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Quantification of *N*-(3-chloro-2-hydroxypropyl)valine in human haemoglobin as a biomarker of epichlorohydrin exposure by gas chromatography–tandem mass spectrometry with stable-isotope dilution☆

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ABSTRACT

Epichlorohydrin (ECH) is an important industrial intermediate for the production of polymers and surface coatings. Animal experiments support the classification of ECH as a carcinogen, and a significant contribution to the cancer risk of ECH exposed humans has to be considered. Upon uptake, epichlorohydrin reacts with nucleophilic moieties of *N*- and *S*-containing macromolecules to form stable adducts, e.g. with haemoglobin. In this article, we describe a GC–tandem MS method for the quantitative analysis of the primary ECH adduct to the *N*-terminal amino acid of human haemoglobin, i.e. of *N*-(3-chloro-2-hydroxypropyl)valine (CHPV), using a globin labelled with d_5-ECH as the internal standard. Incubation of erythrocyte lysate from human blood with ECH or d_5-ECH yielded two reaction products, with CHPV being the major component. The GC–tandem MS method is based on a modified Edman degradation procedure with subsequent *O*-acetylation. The limits of detection and quantification of this method are 10 and 25 pmol/g globin, respectively. Intra- and inter-assay imprecision of the method was about 12 and 15%, respectively, and the mean recovery was 105 and 96% at the levels of 25 and 100 pmol of CHPV per g globin, respectively. The present study reports for the first time on the analysis of CHPV as a haemoglobin adduct of ECH using GC–tandem MS and a stable-isotope labelled internal standard. By this method we quantified haemoglobin adducts of ECH in the blood of subjects potentially exposed to ECH after a freight train accident. Our study points to CHPV in human haemoglobin as a possible biomarker for epichlorohydrin exposure.

1. Introduction

Epichlorohydrin (ECH, 1-chloro-2,3-epoxypropane, CAS-No. 106-89-8) is a chlorinated three-carbon epoxide of particular industrial importance as a basic compound with an annual production volume of 300,000–400,000 tons in Europe as well as in the United States [1,2]. About 50–70% of the total amount of ECH is synthesised for the production of epoxy resins, while another 15–30% serve for the production of glycerol. Another 15–20% is used for additives in the paper industry. A minor fraction is employed in pharmaceutical syntheses, anionic and non-ionic emulgators and brominated flame retardants.

At room temperature, ECH is a colourless liquid with a pungent odour and a vapour pressure of 17 hPa (water: 23.4 hPa). The miscibility of ECH with water is low (65 g/l) [3]. In aqueous solution, epichlorohydrin spontaneously hydrolyses to 3-chloro-1,2-propanediol in a manner depending on the pH value: its half-life ranges from 148 h under neutral conditions to 62–79 h in acidic and alkaline solutions [4–6]. ECH is an irritant to the skin, the eyes and the mucous membranes and sensitising to the skin [7]. Epichlorohydrin has been classified as a possible carcinogen for humans by several national and international scientific committees and agencies such as the Deutsche Forschungsgemeinschaft (DFG) (category 2, "considered carcinogenic for man because sufficient data from long-term animal studies or limited evidence from animal studies substantiated by evidence from epidemiological studies indicate

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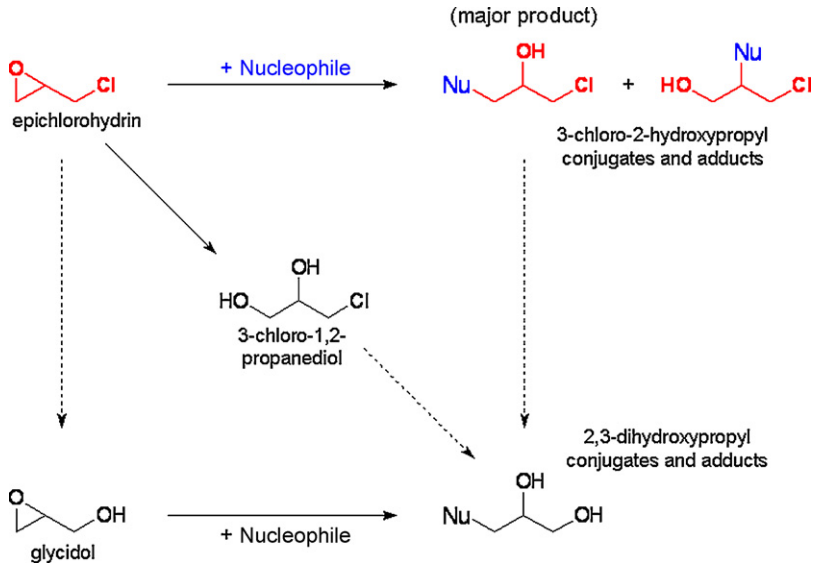

Fig. 1. Reaction pathways of epichlorohydrin to 3-chloro-2-hydroxypropyl and 2,3-dihydroxypropyl adducts and conjugates (solid arrows: known pathways; dashed arrows: putative pathways, Nu: nucleophilic sites in proteins, DNA or reduced glutathione).

that they can make a significant contribution to cancer risk" [8]), the European Union ("substances which should be regarded as if they are carcinogenic", Directive 67/548/EEC), the United States Environmental Protection Agency (category B2, "probable human carcinogen") [9] and by the International Agency for Research on Cancer (group 2A, "probably carcinogenic to humans") [10].

The metabolism of ECH has been intensively investigated in rodents, namely by Weigel et al. [11] and by Gingell et al. [12]. According to these studies, approximately 90% of an inhaled or orally administered dose is retained in the exposed animal and distributed into the body compartments within 2–4 h. The main metabolic pathway of ECH in the body is the epoxide ring

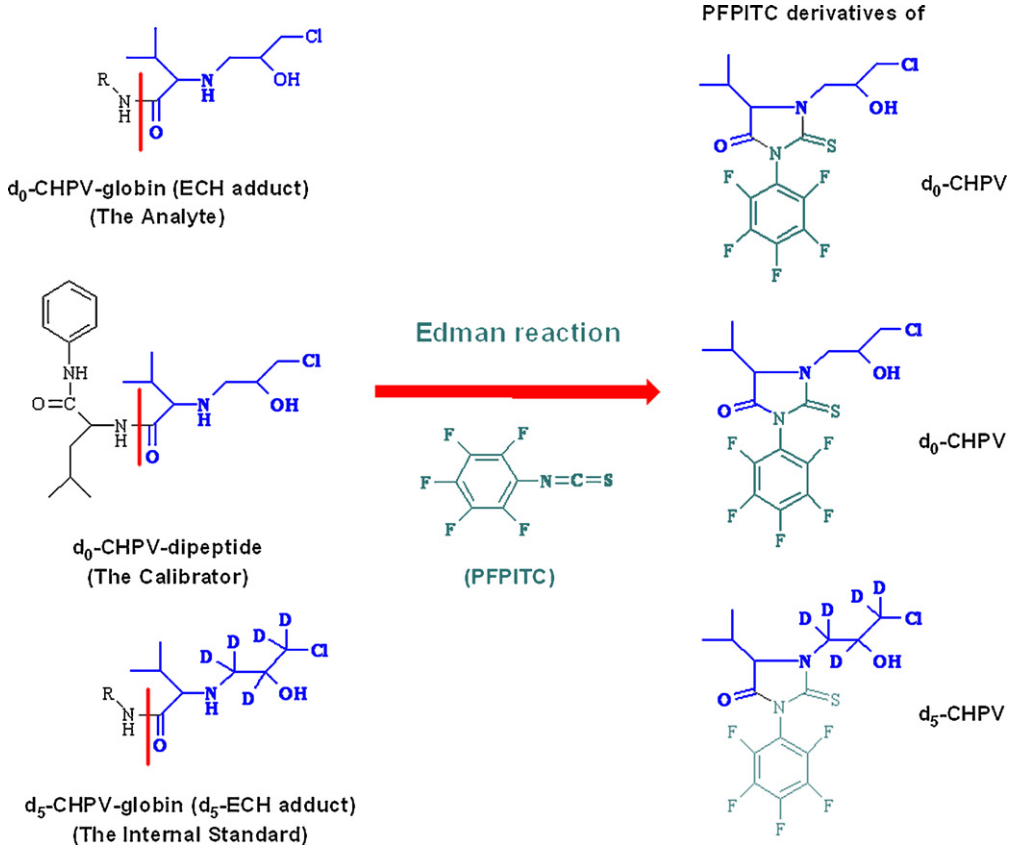

Fig. 2. Reaction products of the *N*-alkyl Edman reaction with pentafluorophenyl isothiocyanate (PFPITC) as the derivatisation reagent for the analytes *N*-(3-chloro-2-hydroxypropyl)valine (d_0-CHPV) in globin, d_0-CHPV in the dipeptide used for calibration and for d_5-CHPV in globin. The *N*-alkyl Edman reaction of CHPV in globin and in the calibrator dipeptide with PFPITC yields the same derivative, i.e. d_0-CHPV–PFPITC.

opening via an S_N2 mechanism, preferably at the more electrophilic C-3 in the epoxide ring (Fig. 1). Reaction of ECH with nucleophiles such as water or reduced glutathione (GSH) yields 3-chloro-1,2-propanediol and the 3-chloro-2-hydroxypropyl GSH conjugate, respectively, while the reaction of ECH with macromolecules, e.g. proteins and DNA, produces ECH adducts. A second metabolic pathway involves the nucleophilic substitution (S_N1) of the chlorine atom at C-1, either before or after the epoxide ring is opened. In this case, conjugates and adducts of the 2,3-dihydroxypropyl type would emerge. The terminal metabolites of ECH in rat urine are mercapturic acids such as *N*-acetyl-*S*-(3-chloro-2-hydroxypropyl)cysteine, *S*-(2,3-dihydroxypropyl)cysteine and *N*-acetyl-*S*-(2,3-dihydroxypropyl)cysteine [7], and the 3-chloro-1,2-propanediol oxidation products 3-chlorolactaldehyde, β-chlorolactic acid, oxalic acid and carbon dioxide [13].

