N-(3-Chloro-2-hydroxypropyl)-valine in blood as haemoglobin adduct of epichlorohydrin

Matrix:	Blood
Hazardous substance:	Epichlorohydrin
Analytical principle:	Capillary gas chromatography with tandem mass spectrometry (GC-MS/MS)
Completed in:	May 2009

Overview of the parameters that can be determined with this method and the corresponding chemical hazardous substances:

Hazardous substance	CAS	Parameter	CAS
Epichlorohydrin	106-89-8	N-(3-Chloro-2-hydroxypropyl)-valine	–

Summary

With this analytical method the primary haemoglobin adduct of epichlorohydrin, N-(3-chloro-2-hydroxypropyl)-valine (CHPV) can be determined quantitatively using gas chromatography-tandem mass spectrometry (GC-MS/MS).

For this purpose, globin is isolated from a blood sample by precipitation, dried and dissolved in formamide. After addition of pentafluorophenyl isothiocyanate, sodium hydroxide and the internal standard (d_5-CHPV-labelled globin), selective cleavage is carried out via a modified Edman degradation including conversion of the adduct to a thiohydantoin. The derivative is extracted with diethyl ether, dried in a stream of nitrogen and resuspended in toluene. After washing using sodium carbonate solution and water the sample is evaporated in a stream of nitrogen. The hydroxypropyl function of the adduct is acetylated by addition of acetic anhydride/triethylamine in acetonitrile. The sample is then evaporated to dryness in a stream of nitrogen, dissolved in n-hexane and extracted with a mixture of methanol and

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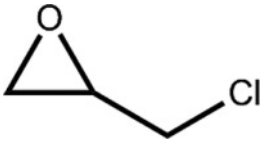
water. The hexane phase is evaporated to dryness in a stream of nitrogen and the residue is dissolved in toluene. Quantitative analysis of the thiohydantoin derivative is carried out using GC-MS/MS in the selected reaction monitoring (SRM) mode after negative chemical ionisation (NCI).

For external calibration the calibration standards of an adduct-modified dipeptide, N-(3-chloro-2-hydroxypropyl)-valine leucine anilide are used.

N-(3-Chloro-2-hydroxypropyl)-valine (CHPV)

Within day precision:	Standard deviation (rel.)	s_w = 12.4% or 9.8%
	Prognostic range	u = 27.6% or 21.8%
	at a spiked concentration of 25 or 100 pmol CHPV per gram globin and where n = 10 determinations	
Day to day precision:	Standard deviation (rel.)	s_w = 15.0%
	Prognostic range	u = 33.4%
	based on the variance of the calibration graph slope and where n = 10 determinations	
Accuracy:	Recovery rate	r = 99.8 ± 12.5%
	at a nominal concentration of 100 pmol CHPV per gram globin and where n = 10 determinations	
Detection limit:	10 pmol CHPV per gram globin	
Quantitation limit:	25 pmol CHPV per gram globin	

1-Chloro-2,3-epoxypropane (epichlorohydrin)



1-Chloro-2,3-epoxypropane (epichlorohydrin, CAS-No. 106-89-8) is a chloromethyl-substituted epoxide with an annual production volume of around 50,000 tons. Epichlorohydrin is mainly used for the production of epoxy resins, glycerol and insecticides and as cross-linking agent in the paper industry [1]. At room temperature, epichlorohydrin is a colourless liquid with a pungent odour, a boiling point of 116°C and a vapour pressure of about 17 hPa. It is poorly soluble in water [2]. In a neutral aqueous solution epichlorohydrin hydrolyses to 3-chloropropane-1,2-diol with a half-life of 5 to 8 days. This takes place more rapidly in acidic or alkaline solutions [1].

In the 2003 MAK documentation, the *Deutsche Forschungsgemeinschaft* (DFG) summarised the state of knowledge on the toxicity of epichlorohydrin. The DFG has classified it in Category 2 for carcinogenic substances [2]. Analogously, the IARC (International Agency for Research on Cancer) has categorised epichlorohy-

drin as probably carcinogenic to humans (Group 2A) [3]. Furthermore, epichlorohydrin has been designated with "Sh" (skin sensitisation hazard) and with an "H" (risk of skin absorption) and it is also classified as a Category 3B germ cell mutagen due to its genotoxicity [2].

Knowledge about the metabolism of epichlorohydrin is mainly derived from animal studies by Weigel et al. [4] and Gingell et al. [5]. According to these studies, about 90% of an inhaled or orally administered dose is absorbed and distributed throughout all body compartments within 2 to 4 hours. Metabolism primarily takes place via reaction of the two electrophilic centres of epichlorohydrin, the C3 in the epoxide ring and the chlorine atom at the C1. Accordingly, conjugation with glutathione leads to the excretion of N-acetyl-S-(3-chloro-2-hydroxypropyl)-L-cysteine, S-(2,3-dihydroxypropyl)-L-cysteine and N-acetyl-S-(2,3-dihydroxypropyl)-L-cysteine as main metabolites. In addition, 3-chloropropane-1,2-diol is formed by epoxide hydrolysis.

In vivo, epichlorohydrin is able to react with macromolecules. Binding to haemoglobin and to lymphocyte DNA has already been described a number of times in the literature and has been considered for biomonitoring [6–13]. In principle, two types of adducts can be distinguished and used for analysis (see Figure 1). These are on the one hand the primary adducts with a 3-chloro-2-hydroxypropyl structure formed by epoxide ring opening and on the other hand the secondary adducts with a 2,3-dihydroxypropyl structure. These secondary adducts are formed either by hydrolytic cleavage of the chlorine-carbon bond after conjugation to glutathione or by direct conjugation of 3-chloro-1,2-propanediol to glutathione under HCl elimination.

