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Excretion of mercapturic acids in human urine after occupational exposure to 2-chloroprene

Elisabeth Eckert · Gabriele Leng · Wolfgang Gries · Thomas Göen

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Abstract A pilot study was conducted for human biomonitoring of the suspected carcinogen 2-chloroprene. For this purpose, urine samples of 14 individuals occupationally exposed to 2-chloroprene (exposed group) and of 30 individuals without occupational exposure to alkylating substances (control group) were analysed for six potential mercapturic acids of 2-chloroprene: 4-chloro-3-oxobutyl mercapturic acid (Cl-MA-I), 4-chloro-3-hydroxybutyl mercapturic acid (Cl-MA-II), 3-chloro-2-hydroxy-3-butenyl mercapturic acid (Cl-MA-III), 4-hydroxy-3-oxobutyl mercapturic acid (HOBMA), 3,4-dihydroxybutyl mercapturic acid (DHBMA) and 2-hydroxy-3-butenyl mercapturic acid (MHBMA). In direct comparison with the control group, elevated levels of the mercapturic acids Cl-MA-III, MHBMA, HOBMA and DHBMA were found in the urine samples of the exposed group. Cl-MA-I and Cl-MA-II were not detected in any of the samples, whereas HOBMA and DHBMA were found in all analysed urine samples. Thus, for the first time, it was possible to detect HOBMA and Cl-MA-III in human urine. The mercapturic acid Cl-MA-III could be confirmed as a specific metabolite of 2-chloroprene in humans providing evidence for the intermediate formation of a reactive epoxide during biotransformation. The main metabolite, however, was found to be DHBMA showing a distinct and significant correlation with the urinary Cl-MA-III levels in the exposed group. The obtained results give new scientific insight into the course of biotransformation of 2-chloroprene in humans.

Keywords 2-Chloroprene · Butadiene · Biomonitoring · Human metabolism · Carcinogenic substances

E. Eckert · T. Göen (✉)
Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, University of Erlangen-Nuremberg, Schillerstrasse 25/29, 91054 Erlangen, Germany
e-mail: Thomas.Goeen@ipasum.med.uni-erlangen.de

G. Leng · W. Gries
Currenta GmbH & Co. OHG, Institute of Biomonitoring, 51368 Leverkusen, Germany

Introduction

The alkylating agent 2-chloroprene (2-chloro-1,3-butadiene) is an important chemical for industrial use, produced in annual volumes of about 350,000 tons (Arpe 2007). 2-Chloroprene is almost exclusively used for the synthesis of polychloroprene. The synthetic rubber (trade names: Neoprene® or Baypren®) exhibits excellent insulation properties and is used in manifold applications, for example, for thermal protection suits (IARC 1999; Arpe 2007). However, exposure to 2-chloroprene may involve significant toxic effects. By the International Agency for Research on Cancer (IARC 1999), 2-chloroprene is classified as possibly carcinogenic to humans (Group 2B). Although, decomposition products of 2-chloroprene have shown bacterial mutagenicity (Westphal et al. 1994), no clear evidence for human mutagenicity of 2-chloroprene exists.

In vivo, 2-chloroprene is apparently detoxified by the formation of glutathione (GSH) conjugates followed by the urinary excretion of mercapturic acids (Summer and Greim 1980). However, apart from that there are no studies on in vivo metabolism of 2-chloroprene. Munter et al. (2003, 2007) conducted comprehensive in vitro studies regarding the biotransformation of 2-chloroprene in liver microsomes of humans and rodents. Based on these studies, they proposed a detailed metabolism scheme of 2-chloroprene. In this scheme, several chlorine and non-chlorine containing GSH conjugates are listed as metabolites. However, prior

to excretion, GSH conjugates are metabolized (Commandeur et al. 1995). Important metabolic products of GSH conjugation in humans are mercapturic acids that are frequently used as biomarkers of exposure to electrophilic substances (Commandeur et al. 1995; De Rooij et al. 1998). According to the scheme proposed by Munter et al. (2003, 2007), metabolism of 2-chloroprene is similar to that of 1,3-butadiene. Initially, an epoxidation of one of the double bonds takes place leading to the intermediate formation of (1-chloroethenyl)oxirane (1-CEO) and 2-chloro-2-ethenyl oxirane (2-CEO), respectively. Though, in contrast to butadiene, there is no evidence for the formation of a diepoxide as an intermediate in chloroprene metabolism, so far (Cottrell et al. 2001; Munter et al. 2007).