In recent years, the search for appropriate biomarkers of ECH exposure has focused on adducts to macromolecules in blood such as haemoglobin [14,15] and lymphocyte or calf thymus DNA [16–20], or on conjugates in urine such as mercapturic acids [21,22]. In most studies, with the exception of Hindsø Landin et al. [14], the 3-chloro-2-hydroxypropyl type adducts or GSH conjugates have been preferred over the analysis of 2,3-dihydroxypropyl adducts due to the higher specificity of these adducts with regard to ECH, because the characteristic chlorine atom of ECH is still present in the adduct. Importantly, 2,3-dihydroxypropyl-containing adducts are not specific for ECH, but may also originate from glycidol, a compound found in tobacco smoke and heated food [14,23].

Nevertheless, Hindsø Landin et al. [14] presented the first successful application of a biomonitoring method to the determination of ECH adducts in humans based on the determination of *N*-(2,3-dihydroxypropyl)valine (DHPV). With the so-called *N*-Alkyl Edman method [24,25], the alkylated *N*-terminal amino acid valine in globin is simultaneously cleaved off of the protein and derivatised for subsequent GC–MS analysis (Fig. 2, Table 1). This approach has been adapted for the analysis of a number of alkylating compounds such as ethylene oxide, acrylonitrile or acrylamide [25–32], and it has become a standard approach to valine adduct monitoring recommended by the DFG [33].

Hindsø Landin et al. [14] have modified this method, in particular by silanisation of all glassware, an additional acetylation to protect the hydroxyl moieties, and the use of a GC–tandem MS to enhance the sensitivity. With a limit of detection of 2 pmol adduct per gram globin (on the basis of a threefold signal-to-noise ratio), they could differentiate between smokers and non-smokers, as well as between ECH exposed workers and non-exposed controls. However, Hindsø Landin et al. [14] did not succeed in the analysis of the primary chlorine-containing adduct of ECH to valine, i.e. *N*-(3-chloro-2-hydroxypropyl)valine (CHPV). They assumed that the chlorine atom of CHPV is readily cleaved off at higher pH values as applied in some extraction steps, and is replaced by a hydroxyl group. Also, the stability of the adduct *in vivo* might be low: in rats treated with ECH, Hindsø Landin et al. [15] observed that the concentration of the globin adduct *S*-(3-chloro-2-hydroxypropyl)cysteine decreased faster in blood than would have been expected from the half-life of rat erythrocytes, while the potential secondary adduct *S*-(2,3-dihydroxypropyl)cysteine slightly increased for some time before dropping due to the erythrocyte turnover. While a straightforward conversion of the primary into the secondary cysteine adduct could not be shown in these experiments, Hindsø Landin et al. [34] supposed that the corresponding *N*-(3-chloro-2-hydroxypropyl)valine adduct in human haemoglobin might be rapidly hydrolysed to *N*-(2,3-dihydroxypropyl)valine under physiological conditions.

Despite the unfavourable perspectives and analytical problems described by Hindsø Landin et al. [14] for the determination of *N*-(3-chloro-2-hydroxypropyl) adducts and in view of the contradictory encouraging results from recent *in vitro* and *in vivo* studies on these specific primary adducts of ECH, our work aimed on the development, validation and application of a GC–tandem MS method for the quantitative determination of CHPV, i.e. the chlorine-containing primary adduct of ECH, in human haemoglobin from red blood cells. For this purpose, we labelled human haemoglobin with d_5-ECH, and isolated and standardised it using the commercially available dipeptide *N*-(3-chloro-2-hydroxypropyl)valine-leucine anilide as the calibrator. The application of the *N*-alkyl Edman method to unlabelled and labelled CHPV in globin as well as to the dipeptide *N*-(3-chloro-2-hydroxypropyl)valine-leucine anilide using pentafluorophenyl isothiocyanate (PFPITC) as the derivatisation reagent yields the same PFPITC derivatives of labelled and unlabelled CHPV species (Fig. 2) and should therefore allow accurate quantitative analysis of ECH adducts to haemoglobin in blood. Our study suggests that quantification of such primary still chlorine-containing adducts of ECH to haemoglobin may provide a toxicant-specific parameter for the biomonitoring and risk assessment of individuals potentially exposed to ECH.

2. Experimental

2.1. Chemicals

Formamide ultrapure was from Amersham Biosciences (Freiburg, Germany) and pentafluorophenyl isothiocyanate from Fluka (Buchs, Switzerland). The dipeptide *N*-(3-chloro-2-hydroxypropyl)valine-leucine anilide was obtained from Bachem (Buchs, Switzerland). Epichlorohydrin (99%) was from Sigma–Aldrich (Deisenhofen, Germany) and d_5-epichlorohydrin (d_5-ECH; declared isotopic purity of 98% at ^{2}H) was from Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals were of highest available analytical grade and purchased either from Sigma–Aldrich (Deisenhofen, Germany) or from Merck (Darmstadt, Germany).

2.2. Blood sampling and analytical procedures

The whole analytical method including blood sampling, derivatisation and extraction procedures are summarised in Table 1 and reported below in detail.

2.2.1. Blood sampling and haemolysis—procedure A

Blood samples for the preparation of a pooled erythrocyte lysate and pooled globin were collected from non-smoking voluntary participants of an earlier study on environmental acrylamide exposure [35]. None of the acrylamide study participants had a known or suspected exposure to ECH. According to the standard operating procedure for *N*-alkyl valine adducts of the Deutsche Forschungsgemeinschaft [33], smoking is generally considered as an exclusion criterion for the preparation of a pooled globin sample as a number of tobacco smoke components form adducts with haemoglobin, thus yielding a background alkylation and a lower diagnostic sensitivity. In the acrylamide study, the smoking status of the participants had been tested by the analysis of urinary cotinine, a metabolite of nicotine, and by the analysis of *N*-2-cyanoethylvaline (CEV), a protein adduct of acrylonitrile in tobacco smoke. Although no CHPV was to be expected in these samples, we accepted only blood samples from study participants with typical non-smoker cotinine (<10 μg/l) and CEV (<10 pmol/g globin) for the preparation of a pooled globin sample for the epichlorohydrin adduct analysis. Briefly, 7.5 ml aliquots of whole blood from each volunteer were collected in EDTA-containing disposable monovettes (Sarstedt, Nümbrecht, Germany). Samples were centrifuged and the plasma fractions discarded. Erythrocytes were washed three times

Table 1
Summary of the procedures involved in the quantification of epichlorhydrin/haemoglobin adducts in blood by GC–tandem MS using d_5-CHPV globin adduct as internal standard (IS).

Procedure/sample	Action
(A) Blood sampling and haemolysis	
Blood (EDTA)	Centrifugation (800 × *g*, 10 min, RT)
Erythrocytes	(1) Washing (0.9 g% NaCl, three times) (2) Centrifugation (800 × *g*, 10 min, RT) (3) Lysis (3–4 ml water) (4) Storage (overnight, −27 °C)
(B) Globin isolation	
Lysate (2 ml, defrosted)	(1) Addition to 12 ml of 50 mM HCl in 2-propanol. (2) Mixing for 15 min. (3) Centrifugation (2000 × *g*, 10 min, RT). (4) Addition of 8 ml ethyl acetate to the clear solution. (5) Storage of the suspension for 1 h at 4 °C. (6) Centrifugation (800 × *g*, 10 min, RT). (7) Washing of the protein with 5 ml ethyl acetate (2×) and *n*-heptane (1×). (8) Drying of the globin precipitate overnight in a vacuum desiccator.
(C) Modified N-alkyl Edman method—first derivatisation	
Globin solution (100 mg in formamide)	(1) Addition of 30 μl 1 M NaOH. (2) Addition of 4 pmol of d_5-CHPV globin (i.e. 100 μl of a 40–100 nM solution). (3) Addition of 20 μl PFPITC. (4) Ultrasonication (30 min, RT).
Clear formamide solution	(5) Derivatisation. (6) Gentle mixing overnight at RT. (7) Incubation in a water bath (2 h, 45 °C).
(D) Extraction and clean up	
Formamide solution of derivatives and reagents	(1) Extraction with 4 ml diethyl ether (2×) (2) Centrifugation (3500 × *g*, RT). (3) Combination of diethyl ether phases. (4) Evaporation to dryness under a stream of nitrogen.
Residue (PFPITC derivatives)	(5) Reconstitution in 1.5 ml toluene. (6) Washing with fresh solution of 0.1 M sodium carbonate (1×) and ultrapure water (1×). (7) Transfer of the toluene phase into 2 ml glass vials. (8) Evaporation to dryness under nitrogen at 35 °C.
(E) Acetylation—second derivatisation	
Residue (PFPITC derivatives)	(1) Addition of 250 μl of a mixture (1:1, v/v) of 12.5 vol.% acetic anhydride and 12.5 vol.% triethyl amine both in acetonitrile. (2) Short agitation and left open for 15 min at RT. (3) Evaporation to dryness under nitrogen at 30 °C.
Residue (acetylated PFPITC derivatives)	(1) Reconstitution in 500 μl *n*-hexane. (2) Extraction with 1 ml of a methanol–water (60:40, v/v) by a vortex-mixer for 1 min. (3) Centrifugation (3500 × *g*, 10 min, RT). (4) Evaporation to dryness under nitrogen at RT. (5) Reconstitution in 30 μl toluene.
(F) GC–tandem MS analysis	
Toluene extracts	(1) Splitless injection of 1 μl aliquots. (2) Selected reaction monitoring (SRM) of *m*/*z* 301 from *m*/*z* 422 for d_0-CHPV. *m*/*z* 301 from *m*/*z* 427 for d_5-CHPV (IS).
(G) Calculation of ECH-content in globin	
Preparation of calibration curves	(1) Complete analysis of globin samples spiked with the dipeptide at 0, 25, 100 and 250 pmol per g globin using d_5-CHPV globin as the IS (40 pmol/g globin). (2) Plotting of the measured peak area ratio of the PFPITC derivatives of CHPV from the dipeptide to d_5-CHPV from d_5-CHPV globin (*y*) versus the added dipeptide content (*x*). (3) Determination of the regression equation $y = a + bx$.
Calculation of the ECH-content of the globin in the blood sample	(4) Insert measured value for *y* into the equation. $x = (y - a)/b$ from the respective calibration curve, calculate *x* and express its value in pmol/g globin.

