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Determination of six hydroxyalkyl mercapturic acids in human urine using hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC–ESI-MS/MS)☆

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ABSTRACT

Mercapturic acids are increasingly used as biomarkers for exposure to certain carcinogenic substances. Glycidol, ethylene oxide, propylene oxide, acrolein and 1,3-butadiene are important intermediates of toxicological concern used in the industrial production of various chemicals. The main urinary metabolites of these alkylating substances are hydroxyalkyl mercapturic acids. Therefore, we developed and validated an analytical method for the simultaneous determination of six hydroxyalkyl mercapturic acids in human urine after solid phase extraction. The mercapturic acids were separated using hydrophilic interaction liquid chromatography (HILIC) and quantified by tandem mass spectrometry using isotopically labelled internal standards. The developed method enables for the first time the determination of 2,3-dihydroxypropyl mercapturic acid (DHPMA), a metabolite of glycidol, in human urine. Additionally, the mercapturic acids of ethylene oxide (hydroxyethyl mercapturic acid, HEMA), propylene oxide (2-hydroxypropyl mercapturic acid, 2-HPMA), acrolein (3-hydroxypropyl mercapturic acid, 3-HPMA) as well as of 1,3-butadiene(3,4-dihydroxybutyl mercapturic acid, DHBMA and monohydroxy-3-butenyl mercapturic acid, MHBMA) can be determined. The limits of detection range from 3.0 to 7.0 μg/L. Intra- and inter-day precision was determined to range from 1% to 9%. Due to the good accuracy and precision and the low limits of detection the developed method is well suited for the determination of occupational exposure to alkylating substances as well as for the determination of background concentrations of the respective mercapturic acids in the general population.

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1. Introduction

A lot of highly electrophilic substances are used in chemical and industrial processes. Whereas their reactivity is crucial for their appliance, this property may be critical from the toxicological point of view. In general, these substances react easily with the nucleophilic sites of biological molecules like DNA, proteins and amino acids. An interesting group of products of these reactions are mercapturic acids. Their formation is introduced by enzymatic or spontaneous conjugation of endogenous glutathione with electrophilic substances [1]. Due to the fact that the chemical structure of mercapturic acids depends on the respective parent compound and that the conjugates usually show a relatively short half-life, mercapturic acids are increasingly used as specific biomarkers for acute exposure to electrophilic substances [2,3].

Important examples of the reactive chemicals, for which the determination of mercapturic acids is used or could be used for biological monitoring are ethylene oxide, propylene oxide, acrolein, butadiene and glycidol.

Ethylene oxide is a high production volume chemical, which was produced in an amount of over 17 million tons in 2004 [4]. As the smallest epoxide, ethylene oxide is an important intermediate in the production of various chemicals like ethylene glycol, polyethylene glycols, polyesters (like polyethylene terephthalate—PET) and ethanolamines. Ethylene oxide is also frequently used for the sterilization of medical devices [4]. A metabolic precursor of ethylene oxide is ethylene, which is formed endogenously [5,6]. Occupational exposure with ethylene oxide mainly occurs during the production of ethylene oxide and its derivatives as well as during operations using ethylene oxide as a gaseous sterilant. Ethylene oxide is also found in cigarette smoke in low concentrations. As a direct alkylating agent ethylene oxide readily reacts with proteins and DNA and was classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (Group 1) [4]. Metabolism of ethylene oxide leads to the urinary excretion of hydroxyethyl mercapturic acid (HEMA, see Fig. 1), which is also a minor metabolite of other volatile substances of toxicological con-

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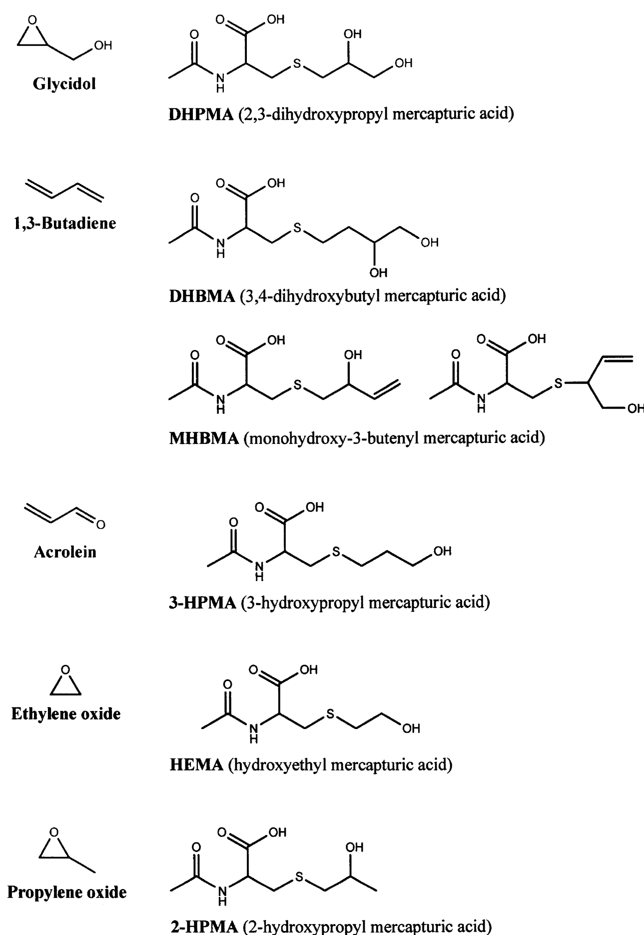

Fig. 1. Chemical structures of alkylating substances and their corresponding mercapturic acids.

cern like vinyl chloride or ethylene dibromide [7,8]. Background levels of HEMA in human urine are reported to be in the lower μg/L-range [7,9].

