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A method for the simultaneous determination of mercapturic acids as biomarkers of exposure to 2-chloroprene and epichlorohydrin in human urine

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ABSTRACT

We developed and validated an analytical method for the simultaneous determination of several chlorine and non-chlorine containing mercapturic acids in urine as specific metabolites of the hazardous chemicals 2-chloroprene and epichlorohydrin. The method involves an online column switching arrangement for online solid phase extraction of the analytes with subsequent analytical separation and detection using LC–MS/MS. The developed method enables for the first time the determination of Cl-MA-I (4-chloro-3-oxobutyl mercapturic acid), Cl-MA-II (4-chloro-3-hydroxybutyl mercapturic acid), Cl-MA-III (3-chloro-2-hydroxy-3-butenyl mercapturic acid) and HOBMA (4-hydroxy-3-oxobutyl mercapturic acid) as potential biomarkers of 2-chloroprene in urine. Additionally, CHPMA (3-chloro-2-hydroxypropyl mercapturic acid) as a specific metabolite of epichlorohydrin in urine and DHBMA (3,4-dihydroxybutyl mercapturic acid) can be determined. The analytical method proved to be both sensitive and reliable with detection limits ranging from 1.4 μg/L (for Cl-MA-III) to 4.2 μg/L (for HOBMA). Intra- and interday imprecision was determined to range from 4.7 to 11.8%. Due to the good accuracy and precision and the low limits of detection the developed method is well suited for application in biomonitoring studies in order to determine occupational exposure to 2-chloroprene and epichlorohydrin.

1. Introduction

The alkylating agents epichlorohydrin and 2-chloroprene are important high production volume chemicals mainly used as intermediates in the industrial synthesis of polymers.

The worldwide production volume of 2-chloroprene totals 350,000 t per year (in 2004) [1] which is almost exclusively used for the synthesis of polychloroprene. The synthetic rubber (also known as Neopren® or Baypren®) exhibits excellent insulation properties and is used in manifold applications, e.g. for thermal protection suits [1,2]. 2-Chloroprene is classified as probably carcinogenic to humans (Group 2) by the Deutsche Forschungsgemeinschaft (DFG) and accordingly, as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer (IARC) [2,3]. In vivo, 2-chloroprene is apparently detoxified by the formation and excretion of mercapturic acids [4]. However, there are few studies on the biotransformation of 2-chloroprene. The most relevant ones are the in vitro-studies by Munter et al. [5,6] who investigated the metabolism of 2-chloroprene in liver microsomes of humans and rodents. They proposed a detailed metabolism scheme of 2-chloroprene that is shown in part in Fig. 1. According to that scheme, the biotransformation of 2-chloroprene involves the intermediate formation of two reactive epoxide forms, 1-CEO ((1-chloroethenyl)oxirane) and 2-CEO (2-chloro-2-ethenyl oxirane). These highly reactive intermediates are likely metabolised by epoxide hydrolases and conjugation with glutathione (GSH) to several chlorine and non-chlorine containing mercapturic acids [5,6].

The annual worldwide production of epichlorohydrin adds up to about 700,000 t (in 1999) [1]. The alkylating substance is used primarily as a monomer for the synthesis of epoxy resins and polymer additives [1,7]. The DFG and the IARC classified epichlorohydrin as probably carcinogenic to humans (Group 2 and Group 2A) [3,7]. In vivo, the formation of DNA and protein adducts has already been shown. Besides, due to its bifunctionality, epichlorohydrin can cause crosslinks between nucleophilic side chains of biological molecules, which illustrates the major genotoxic potential of this substance [8–10].

The proposed biotransformation pathway of epichlorohydrin according to Gingell et al. [11] is illustrated in part in Fig. 2. A direct conjugation of epichlorohydrin with GSH leads to an urinary excretion of the mercapturic acid CHPMA (3-chloro-2-hydroxypropyl