by resuspension in isotonic NaCl solution and subsequent centrifugation. Haemolysis was achieved by the addition of ultrapure water (ASTM type I, prepared in-house with a Millipore Quantum® system from Millipore (Schwalbach, Germany)) and subsequent overnight storage at −27 °C. The lysates were stored in a refrigerator at −27 °C until globin isolation or incubation experiments were commenced.

2.2.2. Isolation of globin—procedure B

For the preparation of globin samples, the frozen lysates were slowly defrosted in a 20 °C water bath and thoroughly mixed before further handling. To prepare about 150 mg of globin, 2 ml aliquots of every lysate were added to 12 ml aliquots of 50 mM HCl in 2-propanol. Samples were thoroughly mixed for 15 min and afterwards centrifuged to remove cell debris (2000 × *g*, 10 min at room temperature (RT)). Aliquots (8 ml) of ethyl acetate were added slowly to the supernatant and the precipitation of globin was completed by maintaining the solution for 1 h at 4 °C. The protein was collected after centrifugation (800 × *g*, 5 min at RT), washed with ethyl acetate and heptane, and then dried overnight in a vacuum desiccator. The globin-containing samples were stored

in a refrigerator at −27 °C until further analysis. For the preparation of a standard globin sample free of ECH adducts, the total protein gain of twenty 7.5 ml blood samples was pooled finally yielding about 20 g of globin and was used in validation experiments and as a biological matrix in quantitative analyses.

2.2.3. Preparation of d_5-ECH-labelled globin

An aliquot (200 μl) of d_5-ECH was added to a 20-ml aliquot of a pooled erythrocyte lysate (each 1 ml erythrocyte lysate from 20 individual samples) and the resulting mixtures were thoroughly mixed by vortexing. The lysate was modestly agitated (180 per min) and maintained for 4 h at RT. Subsequently, the globin was isolated according to the procedure (B) described before, typically yielding a total protein amount of about 3 g.

2.2.4. Preparation of dipeptide calibration standards

An accurately weighed amount of 39.8 mg (0.1 mmol) of the *N*-(3-chloro-2-hydroxypropyl)valine-leucine anilide (in the following referred to as *the dipeptide*) was placed in a 100-ml volumetric flask and dissolved in ethanol to reach a final concentration of 1 mM. Aliquots (100 μl) of this stock solution were transferred into a 100-ml volumetric flask and further diluted in ethanol to achieve a final concentration of 1 μM (the working solution). Dilutions of the calibrator working solution were prepared in ethanol to reach final concentrations of 0, 10, 25, 50, 75, 100, 175, 250 nM; these solutions were used for the calibration of d_0-CHPV in globin samples and for the standardisation of the newly synthesised d_5-ECH-labelled globin (in the following referred to as d_5-CHPV globin). A typical range for the labelled globin preparation was 10–25 μmol of d_5-CHPV per gram of globin (see Section 3). Stock solutions were prepared by adding an accurately weighed amount of d_5-CHPV globin (e.g. 10 mg) to pure formamide (e.g. 50 ml) and sonication for 30 min. Stock solutions (e.g. 5 μM) were further diluted in formamide to achieve a nominal final concentration in the range 40–100 pmol d_5-CHPV globin per g globin (see below).

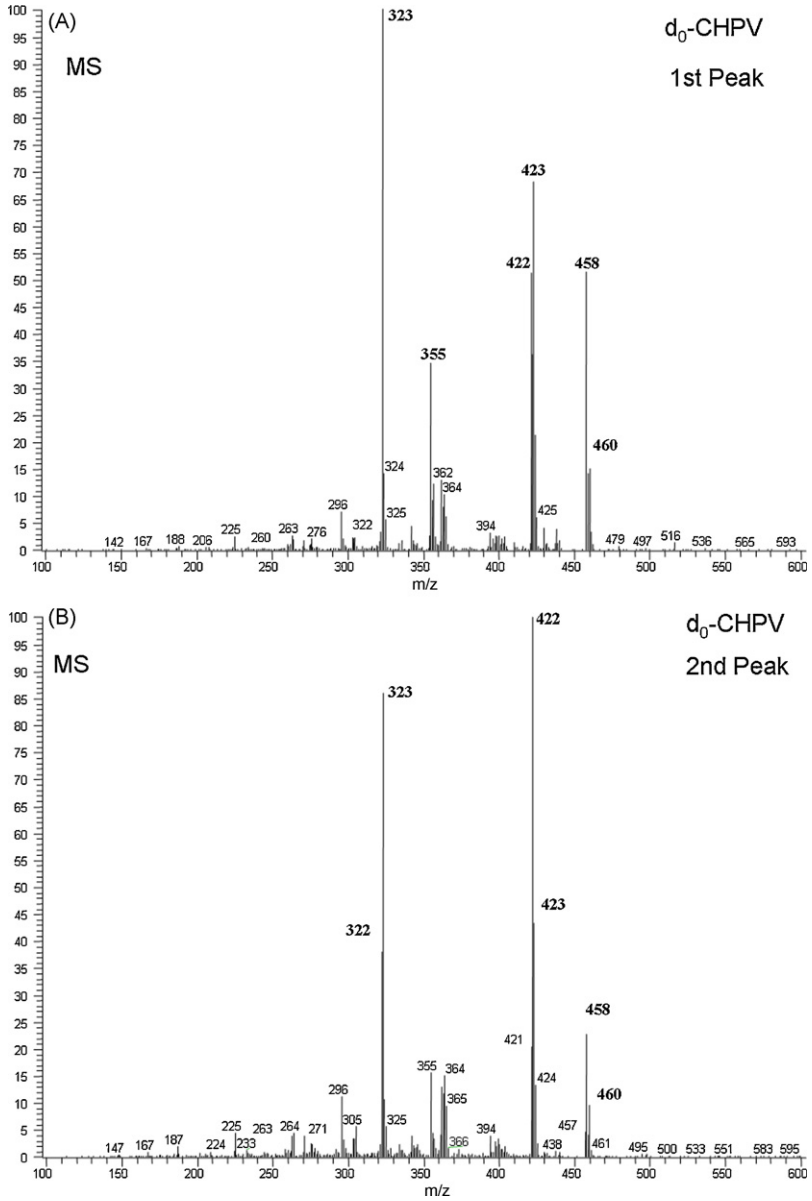

Fig. 3. Electron-capture negative-ion chemical ionisation mass spectra (MS) obtained from the GC–MS analysis of the PFPITC derivatives of d_0-CHPV (A, B) and d_5-CHPV (C, D). The mass spectra were generated from two GC peaks with a retention time of 21.5 min (1st peak) and 21.6 min (2nd peak).