There is some evidence that the intermediate formation of epoxides during metabolism of various unsaturated compounds is linked with the incidence of carcinogenic effects (Gonzalez and Gelboin 1994; Rendic and Guengerich 2012). This mechanism can be explained by the fact that reactive compounds like epoxides may form covalent adducts with biological relevant macromolecules such as DNA. For 1-CEO, the formation of adducts with DNA and haemoglobin, respectively, has already been demonstrated in vitro (Hurst and Ali 2007; Munter et al. 2007). Moreover, due to its bifunctionality, 1-CEO may act as a potent DNA interstrand cross-linking agent, too (Wadugu et al. 2010).

So far, reliable information about the in vivo biotransformation of 2-chloroprene is still missing. Therefore, the purpose of our study was to analyse urine samples of workers occupationally exposed to 2-chloroprene in order to identify urinary metabolites in humans which may confirm the proposed metabolism. Some mercapturic acids examined in our study were selected on the basis of the scheme proposed by Munter et al. (2003, 2007). This applies to 3-chloro-2-hydroxy-3-butenyl mercapturic acid (Cl-MA-III) and 4-chloro-3-oxobutyl mercapturic acid (Cl-MA-I) that were selected as specific products of 1-CEO and 2-CEO, respectively. 4-Hydroxy-3-oxobutyl mercapturic acid (HOBMA) was selected as a further metabolism product of 2-CEO following hydrolysis and rearrangement including dehalogenation (see Fig. 1). In addition, Fig. 1 shows some potential metabolites of 2-chloroprene that may result from the precursors postulated by Munter et al. (2003, 2007), but which were not described in their studies (see arrows with dashed lines in Fig. 1). Thus, our study included 4-chloro-3-hydroxybutyl mercapturic acid (Cl-MA-II) and 3,4-dihydroxybutyl mercapturic acid (DHBMA) as potential biomarkers of 2-chloroprene, too. Their formation can be assumed, based on a potential enzymatic reduction of Cl-MA-I and HOBMA, respectively (see Fig. 1). For example, a similar reduction is described for acrolein that is initially metabolized to 3-oxopropyl mercapturic acid, but mainly excreted in urine as 3-hydroxypropyl mercapturic acid (Draminski et al. 1983; Parent et al. 1998).

Moreover, we examined the potential presence of 2-hydroxy-3-butenyl mercapturic acid (MHBMA) in the urine samples. Based on the assumption that the biotransformation of 2-chloroprene is similar to that of butadiene, we took DHBMA and MHBMA into account as known urinary metabolites of 1,3-butadiene in humans. MHBMA is usually described as a mixture of two regioisomers: 2-hydroxy-3-butenyl mercapturic acid and 1-hydroxymethyl-2-propenyl mercapturic acid. Both regioisomers were found as urinary metabolites of 1,3-butadiene in animal studies (Elfarra et al. 1995; Richardson et al. 1999). However, in human urine, the regioisomer 2-hydroxy-3-butenyl mercapturic acid appears to be by far the predominant metabolite found in human urine of the general population (Ding et al. 2009; Eckert et al. 2011). Therefore, we focused on the determination of 2-hydroxy-3-butenyl mercapturic acid in our study. Both, DHBMA and MHBMA may be formed by several different pathways and may represent derivation products of both 1-CEO and 2-CEO (see Fig. 1).