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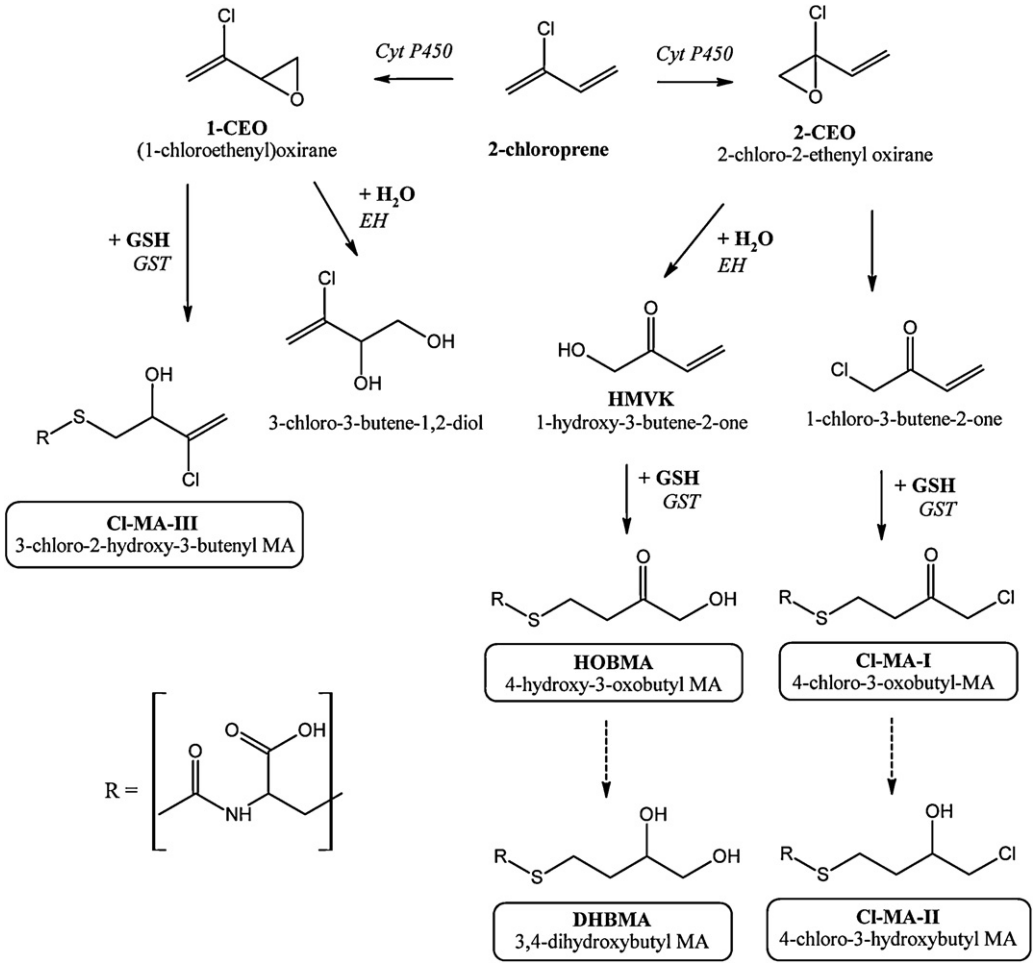

Fig. 1. Proposed biotransformation of 2-chloroprene according to Munter et al. [5,6], modified (Cyt P450 = cytochrome P450; GSH = glutathione; GST = glutathione-S-transferase; EH = epoxide hydrolase; MA = mercapturic acid).

mercapturic acid) and, following a nucleophilic dehalogenation step, of DHPMA (2,3-dihydroxypropyl mercapturic acid) [11–13]. Animal studies demonstrated that the biological half-life of epichlorohydrin averages to only 6–12 h, which lies within the half-life range of other short chain epoxides as ethylene oxide [11,13].

For the biological exposure monitoring of 2-chloroprene analytical procedures for the determination of the specific mercapturic acids do not exist. For the determination of the mercapturic acid of epichlorohydrin, CHPMA, there is only one method available, however, with limited sensitivity [12]. Our study aimed to fill this gap by the development of a suitable LC–MS/MS procedure in combination with online solid phase extraction (SPE). The use of online SPE has already been established for the analytical determination of several biomonitoring parameters [14–17]. The convenient sample cleanup procedure using column switching arrangements is also often applied for the determination of mercapturic acids in human urine [18–21]. Above all, the automation of the sample cleanup and accordingly, the feasible high sample throughput is advantageous for biomonitoring studies. To achieve high sensitivity, online sample cleanup systems are often combined with mass spectrometric detection. Because of similar physical and chemical properties of the mercapturic acids of 2-chloroprene and epichlorohydrin, we developed an analytical procedure for the simultaneous determination of these biomarkers. Thus, the presented method does not only enable further research on human metabolism of these substances but also promises to be a useful tool for a reliable monitoring of occupational exposure.

2. Experimental

2.1. Chemicals

The mercapturic acids CHPMA (N-acetyl-S-(3-chloro-2-hydroxypropyl)-L-cysteine), Cl-MA-I (N-acetyl-S-(4-chloro-3-oxobutyl)-L-cysteine), Cl-MA-II (N-acetyl-S-(4-chloro-3-hydroxybutyl)-L-cysteine), Cl-MA-III (N-acetyl-S-(3-chloro-2-hydroxy-3-butenyl)-L-cysteine) and HOBMA (N-acetyl-S-(4-hydroxy-3-oxobutyl)-L-cysteine) were custom synthesized with a stated purity of at least 95% (Institute for Organic and Biomolecular Chemistry, Göttingen, Germany). DHBMA (N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine, purity 98%) was purchased from Toronto Research Chemicals, Toronto, Canada.