Analogous to ethylene oxide the homologue propylene oxide is mainly used as a reactive intermediate for the production of various chemicals like polyether polyols, propylene glycols and propylene glycol ethers. Worldwide production capacity of propylene oxide covered almost 4 million tons in 1991 [10]. Apart from occupational exposure, propylene oxide is also an environmental pollutant. Another source of exposure is tobacco smoke that contains propylene, a metabolic precursor of propylene oxide [10,11]. The IARC has classified the compound to be possibly carcinogenic to humans (Group 2B) [10]. Due to the fact that propylene oxide is a directly alkylating agent, exposure to this compound has so far been monitored using the hemoglobin adduct of propylene oxide [12–14]. Until now, the determination of the mercapturic acid, 2-hydroxypropyl mercapturic acid (2-HPMA, see Fig. 1) has been described in only one paper [9].

Acrolein is produced in an amount of several thousand tons per year and is mainly used as an intermediate in the production of acrylic acid and for the synthesis of the essential amino acid methionine [15]. Furthermore, acrolein is present at low concentrations as a natural ingredient in several foods, it occurs in automobile exhaust gases and industrial emissions and it is also formed endogenously. Another important source of acrolein is tobacco smoke [15,16]. Exposure to acrolein can therefore occur in several ways. Based on a potential carcinogenic effect [17], acrolein was classified by the Deutsche Forschungsgemeinschaft (DFG) as Group 3B carcinogen [18]. The IARC evaluated acrolein in 1995 as Group 3 carcinogen (not classifiable as to carcinogenicity to humans) due to inadequate evidence [15]. Conjugation of acrolein with glutathione leads to the excretion of 3-hydroxypropyl mercapturic acid (3-HPMA, see Fig. 1), the main metabolite of acrolein found in human urine [16,19]. Determination of 3-HPMA was already described in various reports on the background concentration of this mercapturic acid in urine of occupationally non-exposed individuals [9,17,20].

Another high production chemical is 1,3-butadiene with an annual production volume of over 9 million tons in 2005 [4]. Butadiene is used primarily to manufacture synthetic rubbers and polymers. Environmental sources of butadiene are automobile exhausts, factory fumes and tobacco smoke, hence butadiene is considered as a ubiquitous environmental pollutant [4]. Since there is sufficient evidence for the carcinogenicity of 1,3-butadiene in humans, the IARC and the DFG have classified 1,3-butadiene as Group 1 carcinogen (carcinogenic to humans) [4,18]. Metabolism of butadiene leads to the excretion of two mercapturic acids in urine: 3,4-dihydroxybutyl mercapturic acid (DHBMA) and monohydroxy-3-butenyl mercapturic acid (MHBMA), whereas the latter consists of two isomeric forms (see Fig. 1) [21–25]. In human urine DHBMA is usually found in higher concentrations than MHBMA [26,27]. MHBMA is considered a more specific biomarker of butadiene exposure due to its lower background level [23].

Glycidol is also a highly reactive compound that is increasingly used as a chemical intermediate for pharmaceuticals as well as for the production of functional epoxides. Furthermore, glycidol is used as a reactive diluent in epoxy resins and as an additive for synthetic hydraulic fluids [28,29]. Occupational exposure to glycidol may therefore occur during its production and use. It is also reported that tobacco smoke contains traces of glycidol [30]. Glycidol is a directly alkylating agent that was found to be genotoxic *in vitro* and *in vivo* and has been classified by the IARC as probably carcinogenic to humans (Group 2A) [28]. The major metabolites of glycidol identified in the urine of rats were 2,3-dihydroxypropyl cysteine and the corresponding mercapturic acid N-acetyl-S-(2,3-dihydroxypropyl) cysteine (DHPMA, see Fig. 1) [31]. However, to our knowledge the determination of the mercapturic acid of glycidol in human urine has not yet been reported in literature.

The metabolism of all these chemicals result in the formation of structurally related mercapturic acids (see Fig. 1). Therefore, it was the aim of our study to develop and validate an analytical method for the simultaneous determination of the corresponding hydroxyalkyl mercapturic acids (DHPMA, DHBMA, MHBMA, 2-HPMA, 3-HPMA and HEMA) in human urine.

2. Experimental

2.1. Chemicals

The mercapturic acids HEMA (N-acetyl-S-(2-hydroxyethyl)-L-cysteine, purity 98%), 2-HPMA (N-acetyl-S-(2-hydroxypropyl)-cysteine, dicyclohexylammonium salt, purity 98%), 3-HPMA (N-acetyl-S-(3-hydroxypropyl)cysteine, dicyclohexylammonium salt, purity 98%), DHBMA (N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine, purity 98%) and MHBMA (mixture of N-acetyl-S-1-(hydroxymethyl-2-propenyl)-L-cysteine and N-acetyl-S-2-(hydroxymethyl-3-propenyl)-L-cysteine, purity 98%) were purchased from Toronto Research Chemicals, Toronto, Canada.

The internal standards d_4-HEMA (N-acetyl-S-(2-hydroxyethyl-d_4)-L-cysteine, purity 98%), d_3-3-HPMA (N-acetyl-d_3-S-(3-hydroxypropyl)cysteine, dicyclohexylammonium salt, purity 98%), d_7-DHBMA (N-acetyl-S-(3,4-dihydroxybutyl)-

L-cysteine-d_7, purity 98%) and d_6-MHBMA (mixture of N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine-d_6 and N-acetyl-S-(2-hydroxymethyl-3-propenyl)-L-cysteine-d_6, purity 98%) were also purchased from Toronto Research Chemicals, Toronto, Canada. The internal standard $^{13}C_2$-DHPMA ((R)-2-(1,2-^{13}C)-N-acetyl-S-(2,3-dihydroxypropyl)-L-cysteine, purity >95%) was custom-synthesized by ChiroBlock GmbH (Wolfen, Germany). Isotopic purity of the labelled mercapturic acids was given to be at least 96%.