Experimental procedures

Subjects

Spot urine samples (at least 10 mL each) of 14 individuals occupationally exposed to 2-chloroprene ("exposed group") and of 30 individuals without any occupational exposure to alkylating substances ("control group") were collected at random times and stored at −20 °C until analysis. The control group was a subgroup of the study collective described and examined by Eckert et al. (2011) and consisted of 14 males and 16 females aged 21–63 years (median age 30 years). All subjects of the exposed group were employees of a German company producing polychloroprene. The air contamination with 2-chloroprene at the workplace was estimated to be low due to measurements of 2-chloroprene in workplace air (<0.1 ppm). However, significant dermal exposure was assumed by the occupational hygienist of the plant. A potential co-exposure of the workers to 1,3-butadiene could be ruled out, as all subjects of the exposed group were employed in further processing of 2-chloroprene. 2-Chloroprene itself, however, was not produced in the same plant. The participants were all males, aged 25–57 years (median age 43 years). In each group, half of the participants stated to be smokers. All participants were informed about the aims of our study and gave their written informed consent to their participation. Information

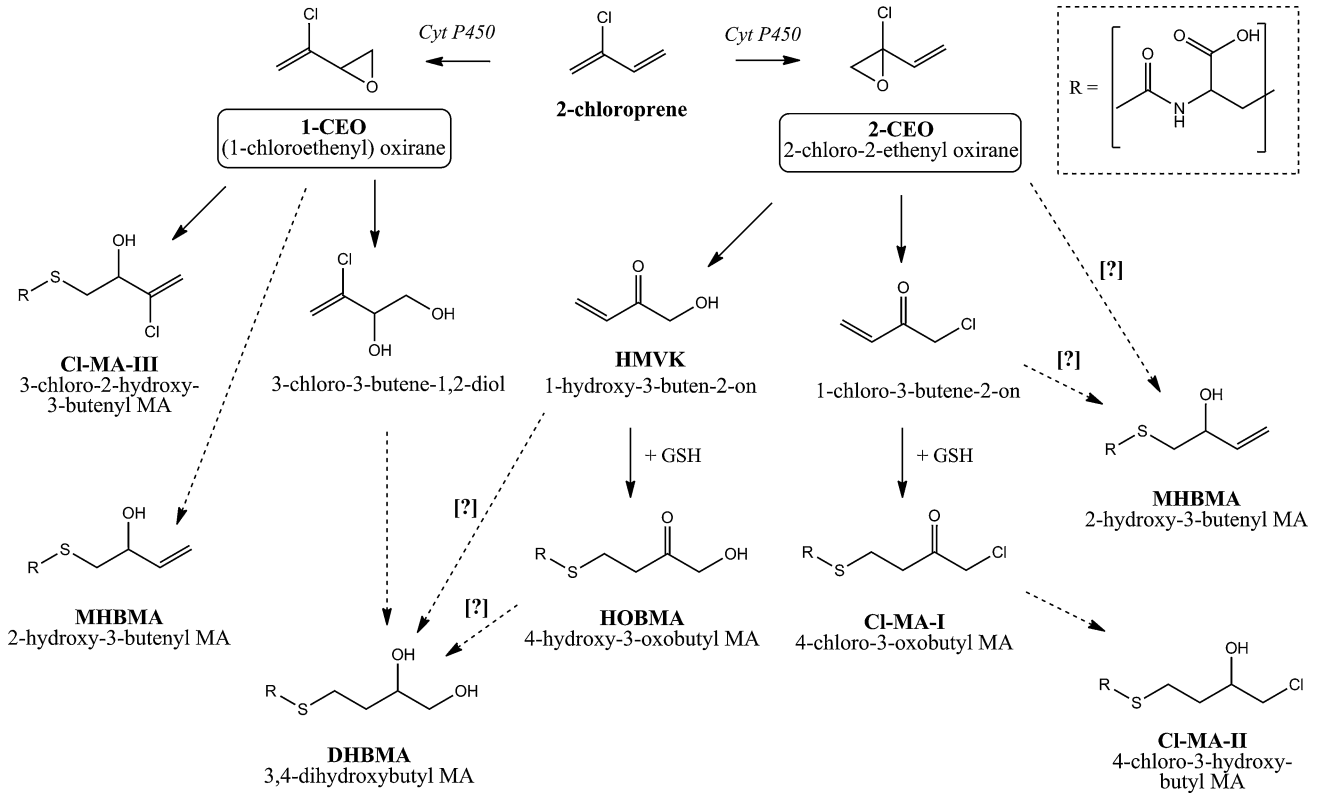

Fig. 1 Proposed biotransformation pathway of 2-chloroprene to mercapturic acids via intermediate formation of the epoxides 1-CEO and 2-CEO (*arrows* with *solid lines* proposed metabolism paths by Munter et al. (2003, 2007); *arrows* with *dashed lines* additionally proposed biotransformation routes; *arrows* with *question marks* unlikely biotransformation routes). *GSH* glutathione, *Cyt P450* cytochrome P450, *MA* mercapturic acid

regarding age, gender and smoking status was collected in a personal interview. Each sample was analysed with regard to mercapturic acid concentration (DHBMA, HOBMA, MHBMA, Cl-MA-I, Cl-MA-II and Cl-MA-III). Additionally, urinary creatinine was determined according to the method by Larsen (1972).

Chemicals

