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An integrated approach to biomonitoring exposure to styrene and styrene-(7,8)-oxide using a repeated measurements sampling design

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Abstract

The aim of this work was to investigate urinary analytes and haemoglobin and albumin adducts as biomarkers of exposure to airborne styrene (Sty) and styrene-(7,8)-oxide (StyOX) and to evaluate the influence of smoking habit and genetic polymorphism of metabolic enzymes GSTM1 and GSTT1 on these biomarkers. We obtained three or four air and urine samples from each exposed worker (eight reinforced plastics workers and 13 varnish workers), one air and urine samples from 22 control workers (automobile mechanics) and one blood sample from all subjects. Median levels of exposure to Sty and StyOX, respectively, were 18.2 mg m^{-3} and 133 μg m^{-3} for reinforced plastics workers, 3.4 mg m^{-3} and 12 μg m^{-3} for varnish workers, and <0.3 mg m^{-3} and <5 μg m^{-3} for controls. Urinary levels of styrene, mandelic acid, phenylglyoxylic acid, phenylglycine (PHG), 4-vinylphenol (VP) and mercapturic acids (M1+M2), as well as cysteinyl adducts of serum albumin (but not those of haemoglobin) were significantly associated with exposure status (controls <exposed workers). Also, levels of VP and M1+M2 were significantly affected by smoking, and levels of M1+M2 were significantly affected by GSTM1 polymorphisms. Multiple linear regression analyses of the subject-specific (logged) metabolite levels across exposed workers showed that Sty was a significant predictor for all urinary analytes while StyOX was a significant predictor of PHG only. Interestingly, the log scale regression coefficients for Sty in these models were significantly less than one for all metabolites except M1+M2. This suggests that the natural scale relationships between levels of all Sty metabolites, except M1+M2, displayed downward concavity with increasing Sty exposure, suggestive of saturable metabolism. Levels of the protein adducts were not associated with exposure to either Sty or StyOX among exposed subjects.

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Introduction

Styrene (Sty) is an important industrial chemical that is used primarily in the production of plastics (IARC 1994a, Miller et al. 1994). Occupational exposures to styrene are accompanied by co-exposures to low levels of styrene-(7,8)-oxide (StyOX), which is produced from oxidation of Sty during the production of plastics, possibly via catalysis by hydroperoxides used as initiators of the polymerization process (Rappaport et al. 1996, Nylander-French et al. 1999, Tornero-Velez et al. 2000). StyOX is an animal carcinogen that is classified as a probable human carcinogen (group 2A) (IARC 1994b). Since StyOX is the primary mammalian metabolite of Sty, it is probably responsible for the carcinogenicity of Sty observed in animal experiments and for designation of Sty as a possible human carcinogen (group 2B) (IARC 1994a).

Occupational exposure to Sty is regulated in many countries: a concentration of 85 mg m^{-3} (20 ppm) as 8-h time-weighted average is currently recommended by the American Conference of Governmental Industrial Hygienists (ACGIH 2007) and the Deutsche Forschungsgemeinschaft (DFG 2007); US OSHA issues a permissible exposure limit for 8-h time-weighted average of 425 mg m^{-3} (100 ppm) (OSHA 2006); NIOSH (1997) indicates a recommended exposure limit for 10-h time-weighted average of 215 mg m^{-3} (50 ppm).

Conversely, little attention has been given to StyOX exposures, probably because its air concentrations are at least two orders of magnitude lower than those of Sty, which is almost entirely metabolized to StyOX *in vivo*. However, based on measurements of StyOX or StyOX-albumin (Alb) adducts in the blood of reinforced plastics workers, Rappaport et al. (1996) and Tornero-Velez and Rappaport (2001) concluded that most of the systemic dose of StyOX came from direct inhalation of StyOX.

Upon inhalation, Sty and StyOX are efficiently absorbed in the lungs/blood. About 20% of absorbed Sty is excreted passively in the breath and urine and can be used for biomonitoring of exposure to Sty (Pezzagno et al. 1985, Ghittori et al. 1987). The remaining 80% of the Sty dose is metabolized, as illustrated in Figure 1. Sty is initially metabolized, via cytochrome P450, to StyOX which exists in two enantiomeric forms, (R)-StyOX and (S)-StyOX. StyOX is subsequently hydrolysed to phenylethylene glycol, which is oxidized and excreted as mandelic acid (MA) and phenylglyoxylic acid (PGA). Together, MA and PGA represent about 95% of the absorbed Sty dose. A very small fraction of PGA is converted to phenylglycine (PHG) (Manini et al. 2002). All other pathways of Sty metabolism collectively contribute only a small percentage of the absorbed dose. One pathway involves the GSTM1-1 catalysed conjugation of StyOX with glutathione to produce a mixture of diastereomeric mercapturic acids, namely, (R,R)- and (S,R)-*N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)-L-cysteine (designated R,R-M1 and S,R-M1, respectively), and (R,R)- and (S,R)-*N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-L-cysteine (designated R,R-M2 and R,S-M2, respectively) (de Palma et al. 2001, Haufroid et al. 2001). Another pathway involves ring oxidation of Sty followed by hydrolysis to 4-vinylphenol (VP), which is typically excreted as a glucuronide (VP-G) or sulfate conjugate (VP-S) (Manini et al. 2003).