Hindsø Landin et al. [9] have been able to show that the concentration of 3-chloro-2-hydroxypropyl-cysteine adducts in rat globin decreases more rapidly ($t_{1/2}$ approx. 4 days) than expected from the lifespan of rat erythrocytes (approx. 30 days). In contrast, the concentration of 2,3-dihydroxypropyl-valine adducts in rats' blood increased after exposure. In 1996, the working group of Hindsø Landin was not successful in analysing 3-chloro-2-hydroxypropyl-valine [14]. From these observations they assumed that the chlorinated primary adduct is not very stable neither *in vivo* nor *in vitro* and is therefore not suitable for biomonitoring [9]. However, more recent studies mentioned above showed that a determination of the 3-chloro-2-hydroxypropyl adduct is possible. At present, human *in vivo* studies are rare. Primarily, occupational medical studies involving employees exposed to epichlorohydrin and investigations following single accidental exposure were carried out.

The DNA adduct N7-(3-chloro-2-hydroxypropyl)-guanine was analysed in a study by Plna et al. [12] and was not detected in 13 samples of controls but in 7 of 16 samples of volunteers potentially exposed to epichlorohydrin.

In 1997, Hindsø Landin et al. determined N-(2,3-dihydroxypropyl)-valine (DHPV) using a GC-MS/MS method (detection limit: 2 pmol/g globin) in 15 German employees exposed to epichlorohydrin (determined DHPV levels were: eight non-smokers: 7.3 ± 2.7 pmol/g globin; seven smokers: 21.1 ± 17.1 pmol/g globin)

as well as in eleven controls from the same company (DHPV concentration: three non-smokers: 6.8 ± 3.2 pmol/g globin; nine smokers: 13.1 ± 12.4 pmol/g globin) [8]. The mean external exposure of the workers exposed to epichlorohydrin during a 12-hour work shift ranged between 0.11 and 0.23 ppm (former Technical Guidance Concentration: 3 ppm). The differences between non-smokers and smokers were significant, whereas no differences were found between exposed and control persons. Thus, Hindsø Landin et al. [8] concluded that the dihydroxypropyl-valine adduct was not substance-specific but could also have been formed from glycidol found in tobacco smoke. Accordingly, tobacco smoke-related adduct levels may interfere with adduct levels resulting from workplace exposure.

Furthermore, this working group analysed six non-smokers and four smokers of a Swedish control collective [14]. With an adduct level of 2.1 ± 1.1 pmol/g globin (non-smokers) or 9.5 ± 2.2 pmol/g globin (smokers), the DHPV level of the Swedish volunteers was significantly lower than that of the controls in the German study.

In a study by Wollin et al. [15] with accidentally exposed individuals it was not possible to detect DHPV neither in smokers nor in individuals potentially exposed to epichlorohydrin (GC-MS, detection limit 10 pmol/g globin).

In a collective of 323 emergency helpers potentially exposed to released epichlorohydrin in an accident involving the transport of hazardous materials, Bader et al. [13] were able to detect the primary adduct N-(3-chloro-2-hydroxypropyl)-valine (CHPV) in a total of 22 samples. The level and duration of each exposure were unknown. Furthermore, CHPV was also found *in vitro* after incubation of human erythrocytes with epichlorohydrin. This implies that CHPV can be used as a biomarker for exposure to epichlorohydrin.

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Contents

1 General principles

With the present analytical method the major haemoglobin adduct of epichlorohydrin, N-(3-chloro-2-hydroxypropyl)-valine can be determined quantitatively based on the so-called modified Edman degradation using gas chromatography-tandem mass spectrometry (GC-MS/MS). For this purpose, globin is initially isolated from a whole blood sample by sequential precipitation, dried and then dissolved in formamide. After the addition of pentafluorophenyl isothiocyanate, sodium hydroxide and an internal standard (d_5-CHPV-labelled globin), selective cleavage and conversion of the N-alkylated terminal amino acid into a thiohydantoin derivative is carried out (Figure 2). The derivative is extracted with diethyl ether, dried in a stream of nitrogen and resuspended in toluene. After washing steps using sodium carbonate solution and water, the sample is evaporated to dryness in a stream of nitrogen. The free hydroxyl function of the adduct is acetylated by the addition of acetic anhydride/triethylamine in acetonitrile. The sample is evaporated to dryness in a stream of nitrogen, dissolved in n-hexane and washed with methanol/water (60:40, v/v). The hexane phase is evaporated to dryness in a stream of nitrogen and the residue is dissolved in toluene. The thiohydantoin derivative is quantitatively analysed using GC-MS/MS in the selected reaction monitoring mode (SRM) after negative chemical ionisation (NCI). Calibration is carried out with a dipeptide standard containing N-terminal N-(3-chloro-2-hydroxypropyl)-valine. A globin labelled with pentadeuterated epichlorohydrin (d_5-epichlorohydrin) is used as internal standard.