3-Chloropropane-1,2-diol (98%), triethylamine (>99%), dicyclohexylamine (p.a.) and L-cysteine hydrochloride (anhydrous, >98%) were obtained from Sigma–Aldrich (Taufkirchen, Germany). Formic acid (100%), acetic acid (100%), ammonium acetate (p.a.), acetic anhydride (p.a.) and ethyl acetate (p.a.) were supplied by Merck (Darmstadt, Germany) as well as acetonitrile, methanol and water (all HPLC grade). Ammonium formate was supplied by Fluka (Taufkirchen, Germany). Columns for solid phase extraction (ENV+, 100 mg, 3 mL) were purchased from Biotage (Grenzach-Wyhlen, Germany).

2.2. Synthesis of N-acetyl-S-(2,3-dihydroxypropyl)cysteine (DHPMA)

The mercapturic acid DHPMA (N-acetyl-S-(2,3-dihydroxypropyl)cysteine) was synthesized as described previously with slight modifications [31]. Briefly, triethylamine (6 g, 59 mmol) and L-cysteine hydrochloride (3 g, 19 mmol) were dissolved in 30 mL water and 3-chloropropane-1,2-diol (3 g, 27 mmol) was added dropwise. The mixture was stirred at room temperature for 2 days and was subsequently evaporated to dryness using a centrifugation evaporator (Speed Vac). The residue was refluxed with 100 mL methanol and after filtration the residue was mixed with 10 mL water. Insoluble material was removed by filtering and the aqueous solution was added to 300 mL of acetone. The resulting precipitate was purified by several reprecipitations from water with acetone. 200 mg of the resulting product was dissolved in water (2 mL) and 1 mL of acetic anhydride was added. The solution was kept at 30 °C for about 17 h and was then evaporated to dryness at 40 °C using a Speed Vac. The oily residue was dissolved in ethyl acetate (20 mL) and crystallized as the dicyclohexylammonium salt by adding a solution of 0.5 mL dicyclohexylamine in 5 mL ethyl acetate. The mixture was kept at 0 °C for 24 h. The precipitated dicyclohexylammonium salt of N-acetyl-S-(2,3-dihydroxypropyl)cysteine was recrystallized from methanol and ethyl acetate. Identity was confirmed by mass spectrometry and ^{1}H NMR. Purity was estimated to be >95%.

2.3. Instrumentation

HPLC was performed using an HPLC system model Agilent series 1100 including a quaternary pump (Agilent G 1311A), a vacuum degasser (Agilent G 1322A) and an autosampler (Agilent G 1313A). 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 and a 10-port valve.

2.4. Standard preparation

Stock solutions of the mercapturic acids 3-HPMA, 2-HPMA, DHPMA, DHBMA, HEMA and MHBMA (1 g/L each) were obtained by dissolving the standard substances in methanol. As a result of different background concentrations of the examined mercapturic acids in human urine, it was necessary to split the calibration range into two groups of mercapturic acids by preparing working solutions with different concentrations for each group. Therefore, the stock solutions of the analytes were diluted with water to obtain working solution I (50 mg/L of 3-HPMA, DHPMA and DHBMA and 10 mg/L of 2-HPMA, HEMA and MHBMA, respectively) and working solution II (10 mg/L of 3-HPMA, DHPMA and DHBMA and 2 mg/L of 2-HPMA, HEMA and MHBMA, respectively).

Stock solutions of the internal standards d_7-DHBMA, d_6-MHBMA, d_4-HEMA, d_3-3-HPMA and $^{13}C_2$-DHPMA (200 mg/L) 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 (15 mg/L of d_7-DHBMA, d_6-MHBMA and $^{13}C_2$-DHPMA, 5 mg/L of d_4-HEMA and d_3-3-HPMA).

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

2.5. 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 of DHBMA, DHPMA and 3-HPMA as well as 5–200 μg/L of 2-HPMA, HEMA and MHBMA. Additionally, pooled urine was used as a blank and included in every analytical series. The standards were processed as described in Section 2.6. 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_3-3-HPMA was used as an internal standard for 3-HPMA and 2-HPMA, since there was no labelled analogue for 2-HPMA available.

2.6. 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 2 mL was transferred to a 13-mL polyethylene tube followed by the addition of 2 mL ammonium formate buffer (50 mmol/L, pH 2.5) and 40 μL of formic acid to adjust the acidity of the samples to a pH value of about 2.5. Subsequently, 30 μL of the working solution of the internal standards was added to the samples. The samples were vortex-mixed and centrifuged at 2000 × *g* for 5 min. 4 mL of the supernatant was then passed through an SPE column (Isolute ENV+, 100 mg, 3 mL), pre-conditioned with 6 mL methanol and 6 mL aqueous formic acid pH 2.5. The flow rate through the SPE-columns was maintained at about 1drop/s. The cartridges were then washed with 3 mL of aqueous formic acid pH 2.5 and 1.5 mL of 5% methanol in aqueous formic acid pH 2.5 (v/v). The cartridges were then completely dried by connecting the SPE station to a vacuum pump. Elution of the analytes into 10-mL glass vials was carried out using 2.5 mL of 2% formic acid in methanol (v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen at 50 °C. The residue was reconstituted in 1 mL of solvent A (see Section 2.7.) and then the solution was transferred to 1.5-mL glass vials. The vials were then centrifuged at 2000 × *g* for 10 min and 15 μL of the supernatant was injected into the LC–MS/MS-system for quantitative analysis.