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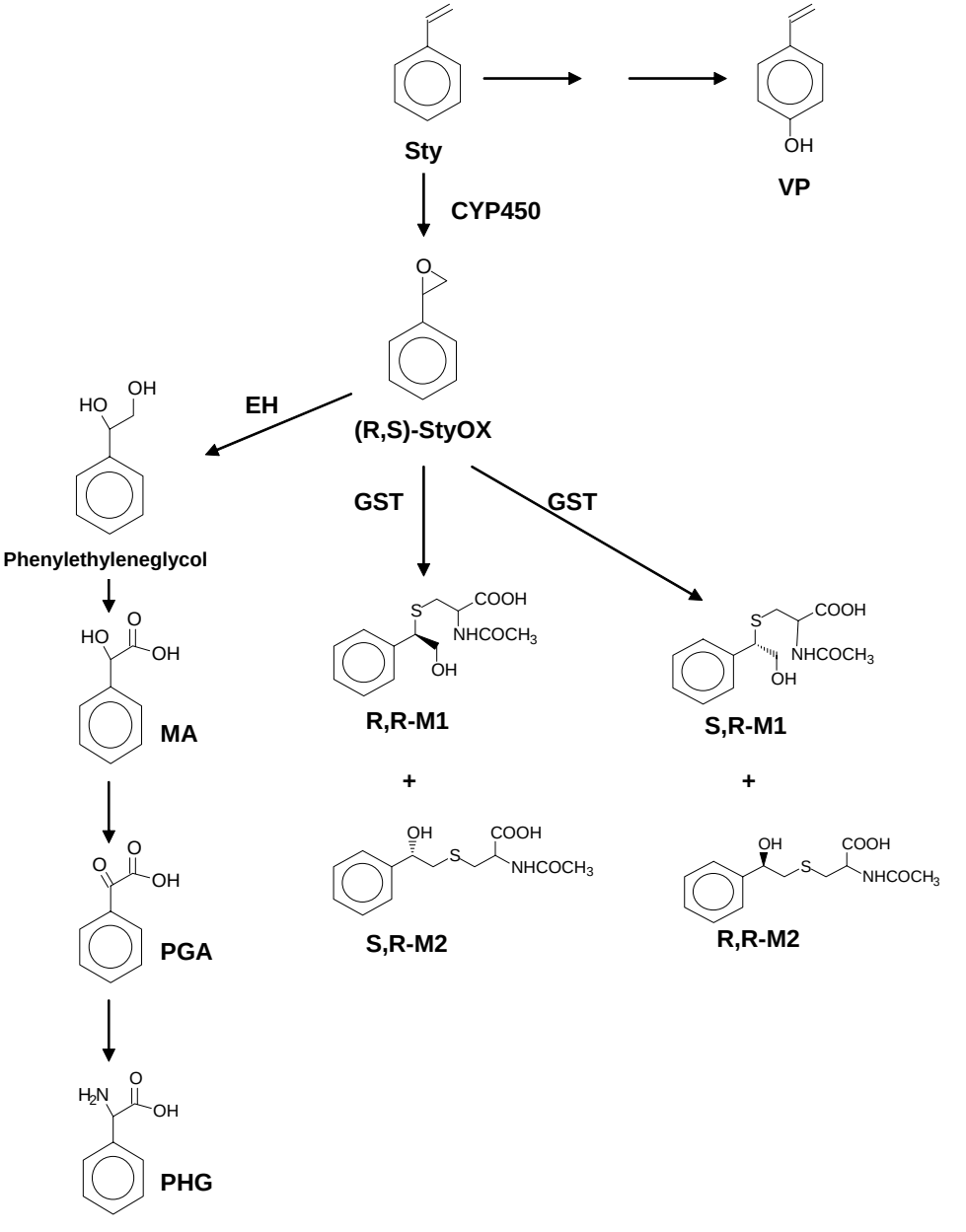

Figure 1. Metabolism of styrene in humans. Sty, styrene; VP, 4 vinylphenol; CYP450, cytochrome P-450 monooxygenase; EH, epoxide hydrolase; (R,S)-StyOX, (R,S)-styrene oxide; GST, glutathione *S*-transferase; MA, mandelic acid; PGA, phenylglyoxylic acid; PHG, phenylglycine; R,R-M1 and S,R-M1, (R,R)- and (S,R)-*N*-acetyl-*S*-(1-phenyl-2-hydroxyethyl)-L-cysteine; R,R-M2 and R,S-M2, (R,R)- and (S,R)-*N*-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-L-cysteine.

For the biological monitoring of occupational exposure to styrene different studies (for example, Gobba et al. 1993 and Guillemin & Berolde 1988) and agencies recommend the use of MA and/or PGA in post shift urine (biological exposure index = 400 mg (MA + PGA) g^{-1} creatinine (ACGIH 2007) and biological tolerance

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value = 600 mg (MA + PGA) g^{-1} creatinine (DFG 2007)). Blood styrene in post shift sample is also recommended (biological exposure index = 0.2 mg l^{-1} (ACGIH 2007)), but the quantitative interpretation of the measurement is ambiguous. Recently other (minor) metabolites of Sty, including VP and M1 + M2, have also been used as biomarkers (Haufroid et al. 2001, de Palma et al. 2001, Manini et al. 2003).

The few attempts at biomonitoring of StyOX exposures have focused upon direct measurements of StyOX in blood (Korn et al. 1994, Tornero-Velez et al. 2001b, Serdar et al. 2006) or measurements of adducts of StyOX with the blood proteins haemoglobin (Hb) and Alb (Rappaport et al. 1996, Yeowell-O'Connell et al. 1996, Fustinoni et al. 1998). Of these options, measurements of StyOX-Hb or StyOX-Alb are generally preferred because they are stable and accumulate in humans over 1 or 2 months (Granath et al. 1992), whereas StyOX is unstable in blood (Yeowell-O'Connell et al. 1997). In one study, levels of StyOX-Alb were significantly correlated with exposures to both Sty and StyOX, as well as with StyOX adducts of DNA, and sister chromatid exchanges (Rappaport et al. 1996). However, the presence of significant background levels of StyOX-Alb and StyOX-Hb (in subjects without occupational exposures) has limited the utility of these adducts as biomarkers of exposure (Yeowell-O'Connell et al. 1996, Fustinoni, et al. 1998).

Aim of this study was to compare several potential biomarkers of exposure to Sty and StyOX in industries where Sty is used. The investigated biomarkers include unmetabolized Sty in urine (StyU), urinary metabolites MA, PGA, PHG, VP, M1 and M2, and StyOX-Alb and StyOX-Hb. To overcome problems that occur when short-term biomarkers (urinary analytes) are compared with intermediate/long-term biomarkers (StyOX-Alb and StyOX-Hb) (Lin et al. 2005), a multiple measurements protocol was employed in which air levels of Sty and StyOX, as well as urine specimens, were repeatedly collected over a period of 6 weeks prior to blood collection. Furthermore, we investigated the influence of cigarette smoking and genetic polymorphisms of GSTM1 and GSTT1 as potential confounders or effect modifiers on the relationships between airborne exposures and biomarker levels.

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Materials and methods

Study population

The study entailed air and biological monitoring of two groups of workers exposed to styrene, namely, 13 varnish workers and eight reinforced plastics workers, and of a group of 22 automobile mechanics, who served as control workers. Styrene is the major solvent and monomer for the resin system used in the production of reinforced plastics and is also used as ingredient for the production of these particular varnishes. All subjects were informed of the aims and protocol of the study and provided written consent to be included as human subjects. Detailed information about work activities, demographic and lifestyle factors, and medical histories was obtained using a questionnaire administered by a physician specialized in occupational health.