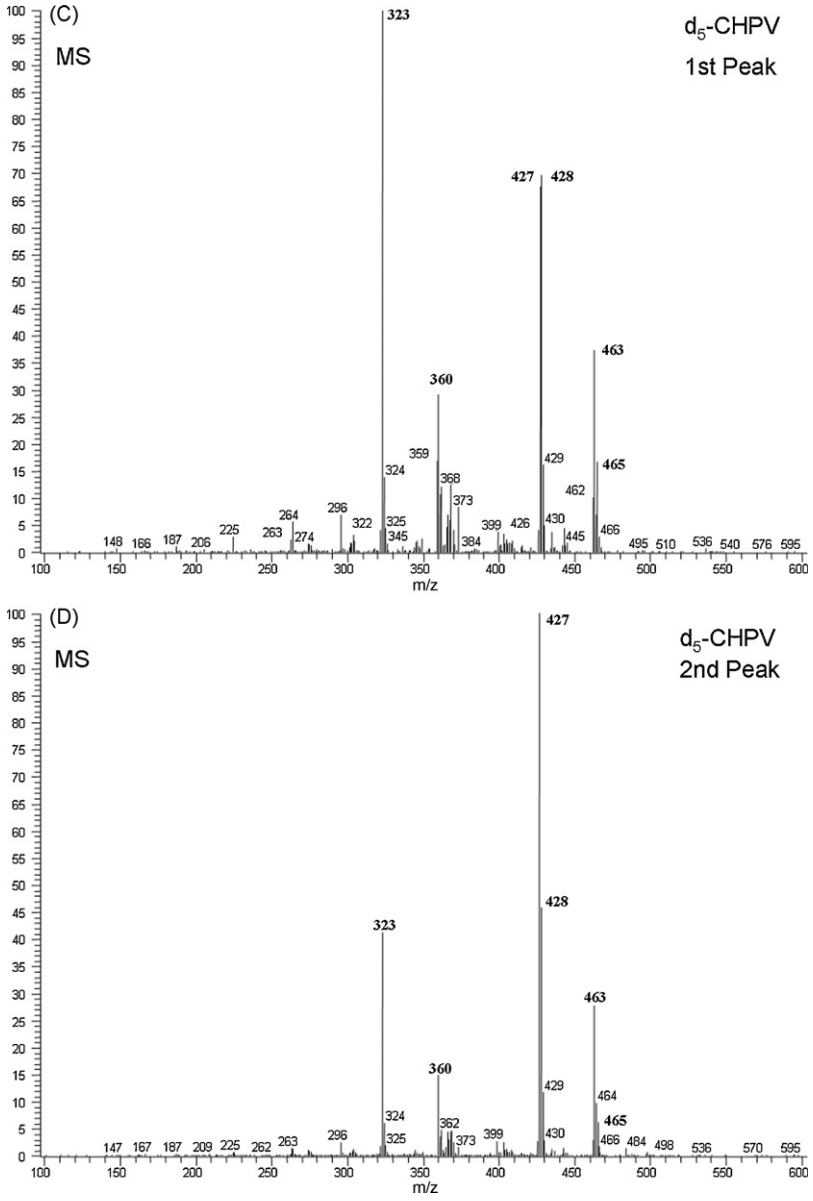

Fig.3. (*Continued*).

2.2.5. The N-alkyl Edman reaction with subsequent acetylation—procedures C–E

The haemoglobin adduct CHPV was analysed according to the *N*-alkyl Edman method [24] after adaptation by Bader et al. [28,32] and Angerer et al. [30] as described below (Fig. 2, Table 1). Additionally, a derivatisation step with acetic anhydride using triethyl amine as the catalyst was employed according to Hindsø Landin et al. [14] to protect the hydroxyl moiety of CHPV.

Globin (100 mg) was dissolved in 3 ml aliquots of formamide followed by addition of 30 μl aliquots of 1 M NaOH in 13 ml screw-capped vials. An aliquot (100 μl) of dilutions of the internal standard d_5-CHPV globin (typically 40–100 nM) was added to achieve typical final contents of 40–100 pmol of d_5-CHPV per gram of globin. The samples were sonified for 30 min in a water bath and 20 μl aliquots of PFPITC were added. The samples were thoroughly mixed and kept overnight at room temperature and under modest agitation. To complete the derivatisation, samples were incubated for another 2 h at 45 °C in a water bath. Calibration standards were prepared by adding 100 μl of the dipeptide standards (see Section 2.2.4) to 100 mg CHPV-free globin in 3 ml formamide, 30 μl 1 M NaOH and 100 μl of the d_5-CHPV globin solution. The preparation of a globin-containing matrix is essential to ensure identical derivatisation and extraction yield in the calibration standards and in samples of unknown globin content. The derivatives were extracted with diethyl ether for 1 min on a laboratory mixer, followed by a centrifugation. The combined ether extracts were evaporated to dryness under a gentle stream of nitrogen, and the residue was redissolved in a 1.5-ml aliquot of toluene. To remove side products and reagents excess, the samples were extracted with a freshly prepared sodium carbonate solution (0.1 M) and ultrapure water. Each extraction involved a 1-min vortex-mixing of the sample, a subsequent centrifugation and a transfer of the upper toluene layer into a 13-ml glass tube. In a final step the toluene samples were transferred into 2 ml glass vials and evaporated to dryness under a gentle stream of nitrogen (Table 1).

Acetylation of the hydroxyl group of the CHPV–PFPITC derivatives was carried out by adding a 250-μl aliquot of a freshly prepared mixture 1:1 (v/v) of acetic anhydride and triethylamine

in acetonitrile (12.5 vol% for each compound). The sample vials were not sealed but left open for 15 min at room temperature. Subsequently, the samples were evaporated to dryness under a gentle stream of nitrogen. The residue was redissolved in a 500-μl aliquot of hexane and extracted with a 1-ml aliquot of a mixture of methanol–water (60:40, v/v), involving a 1-min vortex-mixing with subsequent centrifugation. The hexane layer was then transferred into a 2-ml sample vial and evaporated therein to dryness under a gentle stream of nitrogen. The residue was reconstituted in a 30-μl aliquot of toluene and transferred into a 200-μl micro-insert for subsequent GC–tandem MS analysis.

2.2.6. GC–tandem MS conditions—procedure F

Aliquots (1 μl) of the toluene extracts were injected splitless into a GC–tandem MS instrument from ThermoElectron (Bremen, Germany) consisting of a Trace 2000 gas chromatograph and a TSQ 7000 triple stage quadrupole mass spectrometer equipped with an AS 2000 autosampler and a BEST PTV injector. The capillary column used was an Optima-1-MS (30 m × 0.25 mm, film thickness 0.25 μm) from Macherey-Nagel (Düren, Germany). The carrier gas was helium (5.0) and used under a constant flow rate of 1.0 ml/min. The injector was programmed from an initial temperature of 120 °C with a rate of 10 °C/min to a final temperature of 320 °C which was kept for 4 min. The oven temperature program was: 1 min at 80 °C, then increased with a rate of 8 °C/min to 340 °C which was maintained for 2 min. Transfer line and ion source temperature were kept constant at 300 °C and 180 °C, respectively. Electron-capture negative-ion chemical ionisation (ECNICI) was employed using methane (5.0) as the reagent gas at a pressure of 530 Pa. Electron energy and current emission were 100 eV and 300 μA, respectively. Argon (5.0) at a cell pressure of 0.27 Pa was used for collision-induced dissociation (CID). The collision energy was 15 eV. Quantification was performed by selected-reaction monitoring (SRM) of the common product ion at m/z 301 which was produced by CID of the precursor ions at m/z 422 for d_0-CHPV and m/z 427 for d_5-CHPV (see Section 3). The dwell time was 100 ms for each transition. The electron multiplier was operated with a voltage of 2800 V in quantitative analyses.

2.2.7. Calculation of CHPV in globin samples—procedure G

Calculation of the CHPV content of globin in processed blood samples was carried out by using a daily prepared calibration curve. This approach was necessary because of problems associated with the instability of the internal standard d_5-CHPV globin (see Section 3). To accurately weighed globin samples, which were free of ECH adducts, the dipeptide calibrators were added to achieve final contents of 10, 25, 50, 75, 100, 175 and 250 pmol of dipeptide per gram of globin. Additionally, a pool globin free of ECH adducts was analysed as a blank. Calibration and study samples were spiked with d_5-CHPV-containing globin in a final concentration corresponding to 40 pmol d_5-CHPV per gram globin. It should be noted, however, that the adduct yield from the incubation of d_5-ECH and hemolysate (see Section 2.2.3) may vary and the concentration of the internal standard d_5-CHPV in the globin has to be specified after every synthesis. After complete work-up and GC–tandem MS analysis, the peak area ratio (y) of the PFPITC derivatives of d_0-CHPV to d_5-CHPV was determined and plotted versus the added molar amount of the dipeptide per gram of globin (x). The regression equation