2 Equipment, chemicals and solutions

2.1 Equipment

- GC-MS/MS system with PTV injector, autosampler and negative chemical ionisation (NCI) unit (e.g. ThermoElectron (Dreieich, Germany)) with TSQ 7000 mass spectrometer)
- Gas chromatographic column: length: 30 m; inner diameter: 0.25 mm; stationary phase: 100% polydimethylsiloxane; film thickness: 0.25 µm (e.g. Optima-1-MS, Macherey-Nagel, No. 726205.30)

- 10 mL, 50 mL, 100 mL, 500 mL, 1000 mL Volumetric flasks
- 13 mL and 20 mL Screw cap glass tubes (e.g. Schott)
- Laboratory shaker (e.g. Vibrax, Ika)
- Horizontal shaker (e.g. Janke & Kunkel)
- Laboratory centrifuge (e.g. Megafuge, Heraeus)
- Sample evaporation unit (e.g. ReactiVap, Pierce)
- 1.8 mL Glass vials (e.g. Macherey-Nagel)
- 200 µL Microvials (e.g. Macherey-Nagel)
- Water bath with shaking device (e.g. Thermo Haake)
- Microlitre pipettes, with adjustable volumes between 2 and 20 µL, 10 and 100 µL as well as between 100 and 1000 µL (e.g. Eppendorf)
- Analytical balance (e.g. Mettler Toledo)
- Vacuum desiccator (e.g. Schott)
- Ultrasonic bath (e.g. Bandelin electronic)
- EDTA monovettes (e.g. Sarstedt)
- 250 mL Glass beaker

2.2 **Chemicals**

- 2-Propanol p.a. (e.g. Merck, No. 109634)
- Ethyl acetate p.a. (e.g. Merck, No. 109623)
- n-Hexane p.a. (e.g. Merck, No. 107023)
- Sodium chloride p.a. (e.g. Merck, No. 106404)
- Sodium carbonate, anhydrous p.a. (e.g. Merck, No. 106392)
- 37% Hydrochloric acid (e.g. Merck, No. 100317)
- Sodium hydroxide pellets p.a. (e.g. Merck, No. 106498)
- Highly purified water or bidist. water
- Diethyl ether p.a. (e.g. Merck, No. 100921)
- Formamide ultrapure (e.g. Amersham Life Science, No. US75828)
- Ethanol p.a. (e.g. Merck, No. 100983)
- N-(3-Chloro-2-hydroxypropyl)-valine leucine anilide (e.g. Bachem, No. G-4340)
- Acetic anhydride p.a. (e.g. Merck, No. 100042)
- Acetonitrile ultrapure (e.g. Fluka, No. 00700)
- Triethylamine p.a. (e.g. Merck, No. 808352)
- Methanol p.a. (e.g. Merck, No. 106011)
- d_5-Epichlorohydrin, 98% (e.g. Cambridge Isotope Laboratories, No. DLM-1008-1)
- Pentafluorophenyl isothiocyanate for GC, ≥ 97% (e.g. Fluka, No. 76755)
- Toluene p.a. (e.g. Fluka, No. 34938)
- Nitrogen 5.0 (e.g. Linde)
- Helium 5.0 (e.g. Linde)
- Methane 5.5 (e.g. Linde)
- Argon 5.0 (e.g. Linde)

2.3 Solutions

- 50 mM Hydrochloric acid in 2-propanol:
  4.1 mL 37% hydrochloric acid are pipetted into a 1000 mL volumetric flask containing about 500 mL 2-propanol and made up to the mark with 2-propanol.
- 0.9% Saline solution:
  9 g sodium chloride are weighed into a 1000 mL volumetric flask, dissolved in bidist. water and made up to the mark with bidist. water.
- 1 M Sodium hydroxide solution:
  4 g sodium hydroxide pellets are weighed into a 250 mL glass beaker and dissolved in bidist. water. The solution is transferred to a 100 mL volumetric flask and made up to the mark with bidist. water.
- 0.1 M Sodium carbonate solution:
  1.06 g sodium carbonate are weighed into a 100 mL volumetric flask, dissolved in bidist. water and made up to the mark with bidist. water.
- Wash solution:
  60 mL methanol are transferred to a 100 mL volumetric flask and made up to the mark with bidist. water.

The solutions can be stored in the refrigerator at 4°C for at least one month.

Acetylation reagent:

- 12.5% (v/v) Acetic anhydride:
  1.25 mL acetic anhydride are placed in a 10 mL volumetric flask and made up to the mark with acetonitrile.
- 12.5% (v/v) Triethylamine:
  1.25 mL triethylamine are placed in a 10 mL volumetric flask and made up to the mark with acetonitrile.

For the final preparation of the acetylation reagent, 5 mL 12.5% acetic anhydride solution and 5 mL 12.5% triethylamine solution are pipetted into a 10 mL volumetric flask and are mixed thoroughly.

The acetylation reagent must be freshly prepared on every working day.

2.4 Internal standard

A globin labelled with d_5-epichlorohydrin is used as internal standard (IS). To prepare the IS, 200 µL d_5-epichlorohydrin are added to 20 mL non-smoker's pooled lysate (for preparation see Section 3.1) in a 20 mL screw cap glass tube and shaken on a laboratory shaker at 180 min^{-1} for 4 hours. The globin is then isolated according to Section 3.2.

- Stock solution:
  10 mg of the isolated globin are weighed into a 50 mL volumetric flask, dissolved in formamide (30 min in the ultrasonic bath) and made up to the mark with formamide.
- Working solution:
  1000 µL of the stock solution are pipetted into a 50 mL volumetric flask and made up to the mark with formamide. 100 µL each of this working solution are added to samples, calibration standards and quality controls using pipettes.

2.5 Calibration standards

- Stock solution (1 mM CHPV)
  39.8 mg of the dipeptide standard N-(3-chloro-2-hydroxypropyl)-valine leucine anilide are weighed exactly into a 100 mL volumetric flask and made up to the mark with ethanol.
- Working solution (1 µM CHPV)
  100 µL of the stock solution are pipetted into a 100 mL volumetric flask already containing about 50 mL ethanol and made up to the mark with ethanol.