Cl-MA-I, Cl-MA-II, Cl-MA-III, HOBMA and MHBMA as well as the internal standard substances d_3-Cl-MA-I, d_3-Cl-MA-III and d_3-HOBMA were synthesized with a stated purity of at least 95 % by the Institute for Organic and Biomolecular Chemistry (Göttingen, Germany). DHBMA, d_7-DHBMA and d_6-MHBMA were purchased from Toronto Research Chemicals (Toronto, Canada) with a stated purity of at least 98 %. d_6-MHBMA is described as a 1:1 mixture of deuterated 2-hydroxy-3-butenyl mercapturic acid and deuterated 1-hydroxymethyl-2-propenyl mercapturic acid. 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 standard substances was confirmed by ^{1}H-NMR and LC–MS/MS. All other chemicals were of at least analytical grade.

Determination of mercapturic acids in urine

MHBMA levels in urine were determined using a LC–MS/MS method according to Eckert et al. (2010). Deviating from the described procedure, we used a custom-synthesized standard substance of MHBMA which contains solely the regioisomer 2-hydroxy-3-butenyl mercapturic acid. For this procedure, a detection limit of 0.5 µg/L was estimated. The applied procedure was designed to enable a reliable separation of MHBMA from other regioisomers of monohydroxybutenyl mercapturic acid.

Urinary levels of the other five mercapturic acids were determined by LC–MS/MS using online solid-phase extraction according to a previously published method (Eckert et al. 2012). Calibration was carried out with spiked pooled urine, obtained from non-smoking individuals, for the concentration range of 25–1,000 µg/L each of DHBMA and HOBMA and 5–200 µg/L each of Cl-MA-I, Cl-MA-II and Cl-MA-III. d_3-Cl-MA-III was used as an internal standard substance for both Cl-MA-III and Cl-MA-II. Urine samples with analyte levels above the calibration range were diluted with water and analysed again. Cl-MA-II proved to be unstable in aqueous media, as we observed a rapid reduction in spiked Cl-MA-II levels in urine

samples within a few hours (Eckert et al. 2012). However, an increase in other mercapturic acids was not found associated with the Cl-MA-II decrease. Validation of the method has been carried out for all analytes, with the exception of Cl-MA-II (see Eckert et al. (2012) for full validation data). Limits of detection for DHBMA, HOBMA, Cl-MA-I and Cl-MA-III were determined to be 3.8, 4.2, 2.5 and 1.4 µg/L, respectively.