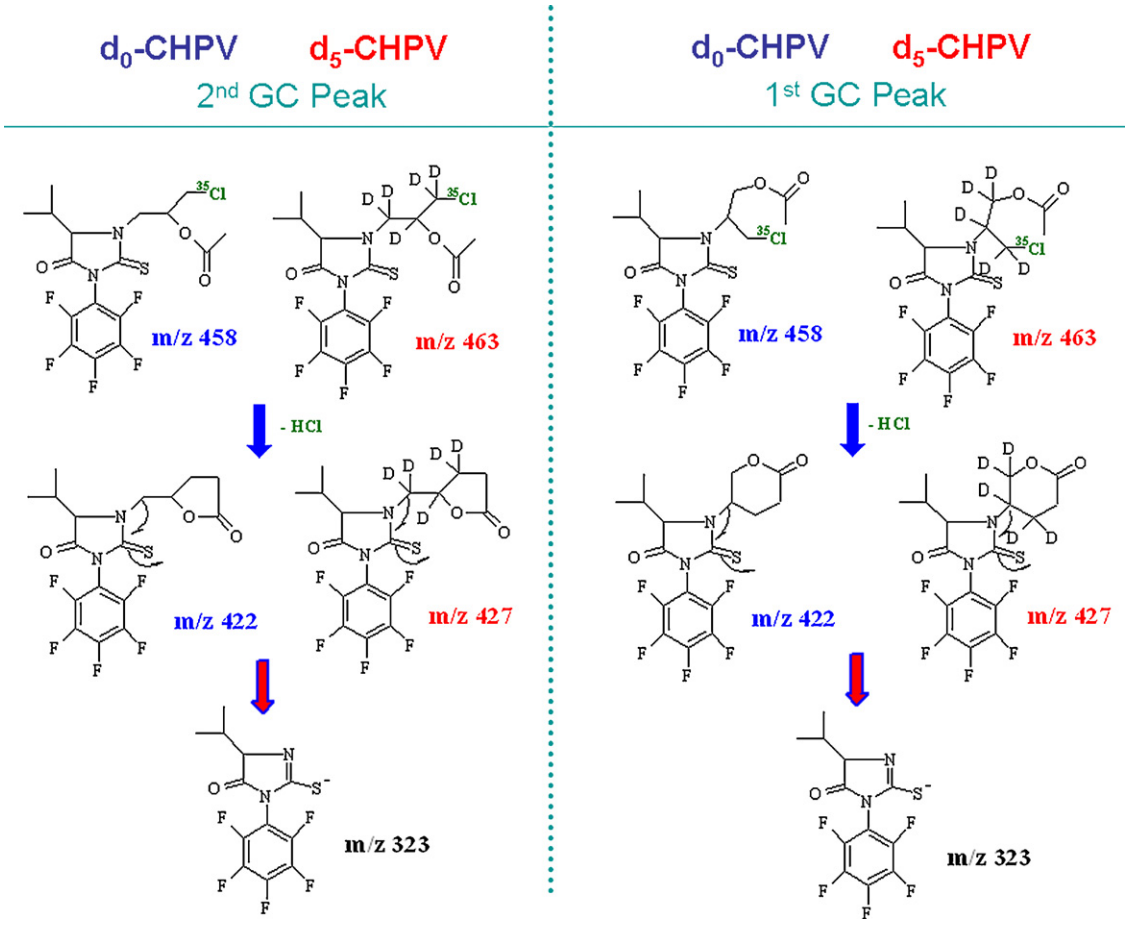

Fig. 4. Proposed structures for the PFPITC derivatives of d_0-CHPV and d_5-CHPV and proposed mechanisms for the formation of the ions m/z 458, 463, 422, 427 and 323.

($y = a + bx$) obtained and the peak area ratio measured (y) were used to calculate the CHPV-content of the globin (x) in the respective co-processed study samples by the formula: $x = (y - a)/b$ (see Table 1).

2.2.8. Validation experiments

The method performance was tested with respect to the limit of detection (LOD) and the limit of quantification (LOQ) following the German guideline DIN 32 645 [36]. In this case, the LOD and LOQ values are calculated from the confidence interval at the blank in the calibration curve. The LOD is equivalent to a concentration that gives a signal distinct from the background noise with a probability for an α-error (wrong positive) of 1%, and of 50% for the β-error (wrong negative). Further, the LOQ is defined as the lowest concentration that can be analysed with a maximum error of 33%. Further performance parameters tested were the intra-assay ($n = 10$) and inter-assay ($n = 10$ different days) imprecision (R.S.D., %) and accuracy (recovery, %). For more details see Section 3.

3. Results

3.1. GC–MS and GC–tandem MS characterisation of CHPV–PFPITC derivatives

GC–MS analysis of the PFPITC derivatives of synthetic d_0-CHPV and d_5-CHPV in the ECNICI mode revealed each two baseline-separated peaks with retention times of 21.5 min (i.e. the smaller peak, here referred to as the *first GC peak*) and 21.6 min (i.e. the larger peak, here referred to as the *second GC peak*), with the d_5-CHPV–PFPITC derivatives eluting a few seconds in front of the d_0-CHPV–PFPITC derivative. The mass spectra of the first (Fig. 3A and C) and the second (Fig. 3B and D) GC peak of the d_0-CHPV and d_5-CHPV derivatives were almost identical (Fig. 3), respectively.

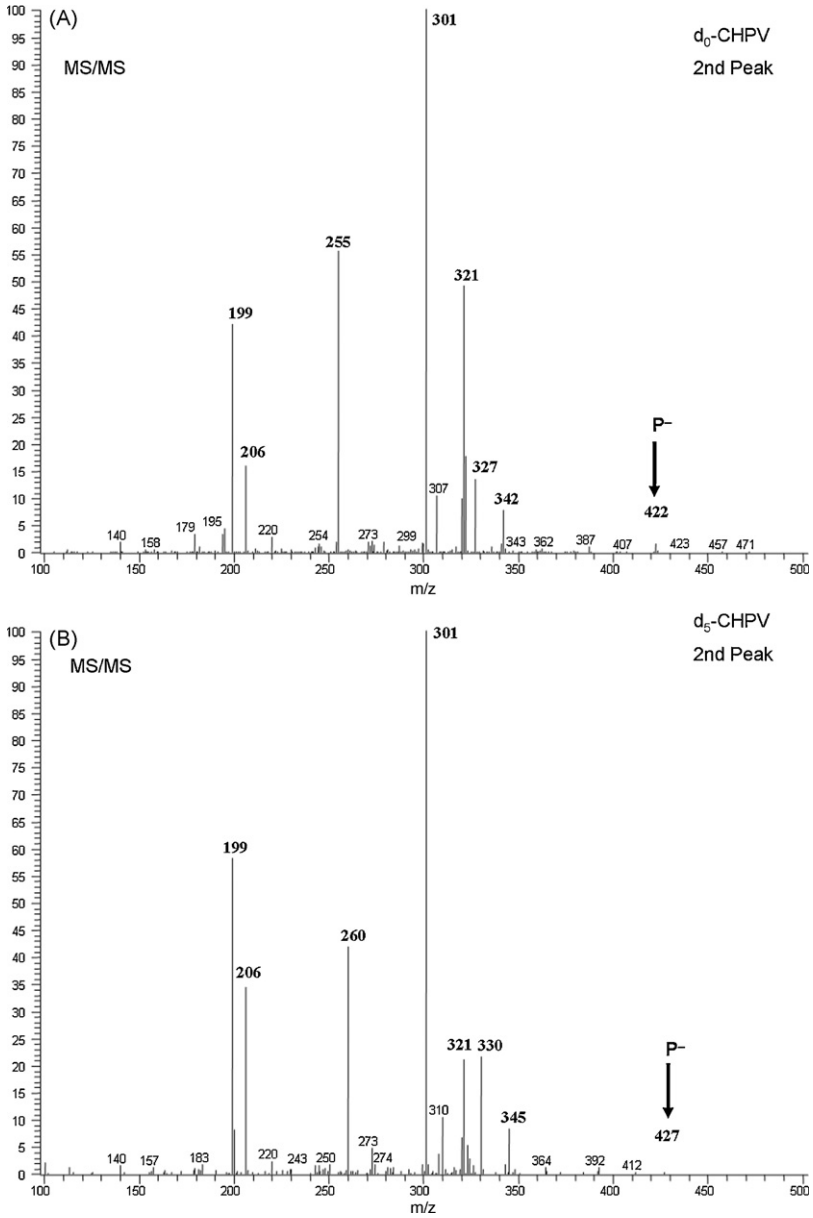

Fig. 5. Product ion mass spectra (MS/MS) from the GC–tandem MS analysis of the PFPITC derivatives of d_0-CHPV (A) and d_5-CHPV (B). The precursor ions at m/z 422 and m/z 427 of the second GC peak (21.6 min) were subjected to CID at a collision energy of 15 eV.

