Accordingly, the highest concentrated sample (resulting from 100 μM SM) did not fit the linear regression for the microbore method and documented 18% ion suppression (82% remaining intensity). Due to the highly limited chromatographic resolution of the step gradient, the same effect was much more prominent for the narrowbore method. Its linear range excluded the 50 and 100 μM samples due to ion suppression of 34% and 51%, respectively (data not shown).

Nevertheless, these findings underline a dynamic range for adduct detection in particular as quantification is not intended. Satisfying LODs of 50 and 100 nM were found for the microbore and the less sensitive narrowbore method.

Ion suppression and sensitivity

In principle, ion suppression was found in both systems but this effect was less dominant in microbore analysis (82% remaining intensity) than in narrowbore measurements (68% remaining intensity). This tendency was expected as the step gradient of the narrowbore method causes low chromatographic resolution and thus coelution of numerous potentially suppressing matrix compounds. Nevertheless, a distinct extent of suppression can also be expected in samples of the FA dilution series as HETE–CP was not present as pure reference compound but embedded in a complex mixture of albumin proteolysis products. These components were present in each sample but their concentrations decreased with higher dilution. Under these conditions, the microbore method was found to be six to seven times more sensitive than the narrowbore system. Peak area ratios of HETE–CP/d8-HETE–CP were constant in both methods with small RSD values (2.4 ± 4% in microbore and 2.2 ± 4% in narrowbore system) documenting stable ionization conditions and the absence of any interferences. As obvious from the constant peak area ratio of quantifying and qualifying ion of HETE–CP (1.85 ± 7% in both methods) fragmentation conditions were reproducible and robust. Therefore, these methods appear highly appropriate for plasma analysis.

With a cycle time of 16 min the narrowbore method is four times faster than the microbore procedure. This will be of importance when numerous samples that might arise from a huge number of poison victims have to be analyzed. Nevertheless, the narrowbore method is less sensitive what might be of relevance when smallest amounts of poison were incorporated.

Stability of HETE–CP in autosampler

HETE–CP appeared to be stable for at least 48 h at 15°C as no time-dependent trend of its peak area was observed thus supporting its use for LC–ESI–MS-MS analysis (data not shown).

Proteolysis kinetics of pronase

As illustrated in Figure 4, the concentration–time profile of HETE–CP formation has a clearly sigmoidal progression, which was fitted with the Gompertz function ($r^2 = 0.99$). The plateau reached after ~60 min indicates that HETE–CP is stable and not further degraded to its single amino acids. Accordingly, time for proteolysis might be reduced to ~60 min still achieving a 95% yield of the analyte. The corresponding period of half-change was found to be 25 min for the pronase lots from both suppliers (dashed line, Figure 4) possibly indicating the same source for both lots. In addition to the major dipeptide product, traces of the alkylated tri- and tetrapeptide were also detected (Figure 4, insert). Both compounds followed a typical curvature of consecutive reactions indicating their progressive degradation possibly to the dipeptide. However, production of the dipeptide directly from albumin might also happen. Furthermore, the concentration–time profiles for the deuterated analogs were identical to the non-deuterated variants thus indicating no isotope-specific discrimination of pronase. The constant peak area ratio of quantifying ions of HETE–CP/d8-HETE–CP (1.8 ± 3%) indicated the absence of any isobaric interference for both compounds.

Dependence of SM concentrations added to plasma on HETE–CP formation

We found that generation of the HETE–albumin adduct and production of its HETE–CP cleavage product were directly proportional to the concentration of SM added to plasma (Figure 5). These results are in accordance to Noort *et al.* (12) and Andacht *et al.* (13) who analyzed the alkylated tripeptide and thus support the use of the HETE–dipeptide as a reliable marker. As obvious from the different slopes obtained when plotting the SM concentration against the absolute HETE–CP peak area the microbore method (Figure 5, curve 1) was about 10 times more sensitive than the narrowbore method (Figure 5, curve 4) indicating a much better chromatographic separation of analyte and ion suppressors and enhanced signal intensity. As expected, sensitivity was further enhanced 4-fold when analyzing more concentrated samples after evaporation and redissolvation (data not shown). Nevertheless, these differences were less obvious when plotting the SM concentrations against the area ratio of HETE–CP/d8-HETE–CP (Figure 5, curves 2 and 3).

Based on SM concentrations added to plasma for incubation a LOD of 50 nM for the microbore and 100 nM for narrowbore method was found. This LOD is higher than those reported by Noort *et al.* (1.5 nM) (12) and Andacht *et al.* (5 nM) (13) based on tripeptide detection. Nevertheless, when considering the different sample preparation procedures the technical sensitivity of our method is as good as those of the tripeptide methods. Noort *et al.* (12) and Andacht *et al.* (13) injected plasma equivalents of ~15 and 13 μL, respectively, for LC–ESI–MS-MS analysis. In contrast, we use an equivalent being about 10 times smaller (1.7 μL plasma). Modifying sample preparation by any concentration step

or injection of larger volumes than 20 μL could thus improve the LOD as documented above, if necessary. However, taking into account the presently available lots of pronase our methods are the only ones allowing adapted and sensitive analysis of SM–albumin adducts.

A dose of 200 μM SM exposed to skin is expected to cause harmful effects of blistering (26). Referring to Noort *et al.* (6) albumin adduct concentrations (HETE–CPF) found in the blood of Iranian victims (blood drawing 8–9 days post-exposure) were equal to *in vitro* plasma incubations applying 0.4–1.8 μM SM. Furthermore, Smith *et al.* reported on HETE–CPF concentrations corresponding to *in vitro* concentrations of 350 nM SM in whole blood after accidental human exposure (10). However, dose of incorporated SM was almost unknown in all cases. Therefore, the presented methods appear appropriate for post-exposure analysis and HETE–CP is a suitable marker as good as the HETE–tripeptide thus underlining the importance of our novel procedures.

Conclusion

We have developed two novel LC–ESI–MS-MS methods reliable for detection of plasma albumin-derived adducts of SM to prove exposure of SM concentrations as low as 50 nM. Our methods make use of current commercially available lots of pronase that in contrast to older lots predominantly or exclusively produce the HETE–CP dipeptide. Even though the methods are optimized for analysis of this dipeptide adduct, simultaneous detection of the formerly established markers HETE–CPF and Q(HETE)–CPF is also possible at least for the microbore method. Therefore, our methods pay attention to the actual situation all laboratories operating verification analysis for SM exposure are faced with. Accordingly, these procedures will also be useful for the methods repertoire the OPCW refers to.

Nevertheless, for significant improvement of sample throughput optimized shortened gradients especially for the microbore method might be elaborated. Furthermore, use of at least two HPLC columns working in parallel will also enable enhanced throughput as described by Noort *et al.* (12). If necessary, the LOD might be further optimized by implementation of simple sample concentration steps or larger injection volumes for LC–ESI–MS-MS. Using more specific enzymes such as trypsin or chymotrypsin that generate less and larger peptide cleavage products might presumably lead to less ion suppression and thus further improve sensitivity and LOD especially for concentrated samples. In addition, application of selective extraction procedures discriminating alkylated and non-alkylated cysteine residues in albumin might be valuable for reduced background signals, less matrix effects and improved LOD as introduced by Funk *et al.* (27).

Acknowledgments

We gratefully thank Dirk Steinritz, Georg Menacher and Mina Zarabi for discussion and technical support.

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