Starting with this working solution, the spiking solutions for calibration are prepared according to the pipetting scheme in Table 1.

Table 1 Pipetting scheme for the preparation of the spiking solutions for calibration.

Spiking solution	Volume of ethanol [mL]	Volume of stock solution [mL]	Total volume [mL]	CHPV level [nmol]
1	20.0	0.0	20	0
2	19.8	0.2	20	10
3	19.5	0.5	20	25
4	19.0	1.0	20	50
5	18.5	1.5	20	75
6	18.0	2.0	20	100
7	16.5	3.5	20	175
8	15.0	5.0	20	250

To prepare the calibration standards, 100 mg standard pooled globin (from non-smokers) are weighed into 13 mL screw cap glass tubes, dissolved in 3 mL formamide (30 min in the ultrasonic bath) followed by the addition of 100 µL of the corresponding spiking solution and 100 µL of the working solution of the internal standard (Table 2).

Table 2 Pipetting scheme for the preparation of the calibration standards.

Calibration standard	Volume of working solution IS [µL]	Volume of the corresponding spiking solution [µL]	CHPV in the sample [pmol/g globin]
1	100	100	0
2	100	100	10
3	100	100	25
4	100	100	50
5	100	100	75
6	100	100	100
7	100	100	175
8	100	100	250
internal standard	100	0	approx. 100*

* Estimated value, as the IS was prepared in the laboratory by incubation of d_5-epichlorohydrin with pooled globin. The exact adduct formation rate is not known.

3 Specimen collection and sample preparation

3.1 Preparation of the erythrocyte lysate

Blood samples (approx. 5 mL) are collected with an EDTA monovette (7.5 mL nominal volume). Globin isolation of the whole blood should take place within 24 hours. For this purpose, every sample is initially centrifuged at 800 *g* for 5 min. The filling level of each monovette is marked on the outside with a permanent marker. The supernatant plasma is pipetted off and 0.9% saline solution is added to the erythrocyte sediment up to the mark and the monovette is mixed thoroughly. The sample is centrifuged again at 800 *g* for 5 min and the supernatant is again pipetted off and discarded. The washing process is repeated until the supernatant is colourless (at least twice). Following this procedure, about 2.5 mL plasma-free erythrocyte sediment is obtained from 5 mL blood. Lysis of erythrocytes is performed by adding bidist. water up to the mark and deep freezing the sample at –20°C for at least eight hours.

3.2 Isolation of the globin

The deep frozen lysate sample is slowly thawed in a water bath at about 37°C and is homogenised by shaking. 2 mL lysate are then transferred with a pipette to a 20 mL screw cap glass tube. After addition of 12 mL 50 mM hydrochloric acid in 2-propanol, the sample is shaken thoroughly and the cell debris are removed by centrifugation (3500 *g*, 10 min). The supernatant is transferred to a new 20 mL screw cap glass tube and the globin is precipitated by slow addition of 8 mL ethyl acetate.

To complete precipitation, the sample is stored at 4°C in the refrigerator for one hour, followed by centrifugation at 3500 *g* for 5 min. The supernatant is decanted off and discarded, whereas the precipitated globin is resuspended thrice using 10 mL ethyl acetate each time and is shaken and centrifuged at 3500 *g* for 5 min each. The supernatant is decanted off and discarded after each centrifugation process. After the final decanting, the precipitated globin is stored overnight in the vacuum desiccator for drying. The dry globin can be stored in glass vials or polypropylene tubes up to 12 months at –27°C without noteworthy adduct losses.

3.3 **Derivatisation of the globin**

The calibration standard and the internal standard are pipetted according to the scheme in Section 2.5.

From the sample to be analysed, 100 mg globin are weighed into a 13 mL screw cap glass tube and dissolved in 3 mL formamide (30 min in an ultrasonic bath). Then, 100 µL working solution of the IS, 30 µL 1 M sodium hydroxide solution as well as 20 µL pentafluorophenyl isothiocyanate are added to the sample. The calibration standards and quality control standards are processed analogously. After mixing of the sample solution on a laboratory shaker, derivatisation is carried out overnight at room temperature using a horizontal shaker (120 min^{-1}) followed by incubation in a water bath for 2 hours at 45°C.

After equilibration to room temperature, the solution is extracted two times with 4 mL diethyl ether each (samples are mixed thoroughly on a laboratory shaker for 1 min per process). After centrifugation (10 min at 3500 *g*) the ether phases are transferred to a new 13 mL screw cap glass tube and evaporated to dryness in a stream of nitrogen. The residue is dissolved in 1.5 mL toluene and washed once with 2 mL 0.1 M sodium carbonate solution and twice with 2 mL water. Each washing process includes 1 min mixing on a laboratory shaker and a centrifugation at 3500 *g* for 5 min. The toluene phase is transferred to a new 13 mL screw cap glass tube after every cleaning step. After the last washing procedure, the toluene phase is transferred as completely as possible into a 1.8 mL glass vial. The sample is evaporated to dryness in a stream of nitrogen at approx. 35°C.

Subsequently, 250 µL acetylation reagent are pipetted into each glass vial. The open sample vials are briefly shaken on a laboratory shaker and then allowed to stand open for 15 min at room temperature. After evaporation to dryness in a stream of nitrogen at 30°C, the residue is dissolved in 500 µL n-hexane and 1000 µL wash solution are added. The sample vial is sealed and mixed thoroughly on a laboratory shaker for about 1 min. After centrifugation (10 min at 3500 *g*) the hexane phase of the sample is transferred to a new 1.8 mL glass vial and evaporated to dryness in a stream of nitrogen at room temperature. The residue is dissolved in 30 µL toluene, transferred to a 1.8 mL glass vial with a 200 µL microinsert, sealed and subjected to GC-MS/MS analysis.