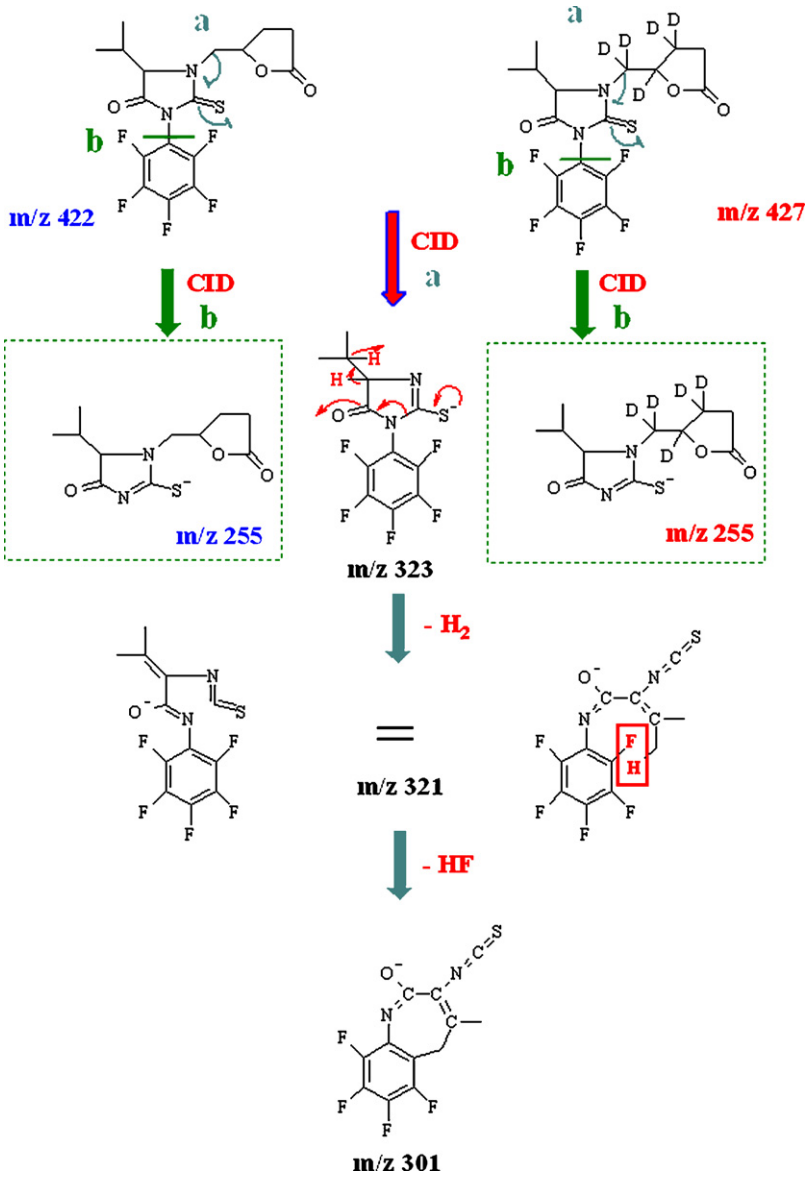

Fig. 6. Proposed mechanisms for the formation of the product ions *m*/*z* 323, 321, 301 (via mechanism a) and *m*/*z* 255 (via mechanism b) from the CID of the precursor ions *m*/*z* 422 for d_0-CHPV and for *m*/*z* 427 for d_5-CHPV.

The largest ions in the mass spectra were *m*/*z* 458 and *m*/*z* 460 for d_0-CHPV and *m*/*z* 463 and *m*/*z* 465 for d_5-CHPV, most likely representing the molecular anions (M^-) containing the ^{35}Cl and ^{37}Cl isotopes, respectively. As proposed in Fig. 4, it is likely that the mass spectra shown in Fig. 3 result from neutral loss of HCl, which is associated with lactone formation (five-membered of the 2nd GC peak; 6-membered of the 1st GC peak), and further loss of the lactone moieties presumably via the same mechanism to form the common thiolate anion with *m*/*z* 323 (Fig. 4). The loss of $H^{35}Cl/H^{37}Cl$, but not of $D^{35}Cl$ and $D^{37}Cl$ from d_5-CHPV points to a participation of one H atom from the acetyl group in the elimination of HCl which is accompanied by the formation of the lactones.

These findings suggest formation of two isomeric derivatives with an approximate molar ratio of 1:7 (see also below) as the result of the nucleophilic attack of the terminal α-amino group of valine in globin on C-2 and C-3 of ECH (1-chloro-2,3-epoxypropane) to produce the *N*-(3-chloro-2-hydroxypropyl)valine globin adduct, i.e. the larger second GC peak, and *N*-(1-chloro-3-hydroxyisopropyl)valine globin adduct, i.e. the smaller first GC peak. In quantitative analyses we considered only the *N*-(3-chloro-2-hydroxypropyl)valine globin adduct, whereas the first peak served as an additional criterion to identify ECH–globin adducts in human blood.

Subjection of the precursor ions (P^-) at *m*/*z* 422 (for d_0-CHPV) and *m*/*z* 427 (for d_5-CHPV, i.e. for the later eluting GC peak) to CID with argon as the collision gas resulted in the product ions mass spectra shown in Fig. 5. Even the relatively low collision energy of 15 eV resulted in complete fragmentation of the precursor ions. The most intense product ion was found at *m*/*z* 301 both for d_0-CHPV and d_5-CHPV, suggesting loss of the lactone moieties in both PFPITC derivatives. This also applies to the product ions *m*/*z* 206 and *m*/*z* 199. Product ions still carrying the lactone moieties but not the pentafluorophenyl (PFP) residues were found at *m*/*z* 255 for d_0-CHPV and *m*/*z* 260 for d_5-CHPV. In Fig. 6, possible mechanisms are proposed for the formation of the product ions *m*/*z* 255 and *m*/*z* 260, as well as for *m*/*z* 323, 321 and 301. For maximum sensitivity in quantification we chose the common product ion at *m*/*z* 301. We used the transitions *m*/*z* 422 to *m*/*z* 301 for d_0-CHPV and *m*/*z* 427 to

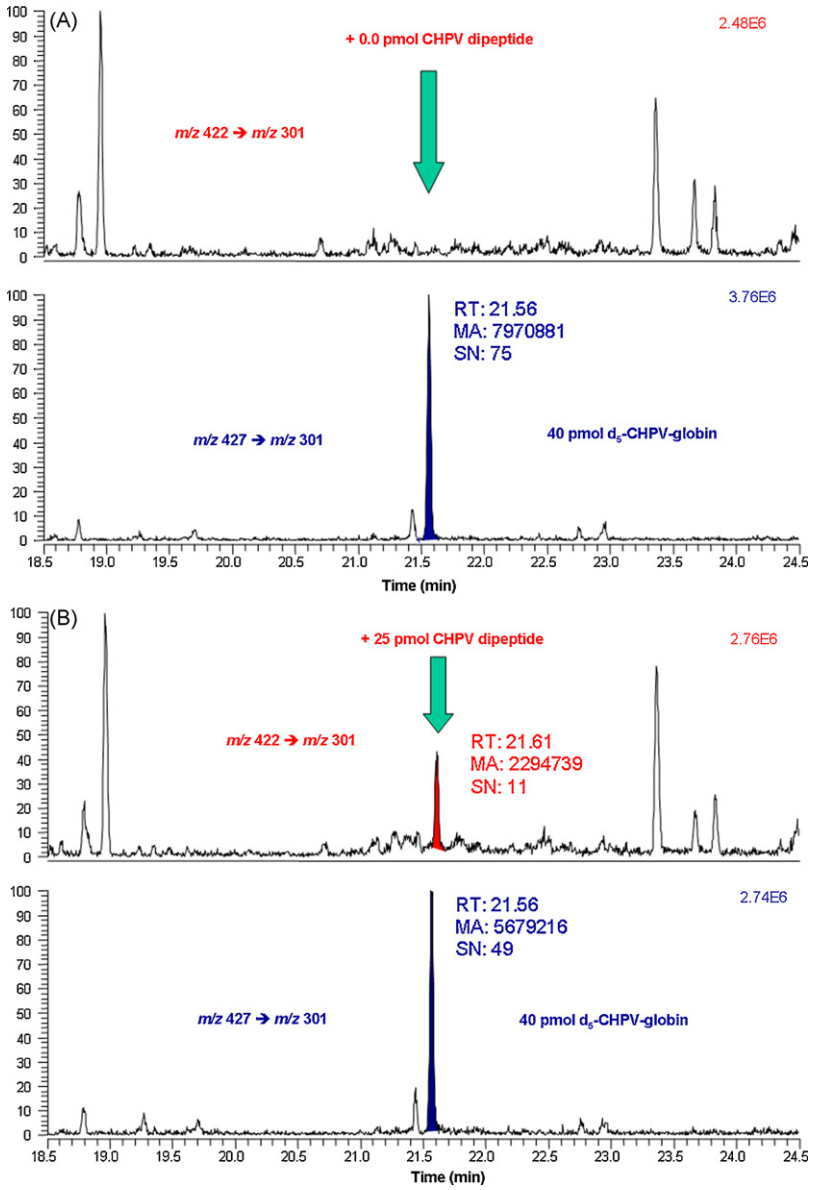

Fig. 7. Partial GC–tandem MS chromatograms from the analysis of a sample from a healthy subject not exposed to ECH before (A) and after (B) addition of d_0-CHPV. In both samples the concentration of the internal standard d_5-CHPV was 40 pmol/g globin.

m/z 301 for the internal standard d_5-CHPV in the SRM mode using a dwell time of 100 ms for each transition. Fig. 7 and Fig. 8 show partial GC–tandem MS chromatograms from quantitative analyses of ECH–globin adducts in blood from two persons.

3.2. Analytical performance of the GC–tandem MS method

3.2.1. Standardisation of d_5-CHPV

Samples containing a fixed known amount of ECH-free globin, the calibrator dipeptide at final concentrations of 0, 10, 25, 50, 75, 100, 175 and 250 pmol/g globin (each $n = 1$), and a fixed unknown amount of a freshly prepared d_5-CHPV globin were analysed by GC–tandem MS following the procedures C to G. Plotting of the measured peak area ratio (y) of d_0-CHPV–PFPITC to d_5-CHPV–PFPITC versus the calibrator content (x) resulted in a straight line with the regression equation $y = -0.0132 + 0.0104\ x$ with $R^2 = 0.9990$. This finding suggests that the content of d_5-CHPV globin in this preparation was 96 pmol per gram of ECH-free globin. Back-calculation showed that the d_5-ECH-labelled globin contained 25 μmol of d_5-CHPV per gram of globin. The linear relationship found between the peak area ratio of d_0-CHPV–PFPITC to d_5-CHPV–PFPITC and the CHPV-content used in this experiment indicates the applicability and sensitivity of the GC–tandem MS method.

The use of freshly prepared d_5-CHPV globin standards is recommended. However, it is worth mentioning that the d_5-CHPV label of globin may vary considerable from preparation to preparation. Thus, a standardisation of every d_5-CHPV globin standard would be necessary. In the freight train accident investigations [37,38] (see below) the freshly prepared internal standard was added to the samples at a final concentration of 40 pmol d_5-CHPV per g of globin.