2.7. LC–MS/MS analysis

In order to achieve sensitive detection of each mercapturic acid it is generally necessary to optimize the MS/MS parameters for every single analyte and internal standard. Therefore, a solution of each analyte and labelled standard in methanol (10 mg/L) was injected directly into the MS/MS-system by continuous infusion via a syringe pump. Compound specific MS parameters were optimized automatically by the Quantitative Optimisation Wizard of the Sciex Analyst™ software. The precursor ion of each

Table 1
Liquid chromatography conditions. Solvent A: 5 mM ammonium acetate pH 4.5 in acetonitrile/water (88/12, v/v); solvent B: 5 mM ammonium acetate pH 4.5 in acetonitrile/water (5/95, v/v).

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (mL/min)
0	100	0	0.3
8.0	100	0	0.3
10.0	65	35	0.3
10.5	65	35	0.4
15.0	65	35	0.4
17.0	100	0	0.4
17.5	100	0	0.3
22.0	100	0	0.3

analyte and internal standard was a $[M-H]^-$ ion in the first quadrupole, as ESI was used in the negative mode. The product ions were selected as the precursor-ion fragments with the highest intensities.

LC separation was carried out using a column for hydrophilic interaction liquid chromatography (XBridge HILIC, 3.5 μm particle size, 2.1 mm × 150 mm, Waters, MA, USA) and a corresponding pre-column (HILIC, 2.1 mm × 10 mm). Chromatography of the mercapturic acids was performed isocratically using 5 mmol/L ammonium acetate pH 4.5 in a mixture of 88% acetonitrile and 12% water (solvent A) at a constant flow rate of 0.3 mL/min. To prevent the MS/MS-system from avoidable contamination, only the analyte-containing fraction that eluted during the runtime of 3–9 min was injected into the detector using a time controlled 10-port valve. The complete solvent program is shown in Table 1. All steps were controlled by Analyst 1.3 software (Applied Biosystems).

The MS/MS-system was operated in negative electrospray ionization mode (ESI). The electrospray needle voltage was set at −4500 V in the negative ion mode. 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 of three instrument units. The mass spectrometer was used in the multiple reaction monitoring mode (MRM). Retention times under the described conditions and analyte specific parameters are shown in Table 2.

2.8. Quality control

Quality control material was prepared by spiking pooled urine (creatinine 0.4 g/L) with different concentrations of mercapturic acids. For a low concentration quality control material (Q_{low}) and a high concentration quality control material (Q_{high}) pooled urine was spiked with 25 and 250 μg/L of each mercapturic acid, respectively. The quality control material was divided into aliquots of 2 mL and stored at −18 °C. One Q_{low}- and one Q_{high}-sample were analysed during each analytical series.

2.9. Validation of the analytical method

The precision of the method was determined using intra- and inter-day relative standard deviations. Intra-day precision was determined by analyzing pooled urine spiked with mercapturic acids at two concentration levels, 25 and 250 μg/L (Q_{low}- and Q_{high}-material), each seven times in a row. By analyzing one Q_{low}- and one Q_{high}-sample on ten different days, inter-day precision was determined.

To investigate the effect of various urine compositions, ten different urine samples with creatinine levels ranging from 0.4 to 2.7 g/L were analyzed unspiked and spiked with 100 μg/L of each mercapturic acid. Relative recovery (accuracy) was calculated by subtracting the background concentration of the respective mercapturic acid obtained by analyzing the blank sample from the analyte concentration in the spiked sample.

Limits of detection were determined by means of a ten-point-calibration in pooled urine according to DIN 32 645 [32]

3. Results and discussion

3.1. Optimization of the method

3.1.1. Mass spectrometry

In contrast to the monohydroxyalkyl mercapturic acids the mass spectra of the two dihydroxyalkyl mercapturic acids (DHPMA and DHBMA) showed two product ions with comparatively high intensities (see Fig. 2). For these two analytes it was therefore possible to use two mass transitions for detection. The mass transition with the higher intensity was used as a quantifier, whereas the mass transition with lower intensity was used as a qualifier. The final settings of the MS/MS detection for every single analyte and internal standard are summarized in Table 2.

3.1.2. Chromatography

Mercapturic acids, especially hydroxyalkyl mercapturic acids are highly polar metabolites. It is therefore difficult to retain these substances on traditional reversed phases (RP). In previous works chromatographic separation of mercapturic acids was predominantly achieved by using more polar RP-columns like "polar embedded" stationary phases or stationary phases with shorter chain lengths (C8-columns) [9,17,20,24,27,33]. Separation on these columns was mostly realized by using high amounts of aqueous mobile phase and a low amount of organic solvents. The use

Table 2
Retention times and MRM-specific parameters of the analytes and their internal standards.

Analyte	Retention time (min)	Precursor ion (Q1)	Product ion (Q3)	DP (V)	FP (V)	EP (V)	CE (V)
MHBMA	4.5	232	103	−21	−340	−10	−20
d_6-MHBMA	4.3 5.1	238	109	−41	−210	−11	−22
2-HPMA	5.3	220	91	−31	−230	−7	−16
3-HPMA	5.6	220	91	−31	−230	−7	−16
d_3-3-HPMA	5.6	223	91	−26	−300	−8	−18
HEMA	5.5	206	77	−26	−320	−10	−20
d_4-HEMA	5.6	210	81	−26	−310	−10	−22
DHPMA quant.	6.8	236	107	−26	−220	−10	−18
DHPMA qual.	6.8	236	89	−26	−280	−10	−28
$^{13}C_2$-DHPMA	6.8	238	107	−26	−310	−9	−20
DHBMA quant.	7.1	250	121	−26	−330	−5	−24
DHBMA qual.	7.1	250	75	−31	−320	−9	−30
d_7-DHBMA	7.2	257	128	−36	−350	−6	−24