The internal standard substances d_3-CHPMA (N-acetyl-d_3-S-(3-chloro-2-hydroxypropyl)-L-cysteine, purity 98%), d_3-Cl-MA-I (N-acetyl-d_3-S-(4-chloro-3-oxobutyl)-L-cysteine, purity 96%), d_3-Cl-MA-III (N-acetyl-d_3-S-(3-chloro-2-hydroxy-3-butenyl)-L-cysteine, purity 98%) and d_3-HOBMA (N-acetyl-d_3-S-(4-hydroxy-3-oxobutyl)-L-cysteine, purity 98%) were custom synthesized (Institute for Organic and Biomolecular Chemistry, Göttingen, Germany). The internal standard substance d_7-DHBMA (N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine-d_7, purity 98%) was purchased

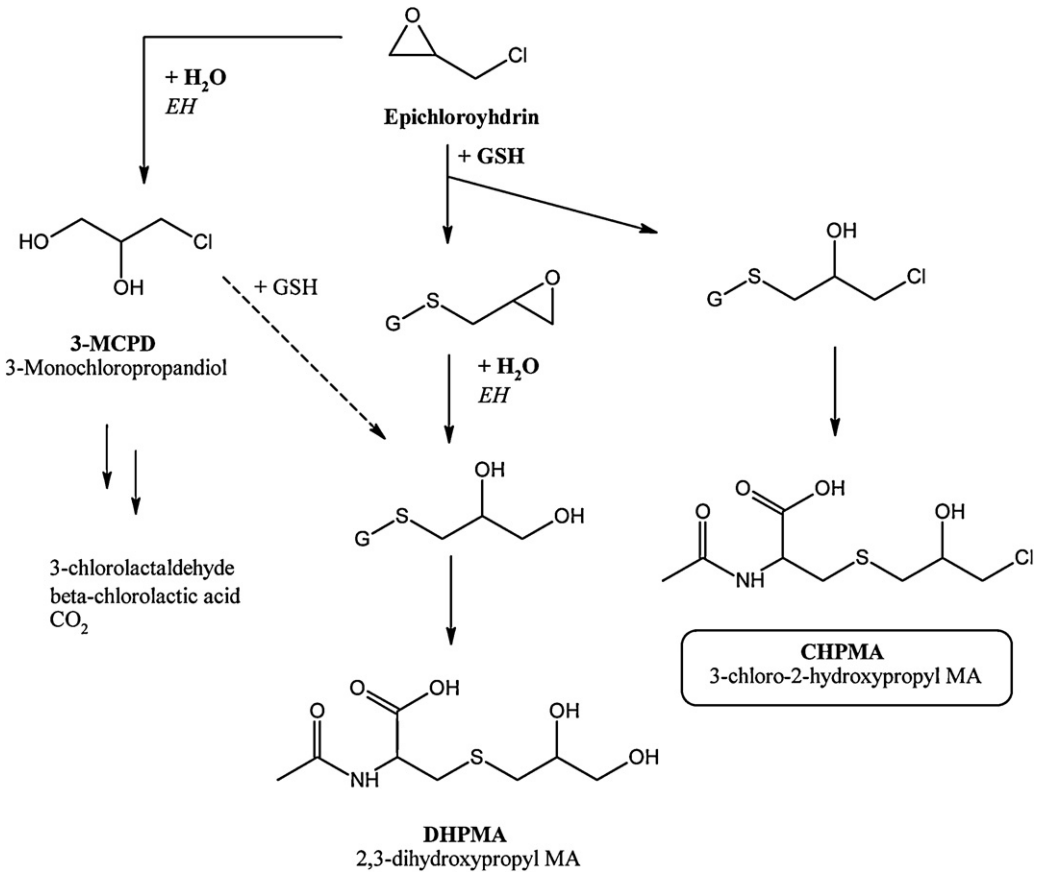

Fig. 2. Proposed metabolism scheme of epichlorohydrin according to Gingell et al. [11], modified (GSH = glutathione; EH = epoxide hydrolase; MA = mercapturic acid).

from Toronto Research Chemicals, Toronto, Canada. Isotopic purity of the labelled mercapturic acids was given to be at least 98%. Identity as well as chemical and isotopic purity of the custom-synthesized standards was confirmed by ^{1}H NMR and LC–MS/MS.

Formic acid (100%), methanol (HPLC grade) and water (HPLC grade) were supplied by Merck (Darmstadt, Germany). Ammonium formiate was supplied by Sigma–Aldrich (Steinheim, Germany).

2.2. Standard preparation

As the examined mercapturic acids are found in different background levels in human urine, the calibration was split into two groups by preparing working solutions with different concentration levels for each group. Stock solutions of the mercapturic acids DHBMA, HOBMA, Cl-MA-I, Cl-MA-II, Cl-MA-III and CHPMA were prepared by dissolving the standard substances in methanol. Working solutions I and II were prepared by dilution of the stock solutions with water. Working solution I contained 25 mg/L each of DHBMA and HOBMA and 5 mg/L each of Cl-MA-I, Cl-MA-II, Cl-MA-III and CHPMA. Concentration levels of 5 mg/L each of DHBMA and HOBMA and 1 mg/L each of Cl-MA-I, Cl-MA-II, Cl-MA-III and CHPMA were contained in working solution II.

Stock solutions of the internal standard substances d_7-DHBMA, d_3-HOBMA, d_3-Cl-MA-I, d_3-Cl-MA-III and d_3-CHPMA were prepared by dissolving the standard substances in methanol.

The stock solutions of the internal standards were diluted with water to obtain a working solution of the internal standards (20 mg/L each of d_7-DHBMA and d_3-HOBMA, 5 mg/L each of d_3-Cl-MA-I, d_3-Cl-MA-III and d_3-CHPMA).