Air and biological sampling

Personal exposures to Sty and StyOX were assessed over the entire work shift (about 8 h), using passive samplers (Model 3500 Organic Vapor Monitors, 3M, St. Paul, MN, USA). Among exposed subjects, air sampling was repeated three or four times

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during 6 consecutive weeks. Personal exposures were measured once among a subset of seven control subjects; all measurements from controls were below the limits of detection.

For exposed subjects, spot urine samples were collected both before the work shift (BS, about 08:30 h) and at the end of the work shift (ES, about 16:30 h) on the same days that air sampling was performed. Six to eight urine samples, consisting of sets of three or four (each) BS and ES specimens, were collected from each exposed subject. A single blood sample (about 10 ml) was collected from the cubital vein prior to the last investigated work shift. For control subjects, single BS samples of urine and blood were obtained.

Urine samples were partitioned in the infirmary of the worksites into two plastic vials (about 7 ml urine each) for the determination of MA, PGA, PHG, S,R-M2, R,R-M1, R,R-M2, S,R-M1, VP-G, and VP-S. For StyU, a further 7 ml aliquot was transferred to a pre-cleaned glass vial (clear glass, 7.9 ml effective volume), promptly closed with a butyl rubber/PTFE septum, and sealed with an aluminium septum cap.

All samples were coded, chilled at 4°C, and delivered to the laboratories within 4 h. Here blood samples were centrifuged to separate plasma from red blood cells for the determination of Alb and Hb adducts, respectively. DNA was prepared from white blood cells. After lysis and proteinase digestion DNA was isolated by a solvent free commercially available kit (Puregene, Gentra Systems, Minneapolis, MN, USA). Samples were stored at −20°C prior to analyses that were performed without knowledge of samples' origins.

Analysis of air and biological samples

Sty and StyOX in air. The air concentrations of Sty and StyOX were determined by eluting the passive monitors with ethyl acetate and subsequent analysis by either gas chromatography with flame ionization detection (Fison series 8000, Fison Instruments, Rodano, Italy) (Sty) or gas chromatography-mass spectrometry (GC-MS) (HP 5890 Series II equipped with a HP 5972 MS detector, Agilent, Cernusco sul Naviglio, Italy) operating in the electron ionization mode (StyOX), as described by Tornero-Velez et al. (2000). Detection limits for Sty and StyOX are 0.3 mg m^{-3} and 5 μg m^{-3}, respectively.

Unmetabolized styrene in urine (StyU). Levels of StyU were determined by headspace solid-phase microextraction (SPME) followed by GC-MS analysis as described by (Fustinoni et al. 1999), with modifications. Briefly, 0.5 ml urine was transferred to a 2-ml autosampler vial containing 300 mg NaCl. One microlitre of the internal standard solution of d_{10}-xylene in methanol (0.475 μg l^{-1}) was added, and the vial was immediately closed with a screw cap equipped with a silicone-PTFE gasket. Styrene was sampled from the urine headspace at room temperature using a 100 μm PDMS fiber (Supelco, Milan, Italy) and a Varian CX8200 autosampler (Leinì, Torino, Italy) and analysed by GC-MS (HP 5890 Series II equipped with a HP 5972 MS detector) operating in the electron impact mode. A BD1 capillary column (J&W, 60-m, 0.25-mm i.d., 1-μm film thickness; CPS Analytica, Milano, Italy) was used for analyte separation. The detection limit is 0.2 μg l^{-1}.

Styrene metabolites in urine. Urinary metabolites were assayed by liquid chromatography with tandem mass spectrometry (LC-MS/MS), as previously described (Manini et al.

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2002). Analyses were performed on a Perkin Elmer liquid chromatograph series 200 coupled to a PE-Sciex API 365 triple quadrupole mass spectrometer equipped with an ionspray interface (Sciex, Thornhill, Ontario, Canada) and an ASPEC XL autosampler (Gilson, Middleton, WI, USA). All analytes but PHG were detected in negative ion mode and quantification was based on selected reaction monitoring. PHG was detected in positive ion mode. Limits of detection are 0.1 mg l^{-1} for both MA and PGA, 0.01 mg l^{-1} for PHG and 0.0004 mg l^{-1} for each mercapturic acid, 0.015 mg l^{-1} for VP-G and 0.005 mg l^{-1} for VP-S. Concentrations of metabolites in urine samples were expressed as a function of creatinine concentration, measured by the method of Jaffe (Kroll et al. 1986). Creatinine levels ranging between 0.3 and 3.0 g l^{-1} were considered acceptable (WHO 1996).

Protein adducts. Cysteinyl adducts of StyOX with Alb and Hb were assayed as described previously (Yeowell-O'Connell et al. 1996, Fustinoni et al. 1998). These adducts were detected as 1-phenylethanol (1PE) and 2-phenylethanol (2PE), representing substitution at the α- and β-carbons of StyOX, respectively, and are designated as 1PE-Alb, 2PE-Alb, 1PE-Hb and 2PE-Hb. In brief, after the extraction and purification of Alb and globin from whole blood, the protein was reacted with Raney-Nickel to cleave the adducts, thereby releasing 1PE or 2PE. These phenylethanols were then extracted and reacted with pentafluorobenzoyl chloride to produce esters that were analysed by GC-MS using negative chemical ionization (5890 Series II-plus gas chromatograph coupled to a 5989B MS engine, Hewlett Packard, Santa Clara, CA, USA). Limits of detection are 0.03, 0.90, 0.03 and 0.60 nmol g^{-1} protein for 1PE-Alb, 2PE-Alb, 1PE-Hb and 2PE-Hb, respectively.

Genotyping. The wide gene deletion polymorphisms of glutathione transferases *GSTM1* and *GSTT1* were determined by polymerase chain reaction (PCR) according to methods described previously (Arand et al. 1996). β-Actin (primers from Stratagene) was used as positive control to verify the presence of amplifiable DNA. The PCR method used for the detection of individuals lacking the *GSTM1* and *GSTT1* genes (the null genotypes) do not differentiate between the heterozygous and homozygous carriers of the functional gene.