4 Operational parameters

4.1 Operational parameters for gas chromatography

Capillary column:	Material:	Quartz glass (fused silica)
	Stationary phase:	Optima-1-MS
	Length:	30 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
Temperature:	Column:	1 min at 80°C; then increase at 8°C/min to 340°C; 2 min at final temperature
	Injector:	Initial temperature 120°C, then increase at 10°C/s to 320°C, 4 min at final temperature
	Transferline:	300°C
Carrier gas:	Helium 5.0 (constant flow: 1.0 mL/min)	
Sample volume:	1 µL (splitless)	

4.2 Operational parameters for tandem mass spectrometry

Ionisation type:	Negative chemical ionisation (NCI)
Source temperature:	180°C
Reagent gas:	Methane 5.5 (pre-pressure 530 Pa);
Collision gas:	Argon 5.0 (0.27 Pa)
Electron energy	100 eV
Electron flow:	300 µA
Collision energy:	15 eV
Detection:	Selected Reaction Monitoring (SRM)
Measuring time per ion:	100 ms
Electron multiplier:	2800 V

All other parameters must be optimised according to the manufacturer's instructions.

5 Analytical determination

On account of the stereoisomery at the C2 atom of the adduct, the derivatives of the unlabelled 3-chloro-2-hydroxypropyl-valine (d_0-CHPV) and of the deuterated internal standard (d_5-CHPV) eluate as two baseline-separated peaks each with reten-

tion times of 21.4 min and 21.6 min, respectively (see chromatogram in Figure 3). The second peak with the longer retention time is the main isomer in each case and was therefore used as reference for quantitative analysis (see Table 3).

Table 3 Retention times of the analytes and ion traces.

Analyte	Retention time [min]	Analysed fragmentation reaction (SRM)
CHPV (analyte)	21.4/**21.6**	*m/z* 422 → 301
d_5-CHPV (IS)	21.4/**21.6**	*m/z* 427 → 301

The mass spectra of the CHPV derivatives are shown in Figure 4. The anions with the mass-to-charge ratios (*m/z*) of *m/z* 458 and *m/z* 460 in the mass spectrum of d_0-CHPV (see Figure 4 top row) correspond to the molecule ions with the isotopes ^{35}Cl or ^{37}Cl. The anions with *m/z* 463 and *m/z* 465 in the mass spectrum of d_5-CHPV (Figure 4 bottom row) also correspond to the molecule ions with the isotopes ^{35}Cl or ^{37}Cl. Elimination of HCl from these derivatives results in the most intensive ions at *m/z* 422 (d_0-CHPV) and *m/z* 427 (d_5-CHPV) (for ^{35}Cl). The loss of $H^{35}Cl$ but not of $^2H^{35}Cl$ indicates that hydrogen atoms from the acetyl function participate in the formation of these ions. The ions at *m/z* 323 occur in the mass spectra of both derivatives, indicating that this fragment no longer contains the original epichlorohydrin side chain.

To obtain product ion mass spectra, the ions with *m/z* 458 (for d_0-CHPV) and with *m/z* 463 (d_5-CHPV) were subjected to a collision-induced dissociation (CID). Figure 5 shows the obtained MS/MS-spectra. The most intensive product ion was obtained at *m/z* 301 in each case and therefore does not contain an epichlorohydrin side chain.

6 Calibration

The calibration standards are prepared and processed according to Section 2.5 and Section 3 and are analysed as described in Section 4 and Section 5. Calibration graphs are obtained by plotting the quotients of the peak areas of the analyte and the internal standard against the spiked concentration in pmol/g globin. The calibration graph is linear between 10 and 250 pmol/g globin.

7 Calculation of the analytical result

To determine the analyte concentration in a sample, the peak area of the analyte is divided by the peak area of the internal standard. The quotient thus obtained is inserted into the corresponding calibration graph (see Section 6). The CHPV con-

centration level of the sample is obtained in pmol/g globin. Use the following algorithm to convert into the unit µg/L blood (c = concentration):

$$c\ [\mu g/L\ \text{blood}] = c\ [\text{pmol/g globin}] \times (209 \times 10^{-6}\ \mu g/\text{pmol}) \times 144\ g/L.$$

8 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) [16]. As the adducts are not stable when dissolved, either pooled globin must be prepared from an excess of unspiked globin and a small quantity of labelled globin and kept dry (for use as quality control material), or the slope of the calibration function determined on a daily basis is used as a parameter in quality control.

9 Evaluation of the method

9.1 Precision

To determine the within day precision ten calibration standards with a CHPV concentration of 25 pmol/g globin or 100 pmol/g globin were prepared and analysed in a row. The relative standard deviations were 12.4% (prognostic range: 27.6%, mean value: 31.8 ± 3.9 pmol/g globin) or 9.8% (prognostic range: 21.8%, mean value: 93.7 ± 9.2 pmol/g globin), respectively. The precision from day to day was determined on the basis of the variances of the slopes of ten four-point calibration curves (0, 25, 100, 250 pmol/g globin) and was 15.0% (prognostic range: 33.4%).

9.2 Accuracy

The accuracy of the method was determined to be 99.8 ± 12.5% (range: 84–129%) and was calculated as relative recovery rate of calibration standards with a CHPV concentration of 100 pmol/g globin (n = 10).