3.2.2. Stability of the dipeptide calibrator and the internal standard

A general problem with the analysis of CHPV occurs with the instability of the analytes in aqueous media, because its chlorine-

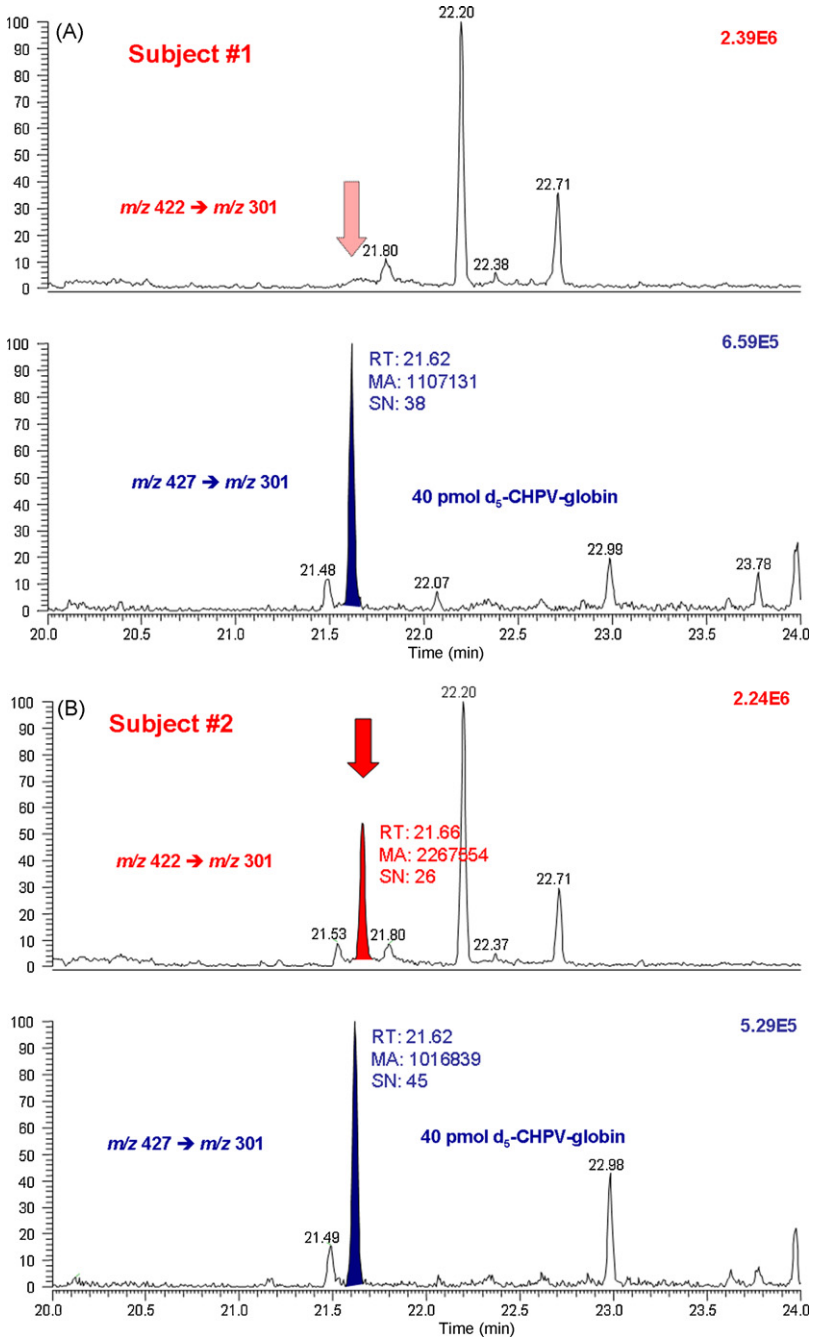

Fig. 8. Partial GC–tandem MS chromatograms from the analysis of blood samples of two subjects who were potentially exposed to ECH during a freight train accident [33,34]. The CHPV adduct was detected in the blood of subject #2 (80 pmol/g globin), but not in that of subject #1 (non-detectable). In both samples the concentration of the internal standard d_5-CHPV was 40 pmol/g globin.

carrying methylene group is susceptible to hydrolysis. We observed that the dipeptide both in water and in alcohol slowly degrades with time. The chlorine atom in CHPV is susceptible to hydrolysis [14] and the isotope-labelled internal standard may exchange deuterium with hydrogen. Table 2 shows a significant increase in the slopes of calibration curves (peak area ratio d_0-CHPV/d_5-CHPV versus d_0-CHPV dipeptide concentration) during a period of 4 weeks due to continuing losses in the concentration of the internal standard stored at 4 °C. Therefore, in the frame of the present study calibration standards were freshly prepared from the stock solution of d_5-CHPV globin in formamide for every single work-up. Obviously, under these conditions, no considerable degradation occurs (Table 3). The repeated preparation of stock solutions and calibration standards over a period of about 6 months did not indicate that CHPV is degraded when the calibrator dipeptide and the d_5-CHPV–globin used kept dry and frozen at −27 °C.

3.2.3. *Precision, accuracy and limits of quantification and detection*

The precision of the whole method was tested by analysing each 10 CHPV-free globin samples from healthy unexposed subjects, which had been spiked with 25 and 100 pmol of the dipeptide per gram of globin. In these samples the ECH adduct concentration was determined to 31.8 ± 3.9 pmol CHPV/g globin and 93.7 ± 9.2 pmol

Table 2
Summary of the GC–tandem MS results[a] from a study on the stability of the dipeptide calibrator[b] and the internal standard d_5-CHPV globin[c].

Day	Slope (g globin/pmol)	*y*-Axis intercept	R^2
Day 1	0.0082	+0.0883	0.9989
Day 9	0.0104	−0.0132	0.9990
Day 22	0.0146	+0.0411	0.9983
Day 24	0.0192	+0.0797	0.9996
Day 28	0.0219	+0.0220	0.9996

[a] Values for slope, *y*-axis intercept and correlation coefficient obtained from the linear regression analysis between *y*, the measured peak area ratio for the dipeptide content (pmol/g globin) and the internal standard content d_5-CHPV globin/g globin, and *x*, the dipeptide content (pmol/g globin).

[b] Each four calibration samples were used with a nominal dipeptide content (pmol/g globin) of 0, 25, 100 and 250 pmol/g globin; the nominal content of d_5-CHPV globin/g globin was 100 pmol/g globin.

[c] Their stock solutions of the dipeptide and the d_5-CHPV globin had been stored at 4 °C in this experiment.

CHPV/g globin using d_5-CHPV globin as the internal standard at 40 pmol/g globin. These data indicate an analytically acceptable intra-assay imprecision of 10–12%. In consideration of the use of a practically CHPV-free globin pool (Fig. 7A) in these first experiments, the measured mean values suggested that the accuracy of the method would be acceptable for lower contents of the order of 25 pmol CHPV/g globin (recovery of 121%) and excellent for higher contents such as 100 pmol CHPV/g globin (recovery of 94%). For these reasons and because of the considerable labour and time associated with this method, we decided to use four-point-calibrations and simple analysis for each content point.

The data from analysing the calibration samples are summarised in Table 3. The slope of the calibration regression equations did not vary considerably, suggesting that the concentration of the internal standard d_5-CHPV–globin remained almost unchanged over the time. The mean value of the slope of 0.028 g globin/pmol suggests that the content of the internal standard d_5-CHPV–globin is 36 pmol/g globin, which is very close to the value of 40 pmol d_5-CHPV/g globin obtained from the standardisation experiment. With these calibration curves, samples spiked with 25 and 100 pmol CHPV/g globin were analysed and gave mean inter-assay recoveries of 105 ± 16% (25 pmol/g globin) and 100 ± 9% (100 pmol/g globin), thus confirming the good recovery data obtained before.

Three calibration curves with CHPV concentrations ranging between 10 and 250 pmol/g globin were used for the determination of LOQ and LOD values of the method. The LOD and LOQ values in globin-containing samples were determined to be 10 and 25 pmol CHPV/g globin, respectively, according to the German guideline 32 645 [36] (see Section 2.2.8).

These results are supported by the data of Table 3 and Figs. 7 and 8. The peak of the internal standard d_5-CHPV at a content of 40 pmol/g globin was measured with signal-to-noise (S/N) ratio values typically ranging between 30:1 and 75:1 (see Figs. 7 and 8). These data suggest that the LOD value of the present method would be in the range of 4–2 pmol/g globin, if the LOD is defined as the analyte concentration that produces a S/N ratio of 3:1; this definition has also been used by others for ECH adducts [14].

3.3. Analysis of blood samples from potentially ECH-exposed individuals

The present GC–tandem MS method was applied to monitor blood samples of intervention forces and residents potentially exposed to ECH after a freight train accident in Bad Münder, Germany [37,38]. In the course of the accident, about 38 tons of epichlorohydrin were released into the environment and intervention forces as well as residents were potentially exposed to epichlorohydrin. CHPV was detected in 36 of 628 blood samples (concentration >10 pmol/g globin) (Bader et al., in preparation). Fig. 8 shows representative GC–tandem MS chromatograms of two blood samples from two subjects potentially exposed to ECH after the freight train accident. No CHPV was detected in the globin of subject #1 (Fig. 8A) but in subject #2 (Fig. 8B). In the chromatogram of Fig. 8B, the first GC peak, i.e. the minor isomer of the CHPV–PFPITC derivative at t_R = 21.53 min, is also present, thus underlining the unequivocal identity of CHPV in the blood sample of subject #2, i.e. the correct assignment of the GC–tandem MS peaks to CHPV. The CHPV content of CHPV in the blood sample of subject #2 was calculated to be 80 pmol/g globin, which is clearly above the LOD/LOQ values of the method. It is worth mentioning that in blood samples of considerably lower d_0-CHPV content, the first minor peak is hardly detectable (see Fig. 7).