Statistical analyses

For subjects with multiple measurements of air and biological levels, statistical analyses were performed using the individual geometric mean values. Since distributions of variables were highly skewed and displayed heteroscedasticity, we conducted analyses (*t*-test for independent or paired samples, ANOVA, Pearson's correlation, multiple linear regression analyses) employing the natural logarithms of the air and biological levels. All subjects were included in multiple regression models to evaluate effects of job, smoking and GST genotypes on biomarker levels. In these analyses, dummy variables were assigned for jobs (reinforced plastics workers or varnish workers with control workers as the reference group), smoking (0 = non-smokers, 1 = smokers), and GST polymorphisms (0 = null genotype, 1 = active genotype). Since air levels of Sty and StyOX among control subjects were either unmeasured or were at or below the limits of detection, control subjects were not included in multiple regression analyses of biomarker levels involving exposures to Sty and StyOX as independent

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variables. Collinearity of exposures to Sty and StyOX was evaluated with condition indices. Air or biological levels for exposed workers that were below the limit of detection were assigned a value of ½ of the detection limit for purpose of statistical analyses. The statistical analyses were carried out using the SPSS 15.0 (SPSS Inc. Chicago, IL, USA) and SAS 9.12 (SAS Institute, Cary, NC, USA) statistical packages.

Results

Study population

Selected characteristics of the three occupational groups are reported in Table I. The workers tended to be male, aged 30–40 years, with about a fourth to a third being cigarette smokers. According to *GSTM1* genotype, 11 controls, six varnish workers and five fibreglass workers had the null genotype. According to *GSTT1* genotyping, three controls, three varnish workers, and three fibreglass workers had the null genotype. These genotype frequencies are in agreement with allele frequencies previously reported for other European populations (Garte et al. 2001). No differences were detected in the frequencies of genotypes among the three study groups (χ^2 test).

Sty, StyOX and biomarkers

Table II summarizes median, minimum and maximum values of personal exposures to Sty and StyOX, urinary analytes before the work shift (BS) and at the end of the work shift (ES), and levels of protein adducts, in subjects divided according to job. Personal exposure to Sty and StyOX decreased in the order: fibreglass reinforced plastic workers >varnish workers >> control workers. Among reinforced plastic workers the ratio of airborne Sty to StyOX was 1000:7.3, which was about twice that observed in varnish workers (1000:3.6). Comparing subjects who used and did not use peroxide catalysts, the ratio Sty:StyOX decreased from 1000:10.9 to 1000:2.7, suggesting that the use of peroxides contributed to StyOX exposure.

Comparing BS urine samples among the three groups of workers, controls always had lower levels of StyU and Sty metabolites than the exposed workers. Median BS

Table I. Selected characteristics of investigated groups.

	Control workers	Varnish workers	Fibreglass reinforced plastic workers	Total
No. of subjects	22	13	8	43
No. male	17	13	8	38
Age (years), mean ± SD	47 ± 8	30 ± 5	41 ± 9	41 ± 10
Smokers (%)	27	38	25	30
No. of cigarettes/day, mean ± SD	22 ± 10	11 ± 2	20	17 ± 8
GSTM1 genotype (%)				
Active	42	54	37	45
Null	58	46	63	55
GSTT1 genotype (%)				
Active	84	75	63	77
Null	16	25	37	23

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Table II. Summary statistics of environmental and biological markers of Sty and StyOX exposure in the investigated subjects classified by job. For environmental and urinary markers, median values were obtained from individual geometric means of air and urine levels for each subject (3 to 4 samples per subject). Protein adducts were measured from a single blood sample for each subject. Values are median (min–max).

	Job title				
Exposure indices	Controls ($n=22$)		Varnish producers ($n=13$)	Fibreglass reinforced plastic workers ($n=8$)	
Airborne pollutants					
Sampling time	8 h workshift		8 h workshift	8 h workshift	
Sty (mg m^{-3})	<0.3 (<0.3)		3.4 (0.55–16.0)	18.2 (2.3–93.4)	
StyOX (μg m^{-3})	<5 (<5)		12.2 (6.7–32.0)	133.5 (39.5–281.5)	
Unmetabolized styrene					
Sampling time	BS	BS	ES	BS	ES
StyU (μg l^{-1})	0.5 (<0.2–1.4)	2.5 (1.9–8.9)	4.3 (1.8–53.6)	1.9 (1.1–6.5)	7.5 (2.1–29.7)
Mercapturic acids					
S,R-M2 (mg g^{-1} creatinine)	<0.0004 (<0.0004–0.002)	0.0209 (0.0004–0.0922)	0.1059 (0.0008–0.2333)	0.0547 (0.0010–0.4531)	0.1378 (0.0189–1.5779)
R,R-M1 (mg g^{-1} creatinine)	<0.0004 (<0.0004–0.002)	0.0336 (<0.0004–0.2307)	0.0968 (0.0009–0.2872)	0.0265 (0.0006–0.9112)	0.0575 (0.0137–1.6211)
R,R-M2 (mg g^{-1} creatinine)	<0.0004 (<0.0004–0.002)	0.0030 (<0.0004–0.0262)	0.0123 (0.0004–0.0300)	0.0053 (0.0004–0.1620)	0.0345 (0.0038–0.2328)
S,R-M1 (mg g^{-1} creatinine)	<0.0004 (<0.0004–0.002)	0.0007 (<0.0004–0.0056)	0.0028 (<0.0004–0.0080)	0.0008 (<0.0004–0.0180)	0.0036 (<0.0004–0.0142)
M1+M2 (mg g^{-1} creatinine)	<0.0016 (<0.0016–0.0080)	0.0937 (<0.0016–0.3878)	0.2059 (0.0032–0.5594)	0.1051 (0.0029–0.6528)	0.2619 (0.0394–3.4641)
Phenylethylene glycol derivatives					
MA (mg g^{-1} creatinine)	0.62 (0.18–1.34)	10.20 (2.58–31.81)	40.01 (7.95–130.71)	32.29 (13.04–242.47)	148.13 (30.64–515.12)
PGA (mg g^{-1} creatinine)	0.52 (0.26–1.57)	15.82 (4.00–38.87)	24.02 (8.13–56.71)	50.05 (17.26–240.31)	77.97 (20.89–248.99)
MA+PGA (mg g^{-1} creatinine)	1.17 (0.44–2.91)	24.95 (8.91–69.21)	69.52 (16.11–178.31)	83.74 (30.34–420.94)	226.30 (51.73–779.61)
PHG (mg g^{-1} creatinine)	0.15 (0.08–0.61)	0.62 (0.20–3.98)	0.97 (0.40–2.21)	2.83 (0.61–5.55)	3.18 (1.30–17.92)
4-Vinylphenol derivatives					
VP-G (mg g^{-1} creatinine)	0.137 (0.031–1.577)	0.468 (0.122–0.681)	0.810 (0.243–1.418)	0.418 (0.210–2.984)	1.597 (0.419–4.920)
VP-S (mg g^{-1} creatinine)	0.166 (0.074–1.085)	0.531 (0.110–1.224)	0.840 (0.149–2.042)	0.952 (0.628–7.448)	2.075 (0.612–11.681)
VP-G+ VP-S (mg g^{-1} creatinine)	0.245 (0.123–2.662)	1.124 (0.235–1.632)	1.721 (0.412–2.976)	1.372 (0.938–10.445)	3.697 (1.033–15.655)
Albumin adducts					
1PE-Alb (nmol g^{-1} protein)	0.19 (<0.03–0.53)	0.48 (0.21–0.75)	n.a.	0.23 (<0.03–1.22)	n.a.
2PE-Alb (nmol g^{-1} protein)	3.57 (<0.90–5.18)	6.18 (2.66–9.53)	n.a.	5.91(4.40–8.14)	n.a.
Haemoglobin adducts					
1PE-Hb (nmol g^{-1} protein)	0.22 (<0.03–0.99)	0.11(<0.03–0.55)	n.a.	0.20 (<0.03–0.74)	n.a.
2PE-Hb (nmol g^{-1} protein)	1.96 (1.01–3.33)	2.80 (<0.60–4.48)	n.a.	2.31 (2.18–5.12)	n.a.