9.3 Detection limit and quantitation limit

According to the DIN method 32645 (German Industrial Standard [*Deutsche Industrie-Norm*]) [17], the detection limit of the procedure (calibration graph method)

was determined as 10 pmol/g globin, corresponding to about 0.3 µg CHPV per litre blood. The quantitation limit obtained accordingly is 25 pmol/g globin (0.75 µg CHPV per litre blood).

9.4 Sources of error

Care must be taken that the toluene phase is completely evaporated to dryness prior to the acetylation step, as traces of water considerably impair derivatisation.

In the course of storage experiments, it was observed that the protein adduct is not stable in solution. This concerns both, the decrease in adduct concentration in the material to be analysed (haemolysate) and in the calibration standards. As regards the storage of the material to be analysed, it is therefore necessary, to carry out isolation of the globin and its subsequent storage in the freezer as soon as possible after specimen collection. Currently, there are no indications that a degradation of the adduct occurs under these conditions. Preparation and processing of the calibration standards on a day-to-day basis is recommended.

10 Discussion of the method

The so-called modified Edman degradation according to Törnqvist et al. [18] is a suitable reaction for the single step release of terminal epichlorohydrin-valine adducts from globin and the chemical conversion of N-(3-chloro-2-hydroxypropyl)-valine to a derivative with properties well suited for analysis using GC-MS/MS. To increase the thermic stability and to improve the volatility of the Edman derivative its hydroxyl group is acetylated in a second derivatisation step. On account of the pentafluorophenyl function, the derivative obtained is a very strong electron withdrawing molecule and therefore well suited to form anions by negative chemical ionisation (NCI). For perfluorated analytes this ionisation method is clearly more sensitive compared to electron impact ionisation [19, 20]. The quantitative analysis of N-(3-chloro-2-hydroxypropyl)-valine as pentafluorophenyl-thiohydantoin derivative under MS/MS conditions is characterised by very high specifity and sensitivity. By detection of characteristic product ions, formed from precursor ions, which contain the characteristic chlorine atom, interference by co-elution of other substances is practically excluded. Therefore, a qualifier ion is not necessarily required. The use of GC-MS/MS instead of GC-MS provides an additional gain in sensitivity due to a lower background noise so that a low detection limit of 10 pmol/g globin is obtained. The quantitative determination of N-(3-chloro-2-hydroxypropyl)-valine as pentafluorophenyl thiohydantoin derivative by means of GC-MS/MS using pentadeuterated epichlorohydrin as internal standard is sufficiently precise in the low pmol/g range. On account of these characteristics, the

present procedure is well suited for the detection and quantitation of globin adducts of epichlorohydrin in blood.

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12 Appendix

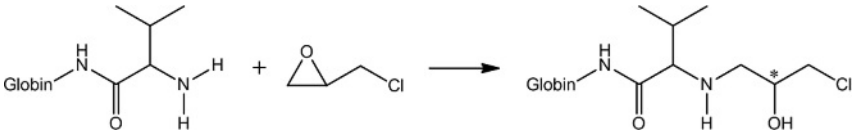

Fig. 1 Formation of the primary adduct N-(3-chloro-2-hydroxypropyl)-valine (CHPV) from the reaction of epichlorohydrin with the N-terminal amino acid of a globin chain.

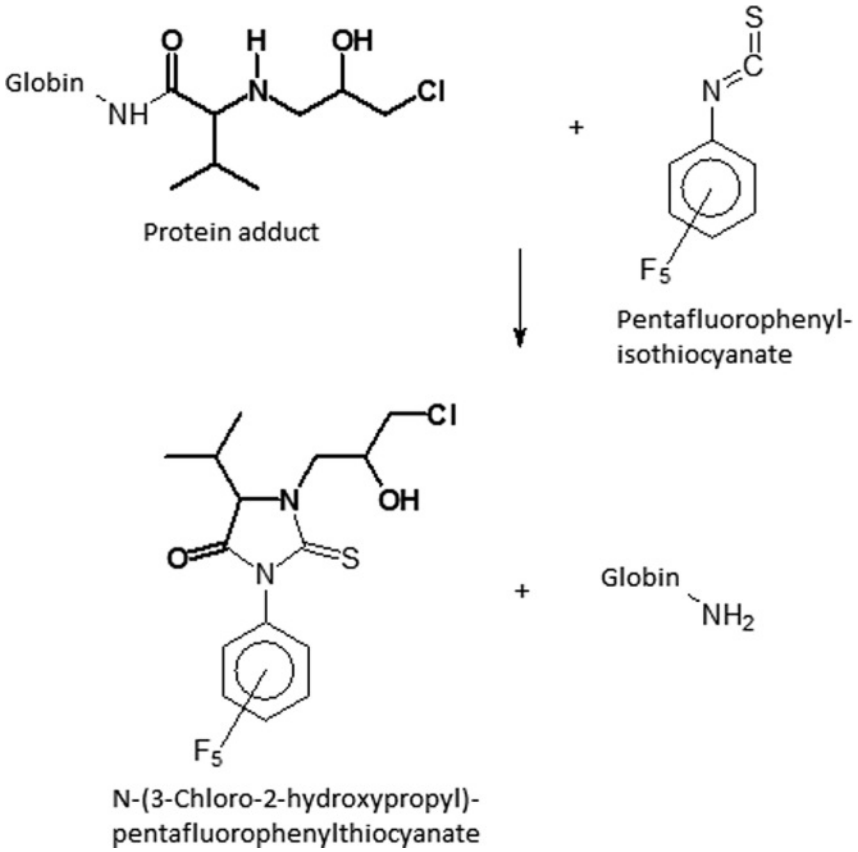

Fig. 2 Cleavage and derivatisation of N-(3-chloro-2-hydroxypropyl)-valine by modified Edman degradation.