4. Discussion

4.1. General remarks concerning ECH adducts analysis in biomonitoring

A well-established procedure to analyse adducts of the *N*-terminal amino acid valine in human haemoglobin is the so-called

Table 3
Summary of the results[a] from the GC–tandem MS analysis of calibration samples[b] on 10 days.

Day	Slope (g globin/pmol)	*y*-Axis intercept	R^2	Recovery (%) for sample[c]	
				25	100
Day 1	0.0271	+0.1209	0.9998	105	101
Day 2	0.0345	−0.0645	0.9984	97	94
Day 3	0.0296	+0.2052	0.9843	74	121
Day 4	0.0330	+0.1166	0.9994	115	99
Day 5	0.0287	+0.0062	0.9999	104	99
Day 6	0.0214	−0.0158	0.9944	129	88
Day 7	0.0297	+0.0039	0.9992	89	104
Day 8	0.0267	−0.0128	0.9971	118	91
Day 9	0.0218	+0.1204	0.9983	113	104
Day 10	0.0274	+0.0195	0.9992	101	194
Mean ± S.D.	0.028 ± 0.004	+0.05 ± 0.08		105 ± 16	100 ± 9
R.S.D.	15%	170%			

[a] Values for slope, *y*-axis intercept and correlation coefficient obtained from the linear regression analysis between *y*, the measured peak area ratio for the dipeptide content (pmol/g globin) and the internal standard content d_5-CHPV globin/g globin, and *x*, the dipeptide content (pmol/g globin).

[b] Each four calibration samples were used with a dipeptide content (pmol/g globin) of 0, 25, 100 and 250 pmol/g globin; the content of d_5-CHPV globin/g globin was 40 pmol/g globin.

[c] Recovery was calculated for samples 25 and 100 pmol/g globin by using the respective regression equation.

"*N*-alkyl Edman degradation" (see Fig. 2), first described and applied by Törnqvist et al. [24] as a modification of a method originally invented for protein sequencing. In recent years, the modified Edman method has become a standard approach for adduct analysis in occupational–medical surveillance. In Germany, the Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area of the Deutsche Forschungsgemeinschaft has evaluated altogether five exposure equivalents or biological guidance values for *N*-terminal protein adducts: acrylamide, acrylonitrile, dimethyl sulphate, ethylene and ethylene oxide [8].

As yet, only one attempt has been made to apply the *N*-alkyl Edman method to the analysis of ECH adducts by Hindsø Landin et al. [14]. While these authors showed the presence of DHPV in ECH-exposed rats, they could not detect CHPV in rat haemoglobin presumably due to analytical problems. Hindsø Landin et al. [14] assumed that CHPV hydrolysed into DHPV under physiological conditions but could not demonstrate this first-hand. Nevertheless, Hindsø Landin et al. [14] concluded that CHPV is not suitable for biomonitoring purposes, because CHPV is either unstable in vivo or CHPV hydrolyses during the sample treatment under alkaline conditions.

The analysis of CHPV in human haemoglobin required some modifications of the basic Edman method for the determination of valine adducts as first described by Törnqvist et al. [24] or of common applications such as the determination of acrylonitrile, acrylamide and methylating chemicals [26,30–32]. In particular, the hydroxyl group in the C-2 position of the adduct moiety requires a derivatisation to avoid low extraction yields and poor chromatography. The acetylation by acetic acid anhydride and triethyl amine as applied in our approach was first suggested by Hindsø Landin et al. [14] for the analysis of the secondary adduct of epichlorohydrin, *N*-(2,3-dihydroxypropyl)valine. A second important difference to the original method was the use of a self-synthesised deuterated CHPV as an internal standard. This approach avoids the use of radiolabelled internal standards [14,24] and of synthetic valine adducts with a different chemical structure than the compound of interest, which may react in a different way and are thus not appropriate for accurate quantification.

To detect CHPV, we introduced tandem mass spectrometry, i.e. triple stage quadrupole (TSQ), instead of the single step quadrupole (SSQ) technique with electron impact or negative ion chemical ionisation to overcome sensitivity and specificity problems. To our experience with the Edman method, both the complex constitution of the injection samples after two derivatisation steps and the loss of analyte during the multiple extraction and washing steps result in a relatively low yield of CHPV and an insufficient chromatographic separation from co-eluting substances. Thus, we observed that single step quadrupole MS has a relatively high limit of detection for CHPV of about 100 pmol/g globin. The use of GC–tandem MS improved the sensitivity of the SSQ–CHPV method by a factor of about 10.

During the development, validation and testing of the present GC–tandem MS method we experienced that the stability of CHPV in aqueous media is limited, as was observed from the increasing slopes of calibration curves with time. In this respect, our findings are in line with those of Hindsø Landin et al. [14]. However, no degradation was observed when the calibrator dipeptide and the d_5-CHPV globin were kept dry and frozen at −80 °C. Also, in contrast to the observations of Hindsø Landin et al. [14], we did not experience loss of CHPV during the work-up of globin samples or degradation of CHPV–PFPITC derivatives from samples ready for injection. It has to be noted, however, that we avoided any harsh work-up conditions and sample treatment with respect to pH value and temperature, in particular during the multiple evaporation steps. We therefore think that careful sample handling during the whole work-up procedure is a crucial aspect of the method. This view is supported by many recent reports on the determination of 3-chloro-2-hydroxypropyl adducts of ECH to DNA bases and amino acids [16–22]. Thus, the hydrolysis of the chlorine-carrying methylene group at C-3 does not seem to be an immanent, but a work-up dependent problem. The in vivo stability of the 3-chloro-2-hydroxypropyl adducts is largely unknown, but the results of Hindsø Landin et al. [34] with *S*-(3-chloro-2-hydroxy)cysteine in rat haemoglobin could support the assumption that the life-time of CHPV in the body may be shorter than adducts of other toxins. This aspect should be considered in sampling strategies for CHPV and other relatives. Also, the general recommendations for globin adduct analysis of the DFG, notably the globin isolation as soon as possible after sampling and the storage of the dry protein in a deep-freezer, should be observed.

4.2. Quantification of CHPV in human blood by GC–tandem MS

The GC–tandem MS method described in this paper reports for the first time the quantitative determination of ECH–haemoglobin adducts of the 3-chloro-2-hydroxypropyl type in human blood. The calibration was based on the use of a commercially available dipeptide standard *N*-(3-chloro-2-hydroxypropyl)valine-leucine anilide as the calibrator, and an newly synthesised stable-isotope labelled analogue, i.e. d_5-CHPV, as the internal standard. The present work demonstrates that this approach in combination with the inherent accuracy of the tandem mass spectrometry as employed here, notably the use of the strongly electron-capturing PFPITC derivative of CHPV alongside ECNICI, allows for a highly accurate and sensitive quantification of the ECH–haemoglobin adduct *N*-(3-chloro-2-hydroxypropyl)valine in human blood. By using this GC–tandem MS method, we were successful in determining CHPV in potentially ECH-exposed individuals during a train accident [37,38]. On the basis of our results, we propose *N*-(3-chloro-2-hydroxypropyl)valine in human blood as a suitable and specific biomarker to monitor the exposure of individuals to ECH.

It is worth mentioning that the GC–MS methodology, which is by nature less specific than the GC–tandem MS methodology, did not allow for accurate quantification of CHPV, as observed by repeated consecutive analysis of the real samples by GC–tandem MS and GC–MS (data not shown). The drastically increased specificity and sensitivity resulting from CID in the GC–tandem MS efficiently compensates the analytical background. At present, it seems that the GC–tandem MS methodology as used in our study is the single method that can be used for accurate quantification of ECH adducts in human blood. In general, the Edman method for CHPV is considerably laborious and time-consuming due to the required extraction and washing steps, an aspect that makes the GC–tandem MS method unfavourable for routine analysis [39]. The LC–tandem MS is increasingly used for the quantitative analysis in biological samples of endogenous and exogenous compounds including drugs and their metabolites. Thus, it might provide an alternative for the GC–tandem MS methodology in the quantification of adducts of toxicants such as ECH to biomolecules including proteins [40]. However, this remains to be demonstrated for CHPV globin. Nevertheless, in consideration of the high molar excess of native "non-adducted" proteins over the "adducted" proteins, for instance 50 pmol/g globin which corresponds to about only one "adducted" molecule per one million "non-adducted" molecules, it is highly questionable that authentic "adducted" proteins could be measured in the presence of their precursors without preceding sample preparation, e.g. by affinity chromatography or ion-exchange chromatography [41]. In our opinion, the Edman method will be indispensable in the quantitative analysis of adducts

such as of ECH to haemoglobin, irrespective of which methodology would finally be applied, i.e. GC–tandem MS or LC–tandem MS. For the present, the GC–tandem MS methodology seems to be superior to LC–tandem MS because of its higher sensitivity.

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