BS, before workshift; ES, end of workshift; n.a., data not available.

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levels of metabolites between varnish workers and reinforced plastics workers were comparable except for MA and PGA, which were much greater in the reinforced plastics workers. Median levels of urinary analytes in ES samples tended to be greater in reinforced plastic workers than in varnish workers; these differences were significant ($p < 0.05$) for all analytes except Sty-U, all but one of the mercapturic acids (levels of R,R-M2 were not different), and VP-S.

Considering only exposed subjects, levels of the urinary analytes were about 2.5 times higher in ES samples than in BS samples. In ES samples among varnish workers and reinforced plastics workers, respectively, MA accounted for 48.1% and 63.4% of total urinary analytes, followed by PGA (48.4% and 33.4%), VP (2.1% and 1.6%), PHG (1.2% and 1.4%), M1+M2 (0.25% and 0.11%) and StyU, (0.005% and 0.003%). In BS samples the relative proportions of MA and PGA were inverted, with the highest excretion for PGA (56.8% and 57.8%), followed by MA (36.6% and 37.2%), PHG (2.2% and 3.3%), VP (4.0% and 1.6%), M1+M2 (0.34% and 0.12%) and StyU (0.009% and 0.002%). The different proportions of MA, PGA and PHG between ES and BS samples reflect the fact that MA is the precursor of PGA, which is the precursor of PHG (see Figure 1).

Among urinary mercapturic acids S,R-M2 and R,R-M1 represented about 84–95% of the total mercapturic acids, with S,R-M2 in ES samples predominating in both reinforced plastic workers and in varnish workers (49% and 59%, respectively).

Considering only styrene exposed workers, and assuming a urinary creatinine concentration of 1.0 g l^{-1}, the daily urine volume was 1.44 l (0.001 l min^{-1} urinary excretion rate $\times$ 1440 min per day). Assuming further that half of the daily urine volume contained biomarkers at median concentrations observed in BS samples while the other half contained biomarkers at median concentrations observed in ES samples, then the median total excretion of urinary biomarkers was 1.53 mmol per day for reinforced plastics workers (i.e. 0.72 l $\times$ (0.58+1.55) mmol g^{-1} creatinine $\times$ 1 g creatinine l^{-1}) and 0.45 mmol per day for varnish workers. These values are similar to estimated median values of Sty uptake, assuming low to moderate rates of exercise, namely 1.01–2.02 mmol daily for reinforced plastics workers (18.2 mg m^{-3} $\times$ 0.9 or 1.8 m^3 h^{-1} $\times$ 8 h per day $\times$ 0.8 (fraction of styrene absorbed)/104 mg styrene $mmol^{-1}$) and 0.19–0.38 mmol per day for varnish workers.

Regarding protein adducts, median levels of 1PE-Alb and 2PE-Alb were significantly higher in exposed workers than in control workers ($p < 0.05$), but did not differ significantly between reinforced plastics workers and varnish workers. Moreover, Hb adducts did not differ significantly across the investigated groups.

Influence of job, cigarette smoking, and GST genotypes

Significant effects of job, smoking and active genotypes of *GSTM1* and *GSTT1* are summarized in Table III, as determined by multiple linear regression of the logged biomarker levels. All urinary biomarkers significantly increased with job category in the order: control workers << varnish workers < reinforced plastics workers. Cigarette smoking was associated with increased levels of the conjugates of VP, as anticipated based upon a previous study (Manini et al. 2003), and also with increased levels of mercapturic acids. Regarding the GST genotypes, subjects with active *GSTM1* had significantly higher levels of mercapturates (except the R,R-M2 isomer). On the other hand, subjects with active *GSTT1* had marginally higher levels of 2PE-Hb. The

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Table III. Screening of effects on biomarker levels of job, smoking and GST genotype by multiple linear regression analysis. Only significant effects are shown (all urinary analytes were measured in end-of-shift urine samples).

Biomarker	No. of subjects	Job	Smoking	*GSTM1* (Active)	*GSTT1* (Active)
Unmetabolized styrene					
StyU	37	***(+)			
Mercapturic acids					
R,R-M1	37	***(+)	*(+)	***(+)	
S,R-M1	37	***(+)	**(+)	***(+)	
R,R-M2	37	***(+)	**(+)		
S,R-M2	37	***(+)	**(+)	***(+)	
M1+M2	37	***(+)	**(+)	***(+)	
Phenylethylene glycol derivatives					
MA	37	***(+)			
PGA	37	***(+)			
MA+PGA	37	***(+)			
PHG	37	***(+)			
4-Vinylphenol derivatives					
VP-G	37	***(+)	***(+)		
VP-S	37	***(+)	***(+)		
VP-S+VP-G	37	***(+)	***(+)		
Albumin adducts					
1PE-Alb	37	**(+)			
2PE-Alb	37	***(+)			
Haemoglobin adducts					
1PE-Hb	37				
2PE-Hb	37		*(−)		*(+)

*$0.05 \leq p < 0.10$; **$0.01 \leq p < 0.05$; ***$p < 0.01$; (+) indicates that the biomarker level increased with the effect; (−) indicates that the biomarker level decreased with the effect.