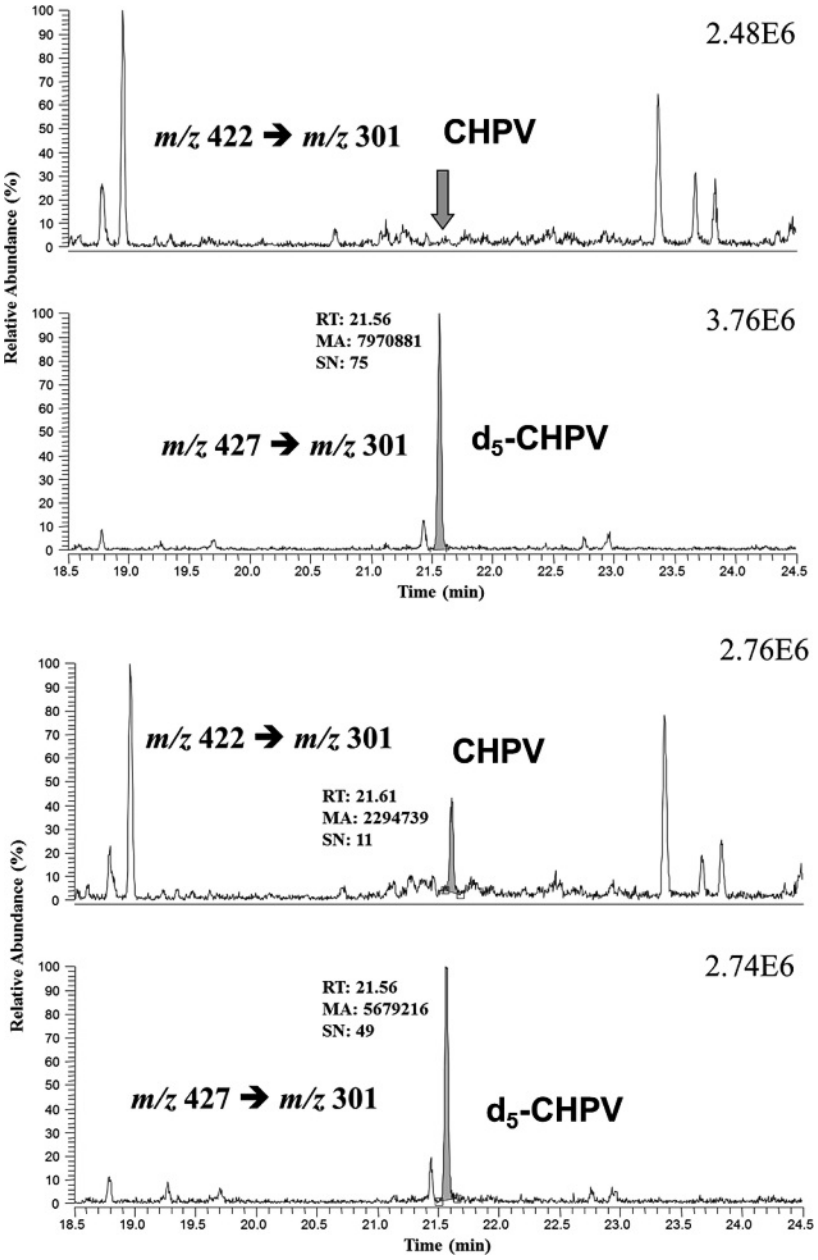

Fig. 3 GC-MS/MS chromatograms of an unspiked pooled globin sample (top row) and of a pooled globin sample spiked with 25 pmol CHPV/g globin (bottom row). The concentration level of d_5-CHPV in both samples was approx. 100 pmol/g globin.