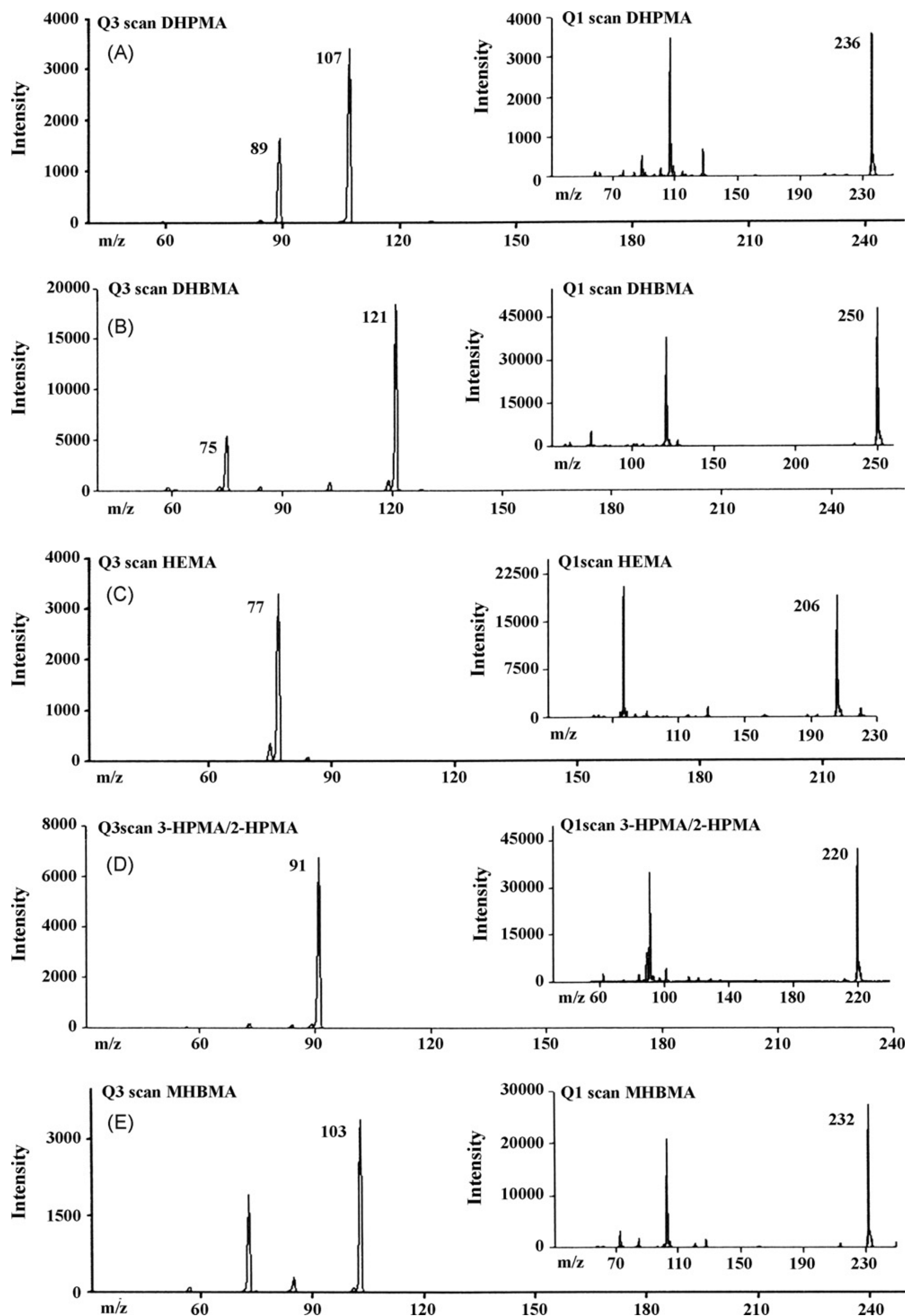

Fig. 2. Mass spectra (Q1 scan) and product ion scans (Q3 scan) of DHPMA (A), DHBMA (B), HEMA (C), 3-HPMA and 2-HPMA (D) and MHBMA (E).

of hydrophilic interaction liquid chromatography (HILIC) offers a useful alternative to RP-columns for the separation of polar compounds. In HILIC, the mobile phase usually consists of a mixture of acetonitrile and aqueous buffer, whereas acetonitrile is used at a rate of 70–90% [34]. It is reported that the high organic content of the solvent may significantly increase the sensitivity in ESI-MS detection [34,35]. In comparison to common RP-columns [17,24,36] best peak shapes, highest intensities and a significantly better separation from urinary interferences for the studied mercapturic acids were obtained on a HILIC column. Retention of the analytes on a HILIC column is strongly dependent on chromatographic conditions like the level of acetonitrile in mobile phase, used buffer-type, its concentration and pH-value. On that account, it is reported that separation in HILIC leads to shifts of analyte retention times and general difficulties with reproducibility when using HILIC with gradient elution [37,38]. The present method applies isocratic elution of the hydroxyalkyl mercapturic acids with 88% of acetonitrile. After the elution of the analytes, the column is rinsed with a decreased percentage of acetonitrile to remove hydrophilic material followed by adequate column equilibration. Under these conditions no inconvenient shifts of analyte retention times were observed even when different urine matrices were applied. With a total run time of 22 min the method enables a rapid, sensitive and reliable simultaneous determination of six hydroxyalkyl mercapturic acids in human urine.