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significant effects of *GSTM1* on mercapturate levels were anticipated based upon a previous study (de Palma et al. 2001). When regression models were run with BS data, the effect of *GSTM1* polymorphisms on mercapturates levels was somewhat higher than for ES levels (results not shown).

Although both Alb adducts were significantly higher in exposed subjects than in controls (Table II), trends were not observed between levels of Hb and Alb and adducts and job category as noted above for the urinary analytes. Levels of 2PE-Hb were negatively associated with smoking status and positively associated with active *GSTT1*.

Pearson correlations

Table IV lists pair-wise Pearson correlation coefficients of the levels of Sty and StyOX in air and those of selected biomarkers in the exposed subjects ($n = 18$–20). Highly significant positive correlations were found between all the urinary biomarkers and Sty, with values of r ranging from 0.789, for M1+M2 vs Sty, to 0.974, for MA vs Sty; slightly lower correlation coefficients were observed between the urinary biomarkers and StyOX. Correlations between Sty or StyOX and protein adducts were not significant ($p > 0.05$). The urinary biomarkers were all highly correlated with each

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Table IV. Pearson's correlations between environmental and selected biological markers across subjects occupationally exposed to styrene ($n = 18$–20) (all urinary analytes were measured in end-of-shift urine samples).

	StyOX	StyU	M1+M2	MA	PGA	MA+PGA	PHG	VP-S+VP-G	1PE-Alb	2PE-Alb
Sty	0.794***	0.810***	0.789***	0.975***	0.931***	0.974***	0.892***	0.941***	−0.121	−0.147
StyOX		0.451*	0.566**	0.766***	0.818***	0.787***	0.846***	0.775***	−0.322	−0.073
StyU			0.660***	0.657***	0.626***	0.653***	0.454**	0.712***	−0.019	−0.272
M1+M2				0.738***	0.773***	0.756***	0.685***	0.820***	−0.167	−0.424*
MA					0.940***	0.995***	0.887***	0.950***	−0.142	−0.118
PGA						0.970***	0.896***	0.940***	−0.160	−0.147
MA+PGA							0.901***	0.958***	−0.142	−0.121
PHG								0.885***	−0.146	−0.078
VP-S+VP-G									−0.045	−0.174
1PE-Alb										0.446**

*$0.05 \leq p < 0.10$; **$0.01 \leq p < 0.05$; ***$p < 0.01$.

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other, with values of r as high as 0.958 (between MA+PGA and VP-S+VP-G). The only significant or borderline significant correlations observed between urinary biomarkers and protein adducts were the negative correlations between the mercapturic acids and 2PE-Alb. Levels of 2PE-Alb were also significantly correlated with 1PE-Alb. Somewhat smaller correlations were observed using BS instead of ES values (results not shown).

Figure 2A–D shows the scatter plots and fitted straight-line regressions (in log scale) between ES StyU, MA, M1+M2 (stratified by *GSTM1* genotype) and VP-S+VP-G, and airborne Sty. For MA the data pair exhibited the highest correlation of any biomarker with Sty or StyOX exposure (Figure 2C). The fitted relationship reinforces the notion that ES MA can be successfully used for biomonitoring of Sty exposures at air concentration as low as 1 mg m^{-3}.

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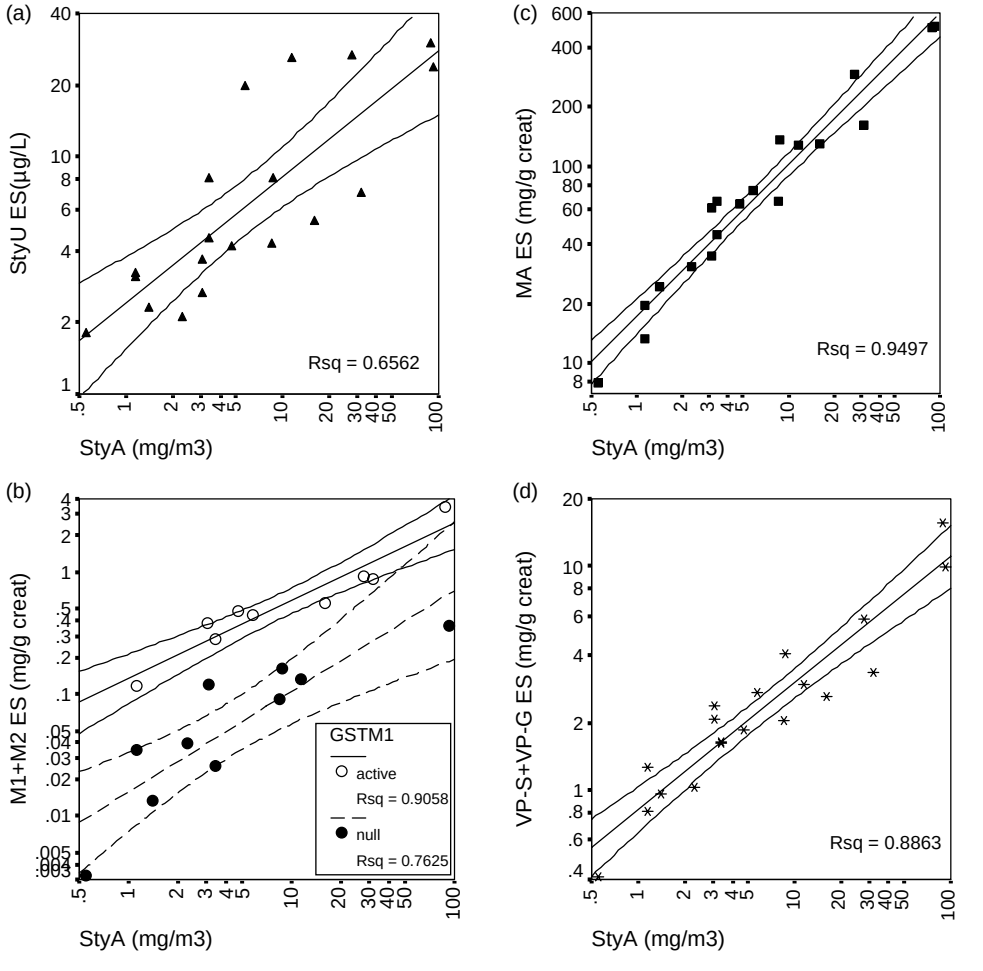

Figure 2. (A–D) Linear regression between end-shift levels of urinary styrene (StyU) (A), mercapturic acids (M1+M2) (B), mandelic acid (MA) (C) and 4-vinylphenol conjugates (VP-G+VP-S) (D), and personal exposure to airborne styrene (Sty) in styrene-exposed workers. Each point in the scatter plots represents a data pair obtained as geometric mean of 3 to 4 determinations for each subject. In (B) data pairs are divided according to *GSTM1* genotype.