All solutions were stored at −18 °C in teflon-capped glass vials.

2.3. Calibration procedure

Calibration was carried out in pooled urine, which was obtained from non-smoking individuals. Five calibration standards were prepared by spiking pooled urine with different volumes of working solutions I and II to achieve final concentrations of 25–1000 μg/L each of DHBMA and HOBMA and 5–200 μg/L each of Cl-MA-I, Cl-MA-II, Cl-MA-III and CHPMA. Additionally, pooled urine was used as a blank and included in every analytical series. The calibration standards were processed as described in Section 2.4. Linear calibration curves were obtained by plotting the quotients of the peak areas of the analytes to the peak areas of the corresponding labelled standards as a function of the spiked concentration. D_3-Cl-MA-III was used as an internal standard substance for both Cl-MA-III and Cl-MA-II.

2.4. Sample preparation

Urine samples were stored frozen at −18 °C until analysis. Initially, urine samples were thawed, equilibrated to room temperature and vortex-mixed. An aliquot of 1 mL urine was transferred to a 13-mL polyethylene tube followed by the addition of 1 mL ammonium formiate buffer (50 mmol/L, pH 2.5) and 40 μL of formic acid to adjust the pH of the sample to a value nearby pH 2.5. Subsequently, 20 μL of the working solution of the internal standards was added. The sample was vortex-mixed and centrifuged at 2000 × *g* for 10 min. After transfer to a 1.8-mL glass vial, 100 μL of the supernatant was injected into the HPLC system for quantitative analysis.

Table 1
Gradient program for liquid chromatography. Mobile phase A: 0.02% formic acid in water/methanol (88/12, v/v); mobile phase B: 0.02% formic acid in water/methanol (10/90, v/v); mobile phase C: 0.5% aqueous formic acid.

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Flow rate (mL/min)
0.0	100	0	0	0.3
2.0	100	0	0	0.3
5.0	75	25	0	0.3
6.5	50	38	12	0.3
11.5	0	88	12	0.3
13.0	0	88	12	0.3
14.5	12	88	0	0.3
19.0	100	0	0	0.3
30.0	100	0	0	0.3

2.5. LC–MS/MS analysis

Liquid chromatography was performed using a HPLC system model Agilent series 1100 (Agilent, Waldbronn, Germany) including a quaternary pump (Agilent G 1311A), a vacuum degasser (Agilent G 1322A) and an autosampler (Agilent G 1313A). The sample-enrichment and cleanup step was carried out using a column switching method including online-SPE. An additional isocratic pump model Merck-Hitachi L-6000 (Merck, Darmstadt, Germany) was applied to carry the injected sample volume (100 μL) to a restricted access material (RAM) phase (LiChrospher RP-18 ADS 4 × 25 mm, 25 μm from Merck, Darmstadt, Germany) using 0.1% aqueous formic acid at a flow rate of 0.5 mL/min. The retained analytes were subsequently transferred in backflush mode to the analytical reversed phase column (Synergi Max RP C12 150 × 3.0 mm, 4 μm from Phenomenex, Aschaffenburg, Germany) using the quaternary pump system. A time controlled ten-port valve was used to accomplish the described column switching arrangement. An illustration of the configuration of the online-SPE-LC-MS/MS system using a six-port-valve is shown in Fig. S-1 of the supplementary data to this manuscript.

Analytical separation of the mercapturic acids was performed using a ternary gradient system composed of mobile phase A (mixture of 88% water, 12% methanol and 0.02% formic acid), mobile phase B (mixture of 10% water, 90% methanol and 0.02% formic acid) and mobile phase C (0.5% aqueous formic acid) at a flow rate of 0.3 mL/min as shown in Table 1. A precolumn filter (3 mm, 0.5 μm from Supelco, Bellefonte, USA) and a guard column (C12 4 × 3 mm from Phenomenex, Aschaffenburg, Germany) were used to extend the life span of the RAM phase and the analytical column, respectively.

The HPLC was directly coupled to a triple quadrupole mass spectrometer (model Sciex API 2000, Applied Biosystems, Langen, Germany) equipped with an electrospray ionization (ESI) interface. Apart form the isocratic pump, all steps were controlled by the Analyst 1.4 software (Applied Biosystems, Langen, Germany). The MS/MS system was operated in negative ESI mode. The electrospray needle voltage was set at −4500 V in the negative ion mode for all analytes. The turbo heater was maintained at 475 °C. Nitrogen was used as nebulizing gas, turbo heater gas and curtain gas and was set at a pressure of 35, 60 and 30 psi, respectively. The collision gas (nitrogen) for the MS/MS mode was set to a flow rate of 3 instrument units. The mass spectrometer was used in the multiple reaction monitoring mode (MRM). To establish the MS/MS operation conditions a standard solution of each analyte and internal standard substance (10 mg/L in methanol) was injected directly into the MS/MS system by continuous flow injection using a syringe pump. It was possible to optimize two mass transitions for each analyte. The mass transition with the higher intensity was used as quantifier, whereas the mass transition with the lower intensity was used as qualifier. The final settings of MS/MS detection for every single analyte and internal standard substance are summarized in Table 2. Retention times under the described conditions and analyte specific parameters are also shown in Table 2.