Statistical analysis

All data analyses were conducted with the statistical software IBM® SPSS® 19.0. Analytical results below the limit of detection (LOD) entered the statistical analyses as LOD/$\sqrt{2}$ according to the statistical model by Hornung and Reed (1990). All statistical analyses were performed using the creatinine-related analyte levels. Correlation coefficients were calculated according to Pearson. The calculation of statistical significances between values of two groups was accomplished using the Mann–Whitney *U* test. For both, comparison of two groups and regression analysis, a probability of error below 5 % ($p < 0.05$) was evaluated as a significant difference and a significant association, respectively.

Results

The chlorine containing mercapturic acid Cl-MA-III was detected exclusively in eleven samples of the occupationally exposed group. In the exposed group, the median level of this analyte was found to be 6.1 µg/g creatinine. MHBMA was also found in eleven urine samples of the exposed group (in six smokers and five non-smokers) with a median level of 3.5 µg/g creatinine. Unlike Cl-MA-III, MHBMA was also found in about half of the urine samples of the control group. However, the MHBMA levels in the control group were significantly lower compared to the levels of the exposed group ($p = 0.004$). In the control group, MHBMA was found in urine samples of smokers, only.

The biomarkers HOBMA and DHBMA were detected in all urine samples, whereas significantly elevated levels of both analytes were found in the exposed group. The median level of DHBMA in the occupationally exposed group was found to be 3,255 µg/g creatinine and thereby being approximately 18-fold higher compared to the median level of the control group (179 µg/g creatinine).

Cl-MA-II was not detected in any of the analysed samples. However, it has to be mentioned that the reliability of the determination of this mercapturic acid was significantly affected due to its instability in aqueous media (Eckert et al. 2012). As well, Cl-MA-I was not found in the urine samples of our study population. All results are summarized in Table 1.

Figure 2 shows the levels of Cl-MA-III, MHBMA, HOBMA and DHBMA as boxplots illustrating the differences in urinary mercapturic acid excretion of both groups ($p < 0.001$ each for DHBMA and Cl-MA-III, $p = 0.004$ for MHBMA, $p = 0.005$ for HOBMA). Furthermore, as illustrated in Fig. 3a, DHBMA showed a strong correlation with the chlorine containing mercapturic acid Cl-MA-III ($r = 0.918$, $p < 0.001$) with an excretion ratio of Cl-MA-III to DHBMA of approximately 1:400. Figure 3b shows the observed correlation of the urinary levels of Cl-MA-III and MHBMA in the exposed group. HOBMA did not show a significant correlation in the exposed group, neither to DHBMA nor to Cl-MA-III or MHBMA (data not shown).

With regard to tobacco smoke exposure, the smoking status of the participants showed no significant impact on mercapturic acid excretion for the subjects of the exposed group. For the control group, however, the analytes MHBMA, HOBMA and DHBMA showed higher median levels in the group of smokers ($n = 15$) compared to non-smokers ($n = 15$) as shown in Fig. 4. However, a statistical significant difference could only be found for DHBMA and MHBMA, respectively ($p < 0.001$ for MHBMA, $p = 0.223$ for HOBMA, $p = 0.001$ for DHBMA).

Discussion

In the present study, Cl-MA-III was detected in human urine for the first time. This finding is consistent with the in vitro studies of Munter et al. (2003, 2007) who identified the precursor glutathione conjugate in microsomal incubations of 2-chloroprene. In the present study, Cl-MA-III was found exclusively in the urine samples of the exposed group, indicating that it represents a highly specific metabolite of 2-chloroprene in humans. In the proposed metabolism scheme of 2-chloroprene, Cl-MA-III is presented as a direct metabolism product of 1-CEO (see Fig. 1). Thus, the detection of Cl-MA-III in human urine of occupationally exposed individuals provides strong evidence for the intermediate formation of the epoxide 1-CEO in human metabolism. This finding is in agreement with the results of the in vitro studies that incubated 2-chloroprene or the epoxide 1-CEO with DNA or haemoglobin. These studies observed the formation of DNA adducts or haemoglobin adducts with a 3-chloro-2-hydroxy-3-butenyl moiety, which is an analogue structure to Cl-MA-III (Munter et al. 2002, 2007; Hurst and Ali 2007; Wadugu et al. 2010).