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Effects of exposure to Sty and StyOX

The multiple regression models of urinary and blood analyte levels, regressed on air levels of Sty and StyOX among styrene-exposed workers, are reported in Table V. For each urinary and blood analyte (dependent variable), values of model R^2 (adjusted) and regression coefficients are indicated for the predictor variables, Sty and StyOX. All urinary analytes were significantly associated with Sty exposure, after adjusting for co-exposure to StyOX ($p < 0.01$), while levels of only StyU and PHG were significantly associated with StyOX exposure, after adjusting for co-exposure to Sty ($p < 0.05$). However, opposite directions of the associations between StyOX exposure and levels of StyU (−) and PHG (+) were observed.

Since these multiple linear regression models were performed on the logged air and biomarker levels, effect coefficients close to one indicate that the biomarker level should be proportional to the particular exposure variable in natural scale, while effect coefficients less than one indicate downward concavity in the relationship between the analyte and the exposure variable in natural scale (Rappaport et al. 2002a,b). As styrene was an important predictor variable for all urinary analytes, it is, therefore, interesting that the regression coefficients for Sty in models of StyU ($\beta_{Sty} = 0.799$) and the mercapturates ($0.845 \leq \beta_{Sty} \leq 1.112$) were not significantly different from one, suggesting linear production of these analytes with increasing levels of Sty exposure.

Table V. Effects on biomarker levels of exposure to styrene (Sty) and styrene oxide (StyOX), determined by multiple linear regression of the logged levels (all urinary analytes were measured in end-of-shift urine samples).

Biomarker	No. of subjects	R^2 (adj.)	β_0	β_{Sty}	β_{StyOX}
Unmetabolized styrene					
StyU	19	0.726	1.691***	0.799***	−0.376**
Mercapturic acids					
R,R-M1	19	0.400	−4.558***	1.112**	−0.143
S,R-M1	19	0.514	−7.018***	0.845***	−0.208
R,R-M2	19	0.826	−6.089***	1.041***	−0.039
S,R-M2	19	0.605	−3.800***	1.096***	−0.222
M1+M2	19	0.587	−3.046***	1.072***	−0.213
Phenylethylene glycol derivatives					
MA	19	0.944	2.891***	0.785***	−0.019
PGA	19	0.868	2.097***	0.543***	0.167
MA+PGA	19	0.943	3.322***	0.710***	0.030
PHG	19	0.826	−0.887***	0.363***	0.250**
4-Vinylphenol derivatives					
VP-G	19	0.738	−1.106***	0.482***	0.050
VP-S	19	0.822	−1.103***	0.544***	0.091
VP-S+VP-G	19	0.874	−0.300	0.529***	0.048
Albumin adducts					
1PE-Alb	18	0.045	0.051	0.287	−0.509
2PE-Alb	18	−0.102	1.755	−0.055	0.031
Haemoglobin adducts					
1PE-Hb	18	−0.064	−3.132***	0.004	0.243
2PE-Hb	18	−0.113	1.006**	0.090	−0.070

β_0, β_{Sty}, β_{StyOX} are the regression coefficients representing the intercept, exposure to styrene and exposure to styrene oxide, respectively. **$0.01 \leq p < 0.05$; ***$p < 0.01$.

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Yet, the corresponding models of the other urinary metabolites, including the products of phenylethylene glycol (i.e. MA and PGA) and of 4-vinylphenol (i.e. VP-G and VP-S), all had values of β_{Sty} that were significantly less than one ($p < 0.05$), suggesting saturable metabolism along the corresponding pathways (Figure 1).

Regarding the protein adducts of StyOX, no significant effects of exposure to Sty or StyOX were observed for levels of Alb and Hb adducts in the exposed workers.

Discussion

In the present study environmental monitoring and biological monitoring of exposure to both Sty and StyOX were performed in two groups of exposed workers, and in a group of unexposed workers. By including a comprehensive set of biomarkers of exposure, including unmetabolized Sty in urine (StyU), all recognized urinary Sty metabolites, adducts of StyOX with Hb and Alb, as well as biomarkers of susceptibility (polymorphisms of *GSTT1* and *GSTM1*), we wished to develop a detailed picture of Sty and StyOX metabolism in humans. By adopting a repeated measures sampling design among exposed workers, we were able to reduce sources of variability that often obscure exposure–biomarker relationships in cross-sectional investigations.

Among exposed workers, the average Sty exposure level was below the accepted occupational exposure limit of 85 mg m^{-3}, for all subjects except one reinforced plastics worker (employed in the lamination process during production of boats) who had a geometric mean exposure of 97 mg m^{-3}. As expected this subject was also the one with the highest level of urinary biomarkers (see Figure 2). StyOX exposure in these workers was about three orders of magnitude lower than that of Sty, and in good agreement with previously published data (Pfäffli & Säämänen 1993, Rappaport et al. 1996, Nylander-French et al. 1999, Tornero-Velez et al. 2000). Moreover, we report for the first time that exposure to StyOX was measured during the production of varnishes that employed styrene as an ingredient. In our investigation, levels of Sty and StyOX were highly correlated across exposed subjects with values of Pearson $r = 0.940$ across reinforced plastics workers and $r = 0.564$ across varnish workers.

Whereas levels of all urinary Sty metabolites in exposed workers were significantly positively associated with airborne Sty exposure (after adjusting for StyOX exposure), only those of PHG were also significantly positively associated with StyOX exposure (after adjusting for Sty exposure) (see Table V). This suggests that the relatively minor metabolite PHG may be a useful biomarker for co-exposures to both Sty and StyOX in air. Actually the biologically plausibility of this observation is not clear, as no direct metabolic connection between StyOX and PHG is recognized (see Figure 1). For this reason this finding needs further evidences before any practical implication for the biomonitoring of StyOX exposure could be foreseen.