2.6. Quality control

Quality control material was prepared by spiking pooled urine (creatinine 0.4 g/L) with different concentration levels of the

Table 2
Retention times and MRM-specific parameters of the analytes and the internal standard substances (DP – declustering potential, FP – focussing potential, EP – entrance potential, CE – collision energy).

Analyte	Retention time (min)	Precursor ion (Q1)	Product ion (Q3)	DP (V)	FP (V)	EP (V)	CE (V)
DHBMA	9.6	250	121	−26	−330	−5.0	−24
			75[a]	−31	−320	−9.5	−30
HOBMA	9.7	248	162	−21	−250	−3.5	−15
			84[a]	−21	−250	−4.0	−25
CHPMA	12.3	254	218	−31	−340	−4.0	−12
			89[a]	−31	−340	−4.0	−25
Cl-MA-I	12.7	266	162	−26	−320	−3.5	−12
			84[a]	−26	−330	−4.0	−25
Cl-MA-II	13.0	268	232	−26	−350	−10	−14
			75[a]	−26	−320	−10	−25
Cl-MA-III	13.5	266	137	−21	−330	−10	−15
			128[a]	−21	−250	−10	−15
d_7-DHBMA	9.5	257	128	−36	−350	−5.5	−24
d_3-HOBMA	9.7	251	165	−31	−230	−7.0	−24
d_3-CHPMA	12.2	257	221	−34	−300	−9.0	−13
d_3-Cl-MA-I	12.7	269	165	−30	−350	−10.5	−11
d_3-Cl-MA-III	13.5	269	137	−51	−210	−11.5	−22

[a] Mass transitions used as qualifier.

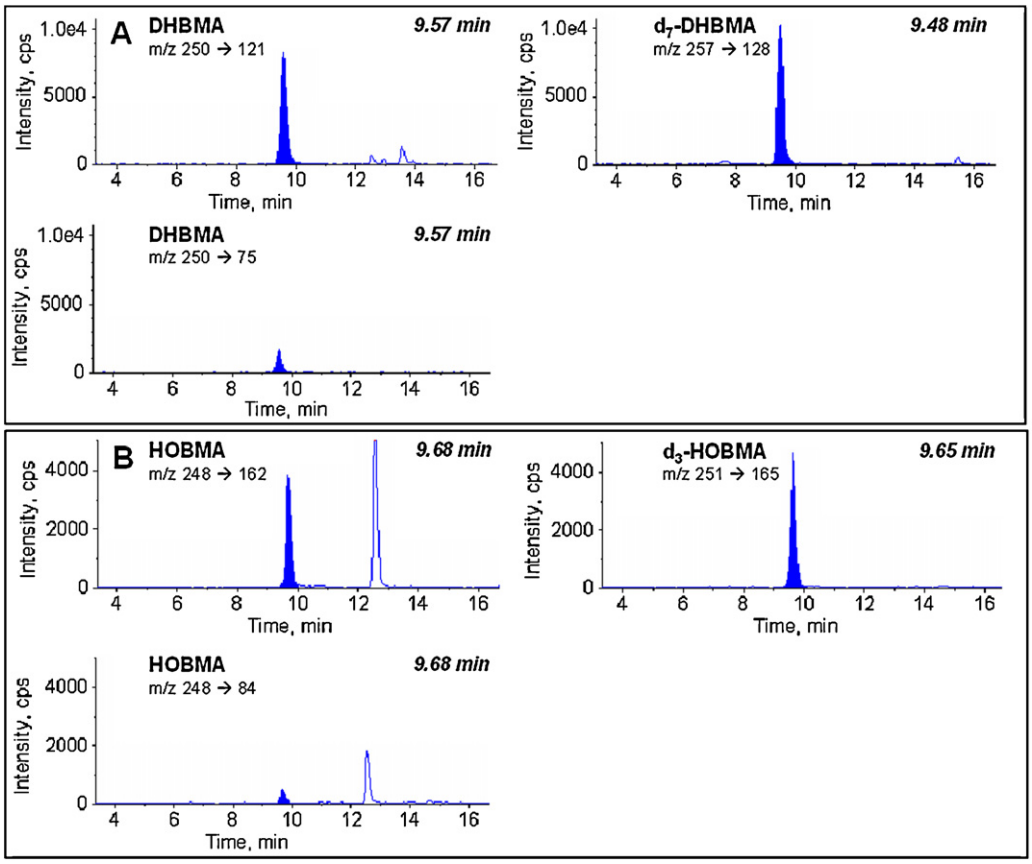

Fig. 3. Chromatogram of a spiked non-smokers urine sample showing the mass transitions of the analytes and internal standards of the analyte group 1. Determined analyte levels were: 180 μg/L of DHBMA (A) and 183 μg/L of HOBMA (B).

mercapturic acids. For a low concentration quality control material (Q_{low}) pooled urine was spiked with 20 μg/L of each mercapturic acid. For preparation of a high concentration quality control material (Q_{high}) pooled urine was spiked separately for both groups of analytes: 500 μg/L each of DHBMA and HOBMA and 100 μg/L each of Cl-MA-I, Cl-MA-II, Cl-MA-III and CHPMA. The quality control material was divided into aliquots of 1 mL and stored at −18 °C. One Q_{low}- and one Q_{high}-sample was analysed during each analytical series.