A remarkable result of the present study is the extremely high level of DHBMA found in the urine samples of individuals exposed to 2-chloroprene. DHBMA is known

Table 1 Urinary mercapturic acid levels in the study population (exposed group and control group)

Analyte	Exposed group ($n = 14$)			Control group ($n = 30$)		
	>LOD (%)[a]	Median (µg/g creatinine)	Range (µg/g creatinine)	>LOD (%)[a]	Median (µg/g creatinine)	Range (µg/g creatinine)
Cl-MA-I	0	<2.5	<2.5	0	<2.5	<2.5
Cl-MA-III	79	6.1	<1.4–25.7	0	<1.4	<1.4
MHBMA	79	3.5	<0.5–42.9	47	<0.5	<0.5–5.9
HOBMA	100	214	97.9–436	100	111	26.8–776
DHBMA	100	3,255	176–12,187	100	179	72.3–523

[a] Percentage of urine samples with analyte levels above the LOD

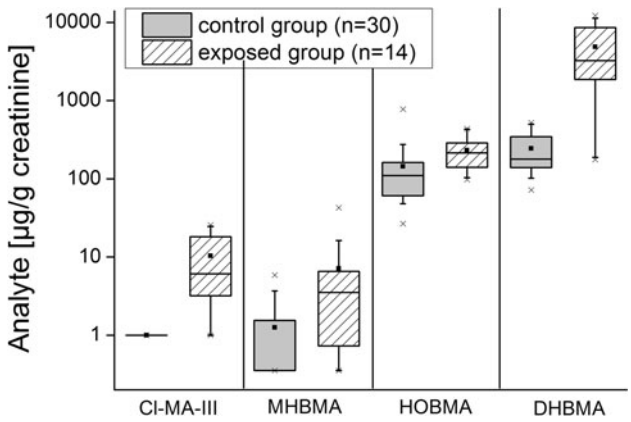

Fig. 2 Levels of Cl-MA-III, MHBMA, HOBMA and DHBMA in urine samples of both groups

to be a major metabolite of butadiene. Many studies examined the urinary background levels of DHBMA in the general population and reported urinary levels in non-smokers of about 150–300 µg/g creatinine and slightly elevated levels in smokers (Urban et al. 2003; Roethig et al. 2009; Eckert et al. 2011). In the present study, the median DHBMA level in the control group was determined to be 179 µg/g creatinine which is in good agreement with the results of other studies. Additionally, we observed a slight but significant difference between the DHBMA excretion levels in smokers and non-smokers ($p < 0.001$, see Fig. 4). However, the determined DHBMA levels in the urine samples of the exposed group were substantially higher with a median level of 3,255 µg/g creatinine and top values as high as 12,000 µg/g creatinine. The elevated levels can only be attributed to an exposure to 2-chloroprene as a co-exposure to butadiene can definitely be excluded for the exposed group. This statement is confirmed by the fact that DHBMA showed a distinct and significant correlation with Cl-MA-III ($r = 0.918$, $p < 0.001$). The excretion ratio between Cl-MA-III, the specific metabolite of 2-chloroprene, and DHBMA was found to be approximately 1:400 (see Fig. 3a). Based on these results, DHBMA is clearly the main urinary metabolite of 2-chloroprene in humans. In the scheme of Munter et al. (2003, 2007), the formation of DHBMA or of the respective glutathione conjugate was not described. However, the metabolism scheme of Munter et al. lists a glutathione conjugate of HOBMA that may represent a hypothetic precursor of DHBMA (see Fig. 1). Munter et al. (2003, 2007) proposed that the epoxide