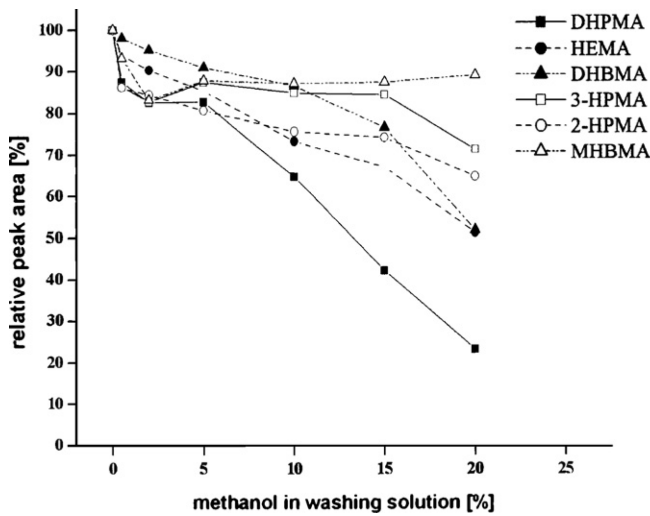

Fig. 3. Relative peak areas of the mercapturic acids in relation to the percentage of methanol in the washing solution used during solid phase extraction.

3.1.3. Solid phase extraction

Sample preparation was accomplished using ENV+ cartridges for solid phase extraction (SPE). ENV+ cartridges are most suitable for the extraction of very polar analytes from aqueous matrices. The good ability of these cartridges to retain mercapturic acids was already reported in previous works [9,20,33]. Our goal was the optimization of the sample preparation to achieve a good retention of the analytes on SPE-material and a satisfying separation of the urinary matrix simultaneously. It was observed that the use of a washing solution with 5% methanol results in the removal of a high portion of interfering urinary matrix. At the same time recovery of the mercapturic acids was hardly affected (see Fig. 3).

Extraction rates of the analytes were determined by comparing the peak areas of pooled urine spiked with 250 μg/L of each mercapturic acid before and after SPE. Analyte losses due to sample preparation with solid phase extraction were determined to be lower than 35% for each analyte. As expected, best retention was found for MHBMA as the least polar analyte with an extraction rate of 81%. Extraction rates of HEMA, 2-HPMA, 3-HPMA, DHPMA and DHBMA were determined to be 74%, 76%, 68%, 65% and 67%, respectively. Thus it becomes apparent, that ENV+ cartridges show excellent retention even of highly polar mercapturic acids as DHPMA and DHBMA. Nevertheless, it turned out to be of exceptional importance to dry the SPE-cartridges thoroughly before the

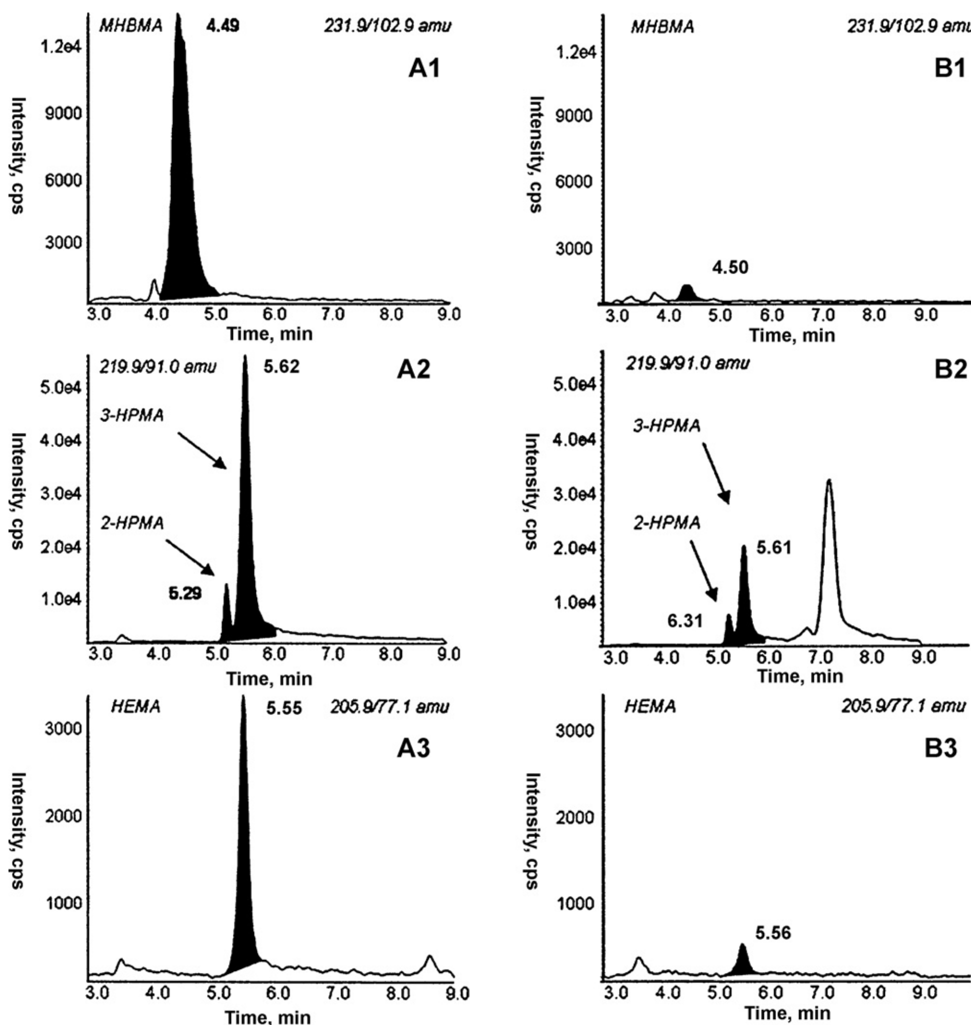

Fig. 4. Chromatogram of a smoker urine sample (A) and a non-smoker urine sample (B), showing the mass transitions of the monohydroxyalkyl mercapturic acids. Determined analyte concentrations were: 115.3 μg/L MHBMA (A1) and 7.1 μg/L MHBMA (B1); 55.6 μg/L 2-HPMA, 432.6 μg/L 3-HPMA (A2) and 31.8 μg/L 2-HPMA, 141.1 μg/L 3-HPMA (B2); 55.7 μg/L HEMA (A3) and 4.2 μg/L HEMA (B3).