Mean analyte levels in Q_{low}-quality control material were determined to be 71.5, 47.5, 23.3, 24.6 and 20.9 μg/L of DHBMA, HOBMA, CHPMA, Cl-MA-I and Cl-MA-III, respectively. Mercapturic acid levels in Q_{high}-quality control material were determined to be 562, 535, 116, 108 and 109 μg/L of DHBMA, HOBMA, CHPMA, Cl-MA-I and Cl-MA-III, respectively.

2.7. Validation of the analytical method

The precision of the method was determined using intra- and interday relative standard deviations. Intraday precision was determined by analyzing pooled urine spiked with mercapturic acids at two concentration levels (Q_{low} and Q_{high}-material), each ten times in a row. By analyzing one Q_{low} and one Q_{high}-sample on seven different days, interday precision was determined.

To investigate the effect of various urine compositions, ten different urine samples with creatinine levels ranging from 0.3 to 1.6 g/L were analyzed unspiked and spiked with 100 μg/L of each mercapturic acid. Relative recovery (accuracy) was calculated by subtracting the background level of the respective mercapturic acid obtained by analyzing the blank sample from the analyte concentration in the spiked sample.

Limits of detection (LOD) and limits of quantification (LOQ) were determined by means of a ten-point-calibration in pooled urine according to ISO 8466-1 [22].

3. Results and discussion

3.1. Selection of biomarkers

Based on the in vitro studies of Munter et al. [5,6] the mercapturic acids Cl-MA-I (4-chloro-3-oxobutyl mercapturic acid), Cl-MA-III (3-chloro-2-hydroxy-3-butenyl mercapturic acid) and HOBMA (4-hydroxy-3-oxobutyl mercapturic acid) were selected as biomarkers of exposure to 2-chloroprene. Additionally, Cl-MA-II (4-chloro-3-hydroxybutyl mercapturic acid) and DHBMA (3,4-dihydroxybutyl mercapturic acid) were included as potential metabolic products of an enzymatic reduction of Cl-MA-I and HOBMA, respectively (see Fig. 1).

Furthermore, the mercapturic acid CHPMA was included in the method. CHPMA is known as the main metabolite of epichlorohydrin in urine [11–13]. Following a dehalogenation step, another metabolite of epichlorohydrin is 2,3-dihydroxypropyl mercapturic acid (DHPMA) (see Fig. 2). However, the formation of the chlorine containing mercapturic acid CHPMA is known to be highly favored [11–13]. Recently, we have already developed a suitable biomonitoring method for several mercapturic acids that includes the determination of DHPMA [23]. The application of this method to urine samples of several occupationally non-exposed individuals revealed a comparatively high urinary background level of DHPMA, which could not be linked to a corresponding exposure to glycidol or epichlorohydrin [24]. Consequently, CHPMA promises