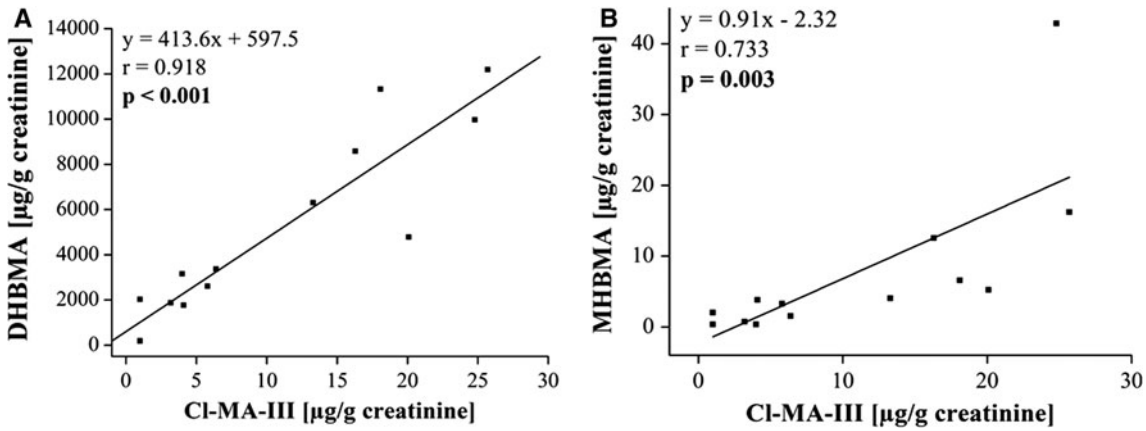

Fig. 3 Correlation of the urinary levels of Cl-MA-III with DHBMA (**a**) and MHBMA (**b**), respectively, in occupationally exposed subjects (exposed group, $n = 14$)

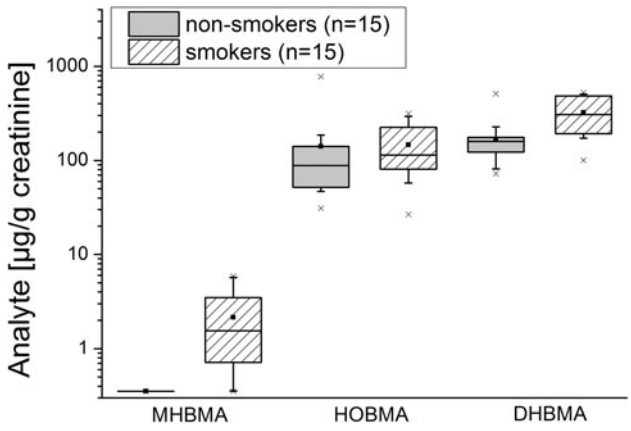

Fig. 4 Urinary levels of MHBMA, HOBMA and DHBMA in smokers and non-smokers of the control group ($n = 30$)

2-CEO is a precursor of hydroxymethylvinyl ketone (HMVK) which may further react with GSH. The formation of HMVK as a reactive intermediate was also frequently proposed for metabolism of butadiene (Krause et al. 2001; Sprague and Elfarra 2004; Barshteyn and Elfarra 2009) and led to the hypothesis that HOBMA is a common precursor of DHBMA (Sabourin et al. 1992; Bechtold et al. 1994; Richardson et al. 1998; Booth et al. 2004) (see also Fig. 1).