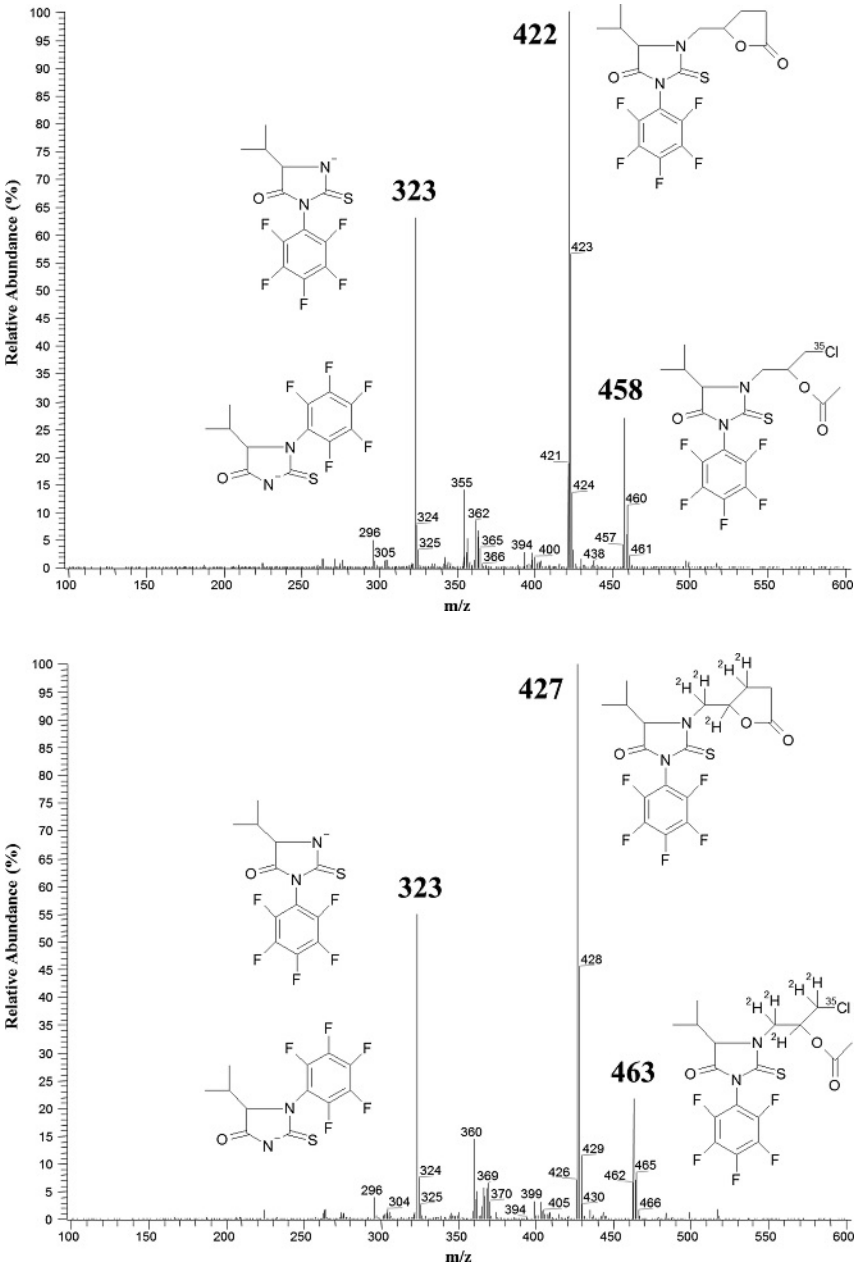

Fig. 4 Mass spectra of d_0-CHPV (top) and d_5-CHPV (bottom).

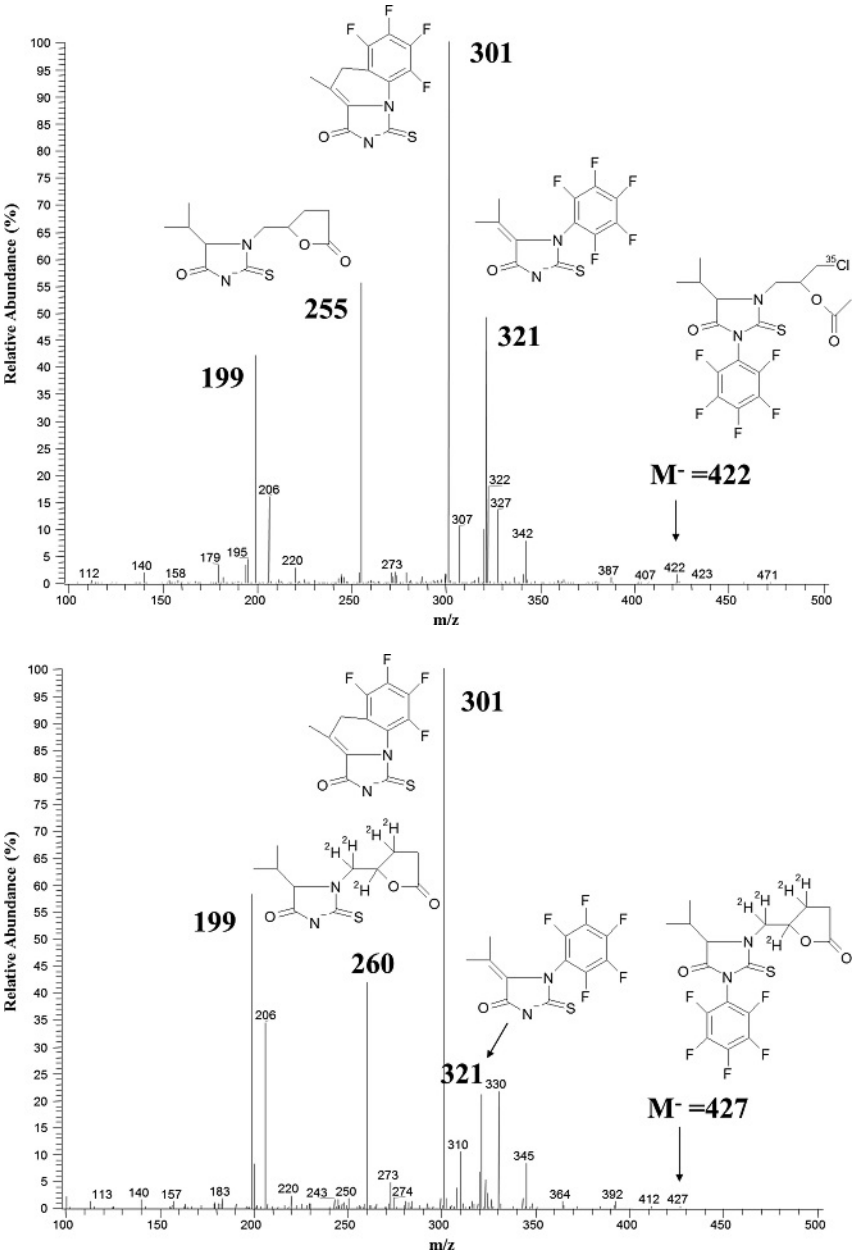

Fig. 5 MS/MS spectra of d_0-CHPV (top) and d_5-CHPV (bottom).