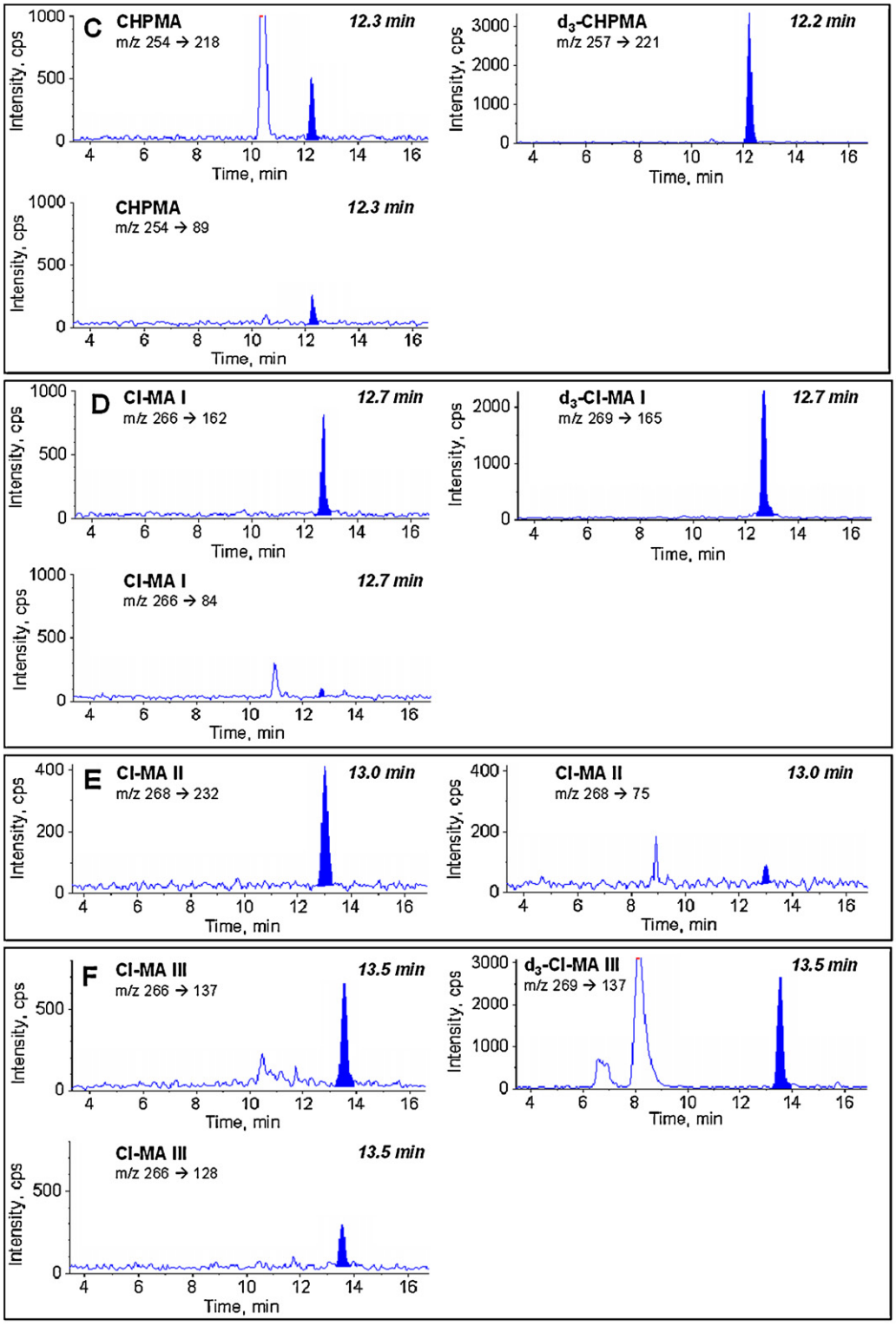

Fig. 4. Chromatogram of a spiked non-smokers urine sample showing the mass transitions of the analytes and internal standards of the analyte group 2. Determined analyte levels were: 26.5 μg/L of CHPMA (C), 30.4 μg/L of Cl-MA-I (D), 50.7 μg/L of Cl-MA-II (E) and 23.6 μg/L of Cl-MA-III (F).

to be a far more suitable and specific biomarker of exposure to epichlorohydrin.

3.2. Optimization of the method

All the analytes could be retained satisfactorily using solid phase extraction on cartridges with stationary phases composed of polystryrol–divinylbenzene-copolymers. However, a higher extraction efficiency was achieved using online enrichment of the mercapturic acids on a restricted access material (RAM) phase. So, the final development of the analytical method was based on the method of Gonzalez-Reche et al. [25] for the determination of the mercapturic acids of xylenes. It was successfully adapted, optimized and finally validated for the determination of the mercapturic acids of 2-chloroprene and epichlorohydrin.

Table 3
Intra- and interday precision data, accuracy, limits of detection (LOD) and limits of quantification (LOQ).[a]

Analyte	LOD/LOQ (μg/L)	c Q_{low} (μg/L)	c Q_{high} (μg/L)	Intraday precision RSD (%)		Interday precision RSD (%)		Accuracy in different matrices (%), (N = 10)	
				Q_{low} (N = 10)	Q_{high} (N = 10)	Q_{low} (N = 7)	Q_{high} (N = 7)	Mean	Range
DHBMA	3.8/12.1	71.5	562	6.5	6.2	11.0	7.3	98.4	77–111
HOBMA	4.2/13.2	47.5	535	10.4	4.7	8.1	8.9	102.8	83–123
CHPMA	2.7/8.7	23.3	116	10.4	7.2	11.3	8.8	99.5	86–112
Cl-MA-I	2.5/8.3	24.6	108	8.5	6.8	11.8	9.4	101.2	84–119
Cl-MA-III	1.4/4.5	20.9	109	7.0	6.7	9.4	7.3	98.9	80–114

[a] Validation data of Cl-MA-II could not be determined due to its instability in aqueous solutions (see Section 3.3).

For optimization of analyte extraction, several enrichment columns (RAM-phases) with different chain lengths of the stationary phase (LiChrospher ADS RP-18, RP-8, RP-4 by Merck, Darmstadt, Germany) were tested for applicability. The chlorine-containing mercapturic acids were retained well on all examined columns, whereas the more polar analytes HOBMA and most notably DHBMA showed a significantly lower affinity towards the stationary phases. A satisfactory enrichment of these mercapturic acids could only be achieved using a RP-18 RAM phase. Still, the loss of HOBMA and DHBMA was significant even when using a purely aqueous mobile phase for enrichment. As a feasible compromise between effective separation of matrix compounds and minimizing losses of the polar analytes, the enrichment time on the RAM phase in this method was limited to 1.5 min. The enrichment time can be extended to 3–4 min, if only the chlorine containing analytes are to be determined. If, however, the analysis of DHBMA and HOBMA has priority, a further reduction of the enrichment time may be indicated.