Table 3
Intra- and inter-day precision data, accuracy and limits of detection.

Analyte	LOD (μg/L)	Conc. Q_{low} (μg/L)	Conc. Q_{high} (μg/L)	Intra-day precision RSD (%)		Inter-day precision RSD (%)		Accuracy in different samples (%), (N = 10)	
				Q_{low} (N = 7)	Q_{high} (N = 7)	Q_{low} (N = 10)	Q_{high} (N = 10)	Mean	Range
DHPMA	5.5	109	341	3.4	3.3	3.7	4.6	102	88–136
HEMA	4.0	25	250	6.3	0.9	6.9	4.0	96	88–107
2-HPMA	7.0	36	283	6.0	6.0	9.0	9.2	60	24–83
3-HPMA	3.0	71	318	2.0	3.6	5.3	3.3	99	91–110
DHBMA	4.5	115	340	3.8	5.0	5.2	4.5	101	82–121
MHBMA	5.0	31	278	9.0	7.0	5.8	4.6	110	96–125

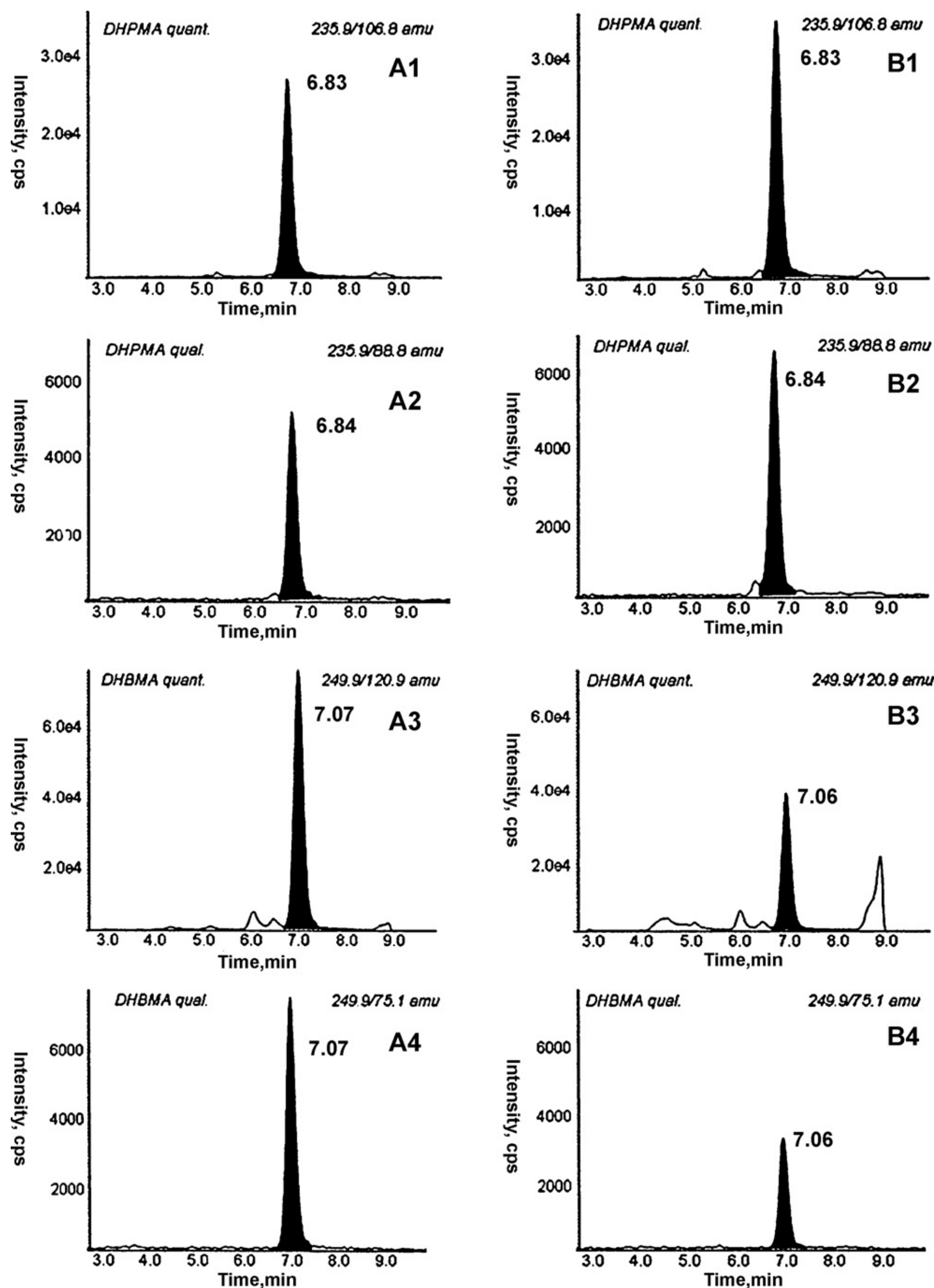

Fig. 5. Chromatogram of a smoker urine sample (A) and a non-smoker urine sample (B), showing the mass transitions of the dihydroxyalkyl mercapturic acids DHPMA m/z 236 → 107 (A1 and B1) and DHBMA m/z 249 → 120 (A3 and B3) as well as the second mass transition used as a qualifier: DHPMA m/z 236 → 89 (A2 and B2) and DHBMA m/z 249 → 75 (A4 and B4). Determined analyte concentrations were: 356.7 μg/L DHPMA (A1) and 432.6 μg/L DHPMA (B1); 364.4 μg/L DHBMA (A3) and 222.5 μg/L DHBMA (B3).

elution of the analytes takes place. Due to the fact, that in HILIC the mobile phase contains only low amounts of water, excessive water in the sample has to be removed carefully.