In the present study, HOBMA was detected in human urine for the first time and was found in all analysed urine samples with median levels of 111 and 214 µg/g creatinine in the control group and the exposed group, respectively. In the control group, the urinary excretion level of HOBMA was similar to that of DHBMA. Additionally, excretion of HOBMA is only modestly affected by exposure to tobacco smoke (see Fig. 4). In the exposed group, HOBMA did show significantly elevated levels. In comparison with DHBMA, however, the difference in HOBMA excretion seems rather small (see also Fig. 2). Based on our results in the control group, a shared biotransformation pathway of DHBMA and HOBMA as shown in Fig. 1 might be possible. For the exposed group, however, this appears to be questionable. This assumption is supported by the fact that the urinary levels of HOBMA and DHBMA did not correlate at all in the exposed group ($r = 0.092$, $p = 0.755$). That may indicate that HOBMA is not, as first hypothesized, the precursor of DHBMA in human metabolism of 2-chloroprene. In contrast, DHBMA may rather be derived as a secondary product of 1-CEO as proposed in Fig. 1. This is supported by the fact that the urinary levels of DHBMA showed a significant and tight correlation with those of Cl-MA-III in the exposed group indicating the same biotransformation route of both compounds. In humans, the hydrolysis of epoxide compounds, for example, derived from butadiene, is highly favoured (Bechtold et al. 1994; Henderson et al. 1996; Boogaard et al. 2001). For this reason, it can be assumed that 1-CEO will preferably be metabolized to 3-chloro-3-butene-1,2-diol that may react via a dechlorination step with GSH finally leading to the formation of DHBMA (see Fig. 1). Based on our observations, this seems to be the most likely biotransformation route for DHBMA.

However, in our study, HOBMA levels were significantly elevated in the exposed group. Provided that 2-CEO is a precursor of HOBMA as proposed by Munter et al. (2003, 2007), our observations may also indicate a formation of 2-CEO in human metabolism of 2-chloroprene, albeit to a lesser extent than 1-CEO. Though, it should be noted that the difference in HOBMA excretion in both groups was rather small (see Fig. 2). For a definitive statement on the formation of 2-CEO, further investigations are necessary.

In addition, MHBMA was also found to be a metabolite of 2-chloroprene in the present study. Moreover, all samples were checked for the second regioisomer 1-hydroxymethyl-2-propenyl mercapturic acid, which was not detected in any of the analysed samples. MHBMA may be formed via several pathways (see Fig. 1). Nevertheless, the observed correlation of the urinary levels of MHBMA and Cl-MA-III indicates that MHBMA may rather be a derivation product of 1-CEO, too. Moreover, these considerations are supported by the fact that in direct comparison with 2-CEO, 1-CEO has been confirmed as the intermediary main metabolite of 2-chloroprene in vitro (Cottrell et al. 2001; Himmelstein et al. 2001; Munter et al. 2003).

In general, the occurrence of dehalogenations during biotransformation is not unusual and has already been described for several halogenated substances in animal studies (Bernauer et al. 1996; Gingell et al. 1985; Jones 1975). However, a detailed mechanism for this reaction is rarely discussed. There are various possible mechanisms for dehalogenations in vivo as nucleophilic substitution or the intermediate formation of episulfonium ions. Cottrell et al. (2001) have already proposed a detailed dechlorination pathway for 2-chloroprene. However, this scheme involves HMVK and 2-CEO whose formation could not be reliably confirmed by the present study.

Conclusion

The results of the present study revealed that the mercapturic acids Cl-MA-III, HOBMA, DHBMA and MHBMA are human metabolites of 2-chloroprene. The occurrence of Cl-MA-III in the urine of 2-chloroprene exposed individuals provides evidence for the intermediate formation of the epoxide 1-CEO in human metabolism of 2-chloroprene. The main metabolite, however, was found to be the

non-chlorine containing mercapturic acid DHBMA. Theoretically, DHBMA may be derived via both precursors 1-CEO and 2-CEO. However, the strong association between the urinary levels of DHBMA and Cl-MA-III as well as the missing correlation between DHBMA and HOBMA levels in the exposed group support the hypothesis that 1-CEO might be the precursor of DHBMA. Nevertheless, further studies are required to collect more evidence for this hypothesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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