For optimization of the analytical separation of the mercapturic acids we examined different HPLC columns including reversed phase (RP) and hydrophilic interaction chromatography (HILIC) columns. The lower polarity of the chlorine containing metabolites led to a short retention time associated with inferior matrix separation on the examined HILIC columns. The use of a RP column with medium chain length of the stationary phase (Synergi Max RP C12 150 × 3.0 mm, 4 μm from Phenomenex, Aschaffemburg, Germany) resulted in a good separation of all analytes even of those with very high polarity, like HOBMA and DHBMA. Particularly in conjunction with the applicability for online enrichment on a RAM phase, the analytical separation using a RP column proved to be highly advantageous.

Regarding analytical separation and MS/MS detection, it turned out that the amount of the acid concentration in the mobile phase had a significant influence not only on analyte signal intensities but also on reproducibility of the retention times. To guarantee a high sensitivity for the polar analytes HOBMA and DHBMA as well as a stable retention of the chlorine containing mercapturic acids, the presented method applies a pH gradient for analytical separation. At first, HOBMA and DHBMA are eluted with 0.02% formic acid in the mobile phase followed by an increase of the acid concentration to 0.08% formic acid for elution of the chlorine containing analytes.

All in all, run time of the method adds up to only 30 min leading to a rapid, sensitive and reliable simultaneous determination of the presented mercapturic acids as metabolites of 2-chloroprene and epichlorohydrin.

A chromatogram of a spiked non-smokers urine sample is shown in Figs. 3 and 4. Identity of the peaks was additionally checked by calculating the area ratio of qualifier and quantifier mass transition (see Table 2) for every analyte. An isotopically labelled specific internal standard substance was used for every analyte, but for Cl-MA-II. As Cl-MA-II proved to be instable in water and urine (see Section 3.3), we did not purchase a specific internal standard substance for this mercapturic acid. Instead, d_3-Cl-MA-III was used as an internal standard for both, Cl-MA-III and Cl-MA-II.

3.3. Reliability of the method

The mercapturic acid Cl-MA-II was stable in methanolic solutions, but not in aqueous solutions or urine. In the latter, we observed a rapid reduction of spiked Cl-MA-II levels within a few hours, particularly when stored at room temperature (e.g. in the autosampler). Hence, Cl-MA-II is obviously not a suitable biomarker for determination in urine.

Due to this instability, we could not gather any validation data for this mercapturic acid. Thus, the following validation data is related exclusively to the five mercapturic acids HOBMA, DHBMA, Cl-MA-I, Cl-MA-III and CHPMA.

The coefficients of correlation of the calibration curves were higher than r = 0.995 for all analytes. These graphs were used to ascertain the unknown concentration of the mercapturic acids in urine samples. Intraday precision was determined by analysis of Q_{low} and Q_{high}-material (see Section 2.6) and ranged from 6.5 to 10.4% and from 4.7 to 7.2%, respectively.

As expected, slightly higher results were found for interday precision, which ranged from 8.1 to 11.8% relative standard deviation for Q_{low}-material. For analysis of Q_{high}-material similar results were found with relative standard deviations of 7.3–9.4%.

Additionally, the effect of various urinary matrices was investigated. Accuracy was found to be very satisfactory with a mean relative recovery of 98% for DHBMA to 103% for HOBMA. Consequently, the use of labelled internal standards proved to be very efficient for compensation of influences due to different urinary matrices. The limits of detection (LOD) and the limits of quantification (LOQ) were determined according to ISO 8466-1 [22]. LOD were found to range between 1.4 μg/L for Cl-MA-III and 4.2 μg/L for HOBMA. Detailed validation results for every single mercapturic acid along with the respective LOD and LOQ are summarized in Table 3.

Additionally, the presented method was submitted to and approved by the DFG working group “Analyses in biological materials”. The working group is part of the Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area and deals with the development and examination of human biomonitoring methods [26].

During this process, the practicability and validity of the method was confirmed by two external laboratories that successfully reproduced the presented analytical method. Moreover, the resulting examination reports did confirm the above given validation data in full. All in all, this proves the excellent suitability and reliability of the presented analytical method in order to be used in routine human biomonitoring applications.

4. Conclusion

An analytical method was developed for the determination of the urinary metabolites of the chemical agents 2-chloroprene and epichlorohydrin that are widely used as monomers in industrial production of synthetic rubbers and epoxy resins, respectively.

However, suitable methods for the determination of biomarkers of exposure to these alkylating agents have been missing. The presented biomonitoring method allows the determination of CHPMA as mercapturic acid of epichlorohydrin as well as the determination of five potential mercapturic acids of 2-chloroprene (Cl-MA-I, Cl-MA-II, Cl-MA-III, HOBMA, DHBMA). It applies online-SPE-HPLC-MS/MS analysis and is characterized by a robust chromatography, high sensitivity and reproducibility. Determination was accomplished using isotopically labelled internal standards of the analytes (except for Cl-MA-II), which proved to be very efficient in order to compensate matrix related effects as well as analyte losses due to sample preparation. Herewith, it is possible, for the first time, to determine the biomarkers Cl-MA-I, Cl-MA-II, Cl-MA-III and HOBMA in human biomonitoring studies. The presented method promises to be a useful tool in occupational medicine as well as for exploratory research studies on human chloroprene metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.01.032.

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