3.2. Reliability of the method

The coefficients of correlation for all calibration curves were higher than $r = 0.998$ for all analytes. These graphs were used to ascertain the unknown concentration of the mercapturic acids in urine samples. Intra-day precision was determined by analysis of pooled urine spiked with 25 and 250 μg/L (Q_{low}- and Q_{high}-material), respectively. Due to background concentrations of mercapturic acids in pooled urine the actual analyte concentrations were slightly higher (see also summarized results in Table 3). Relative standard deviation for Q_{low}- and Q_{high}-material ranged from 2.0% to 9.0% and from 0.9% to 7.0%, respectively.

Only slightly higher results were found for the inter-day precision, which ranged from 3.7% to 9.0% relative standard deviation for Q_{low}-material. For the analysis of Q_{high}-material similar results were found with a relative standard deviation of 3.3–9.2%.

Additionally, the effect of various urinary matrices was investigated. With the exception of 2-HPMA accuracy was very satisfactory with a median recovery of 96% for HEMA to 110% for MHBMA. Median recovery of 2-HPMA in different urine samples was determined to be 60% with a high margin of deviation. As inter- and intra-day precision of 2-HPMA was determined to be fairly satisfactory, a likely reason for the missing accuracy and precision in the case of different urinary matrices is that no individual internal standard in form of a labelled analogue was available for 2-HPMA. With regard to the other mercapturic acids the use of labelled internal standards has been proven to be very efficient for compensation of influences due to different urinary matrices. Therefore, for future work it appears to be necessary to synthesize labelled 2-HPMA to guarantee a reliable determination of this mercapturic acid in different human urines.

The limits of detection (LOD) according to DIN 32 645 [32], were determined to be 3.0 μg/L for 3-HPMA, 4.0 μg/L for HEMA, 4.5 μg/L for DHBMA, 5.0 μg/l for MHBMA, 5.5 μg/L for DHPMA and 7.0 μg/L for 2-HPMA.

Detailed validation results for every single mercapturic acid along with the respective LODs are summarized in Table 3.

Calibration was always carried out in pooled urine, as slopes of calibration curves in water and urine proved to be disproportionate for 2-HPMA, HEMA and MHBMA (data not shown).

3.3. Application of the method

The method was applied to determine the background concentration of hydroxyalkyl mercapturic acids in urine samples of occupational non-exposed smokers and non-smokers. The identity of the analytes in urine samples was confirmed using the specific mass transitions summarized in Table 2 and by checking the correct retention times in association with the internal standards. For DHBMA and DHPMA the identity of the peaks was additionally checked by calculating the ratio between the peak areas of quantifier and qualifier, which is supposed to be constant in every run. A chromatogram of a smoker and a non-smoker urine is shown in Fig. 4 and Fig. 5. Fig. 4 shows the mass transitions of the determined monohydroxyalkyl mercapturic acids whereas Fig. 5 shows the mass transitions (qualifier and quantifier) of the dihydroxyalkyl mercapturic acids. A detailed report of the results will be published soon.

Furthermore, it is remarkable, that in human urine samples only one MHBMA peak with a retention time of 4.5 min is observed (see Fig. 4), although the applied method enables the separation of the two regioisomers of MHBMA (see Fig. 1). Schettgen et al. [36] noticed as well that in human urine samples one MHBMA peak is clearly predominant and they conclude that this might be due to differences between the purchased standard substance and the metabolic *in vivo* reaction. Ding et al. [39] observed similarly only one MHBMA peak in human urine. They worked with a custom-synthesized MHBMA standard substance and they discovered that the MHBMA regioisomer N-acetyl-S-(1-hydroxymethyl-2-propenyl)-L-cysteine was not detectable in the examined urine samples. Altogether, further research in this field seems to be necessary.

The detection of the analytes in occupationally non-exposed non-smokers urine demonstrates the high sensitivity of the method and its potential to determine the background concentration of hydroxyalkyl mercapturic acids in urine samples of the general population. These results are plausible since the occurrence of background concentrations of hydroxyalkyl mercapturic acids like 3-HPMA [17,20], DHBMA and MHBMA [22,24,40], 2-HPMA [9] as well as HEMA [7] in the general population was already reported in several studies. To our knowledge, determination of DHPMA in human urine has not yet been reported.

4. Conclusion

The developed analytical procedure enables the simultaneous determination of six hydroxyalkyl mercapturic acids which are important metabolites of alkylating agents used in industrial production. These metabolites are suitable biomarkers of occupational exposure for glycidol, ethylene oxide, propylene oxide, acrolein and 1,3-butadiene. The presented method allows for the first time the determination of these biomarkers in one analytical run and to our best knowledge this is the first analytical method that enables the determination of DHPMA (metabolite of glycidol) in human urine. This is of particular importance as glycidol is a chemical substance classified by the IARC as a probable human carcinogen (Group 2A) [28], that is widely used in industrial production.

The reliability of the method is characterized by a robust chromatography, high sensitivity and high reproducibility. Determination was accomplished using isotopically labelled internal standards of the analytes (except for 2-HPMA), which proved to be a successful method to compensate for matrix-related effects as ion suppression or ion enhancement as well as analyte losses due to sample preparation. In conjunction with the wide linear range (up to 1000 μg/L) it is possible to apply the method for the determination of individual occupational exposure to several alkylating agents as well as for the determination of background concentrations of the respective mercapturic acids in the general population.

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