The most abundant Sty metabolites were MA and PGA, which together accounted for about 95% of all urinary metabolites, both at the beginning and end of the work shift (BS and ES, respectively). Neither smoking nor genetic polymorphisms of GST had significant effects on urinary levels of MA and PGA (Table III). The high levels of MA and PGA observed in exposed workers, plus the relatively low levels of these metabolites observed in control workers (see Table II), and the lack of smoking effects, supports the use of MA and PGA as biomarkers of occupational exposure to Sty. This

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conclusion is reinforced by the simplicity and convenience of the assay for these urinary analytes (for example see Buratti et al. 1989).

When VP conjugates are considered, we found that they accounted for 2–5% of all urinary Sty metabolites. Although these metabolites were also strongly associated with Sty exposure (Table V), smoking significantly increased urinary levels of VP metabolites (Table III) as anticipated (Manini et al. 2003). Given the relatively minor abundance of VP conjugates in exposed subjects and the potential for confounding by cigarette smoking, the VP metabolites would appear to offer little utility as biomarkers of occupational exposure to Sty.

Although multivariable analyses indicated that airborne Sty was a strong predictor of urinary levels of MA, PGA, and the VP conjugates, the log-scale regression coefficients for Sty exposure were significantly less than one in all cases (upper 95% confidence limits of β_{Sty}: MA = 0.940, PGA = 0.756, VP-G = 0.731, VP-S = 0.776), suggesting downward concavity in the relationship between each metabolite and Sty exposure in natural scale (Rappaport et al. 2002a,b). This points to partial saturation of the predominant CYP-mediated metabolic pathways for Sty. Partial saturation of Sty metabolism beginning at about 200 mg m^{-3} has been reported previously in human volunteers following experimental exposure to Sty (reviewed in Löf & Johanson 1998). Our results indicate that such saturable effects are observed at much lower air concentrations of Sty since the exposed subjects in our study had geometric mean Sty exposures between 0.55 and 93.4 mg m^{-3}.

The mercapturic acids were the least abundant metabolites measured in our study, collectively accounting for about 0.1% of the total urinary metabolites. The correlations between levels of the mercapturic acids and those of airborne Sty and StyOX were weaker than those for the other Sty metabolites (Table IV). The mercapturates were also the only urinary analytes that were significantly affected by genetic polymorphisms of *GSTM1* (Table III), with subjects bearing the null genotype in a given job having lower level of mercapturic acids than their co-workers with the active genotype, consistent with earlier reports (de Palma et al. 2001, Haufroid et al. 2001). Moreover, for the first time, increased levels of these metabolites were also found following cigarette smoking. Given their low abundance and the difficulties related to the separation, identification and quantitation of the four different mercapturates stereoisomers, these metabolites are not good candidates for use in routine biomonitoring of occupational Sty exposure.

On the other hand, the mercapturates offer potentially useful information regarding stereochemistry of the metabolism of Sty in humans. In fact, both of the most abundant stereoisomers S,R-M2 and R,R-M1 are derived from less genotoxic (S)styrene-(7,8)-oxide (Linhart et al. 1998). Because previous evidence demonstrated that inhaled Sty is transformed via both (S) and (R)styrene-(7,8)-oxide (Kežić et al. 2000, Wenker et al. 2000), the low availability of (R)styrene-(7,8)-oxide to form the mercapturic acid may be related to the higher efficiency of microsomal epoxide hydrolase toward hydrolysis of this stereoisomer to give phenylethylene glycol (Wenker et al. 2000). We observed that S,R-M2 accounted for 52% of the total ES M1 + M2, whereas R,R-M1 accounted for only 29%. This suggests that conjugation of glutathione with the less hindered C8 of (S)styrene-(7,8)-oxide to form S,R-M2 is favoured over conjugation with the more hindered C7.

It is also intriguing that the log-scale regression coefficients for Sty exposure in our multivariable models of mercapturates levels were all very close to one. This indicates

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that, in contrast to the derivatives of phenylethylene glycol and VP discussed above, there was no evidence of partial saturation of the pathway(s) leading to the mercapturates. Since the accepted pathways of Sty metabolism shown in Figure 1, indicate that the mercapturates should be derived from StyOX formed from an initial CYP oxidation of Sty, this suggests that an alternative, unrecognized pathway may exist for production of the mercapturates. Interestingly, similar results have recently been published, which indicate that *S*-phenylmercapturic acid, derived from metabolism of benzene, is not subject to the saturable effects of benzene metabolism that affect the major benzene metabolites (Kim et al. 2006a,b).

When StyU is considered, we found that it accounts for less then 0.01% of all the urinary analytes. The correlation of StyU with Sty in ES samples ($r = 0.810$ in exposed workers) was weaker that that observed for MA, PGA and VP, but stronger than that of the mercapturic acids. The correlation of StyU with StyOX exposure was not significant. Although it is known that Sty is present in cigarette smoke (Smith & Hansch 2000), no significant effect of smoking was found on the urinary levels of StyU in our subjects (Table III). Moreover, the assay for StyU requires special precautions, regarding the sampling and handling of urine, to avoid loss and contamination of the analyte. Given these constraints, there are no obvious advantages for using StyU as a biomarker of occupational Sty exposure, particularly given the convenience of measuring MA and PGA, as noted above.

Regarding levels of protein adducts in exposed workers, levels of both Alb adducts were positively associated with exposure status (i.e. controls < exposed workers) indicating effects of exposure on adduct production. However, no effects were observed for the corresponding adducts of Hb, and none of the protein adducts was significantly associated with individual exposure to Sty or StyOX among exposed workers (Table V). Furthermore, we observed high levels of background protein adducts as previously reported in other studies (Yeowell-O'Connell et al. 1996, Fustinoni et al. 1998). Such background adducts tends to obscure exposure–adduct relationships unless large numbers of subjects are investigated. Interestingly, we observed significant or borderline significant negative correlations between 2PE-Alb and both BS and ES mercapturic acids (Table IV; $r = -0.468$ and -0.424, respectively). These correlations suggest a competition toward the reaction of StyOX with cysteinyl residues of Alb and glutathione.

In designing the study we were aware of the limited sample size, and the multiple sampling protocol was set also to overcome this limit. Our results confirm that this approach is very useful in explaining the observed variability, in fact multiple regression models, summarized in Table V, had relatively large coefficients of determination for each of the urinary analytes in our study ($0.400 \leq R^2$ (adj.) ≤ 0.944). While some of the reduction in random errors can be attributed to the good precision of the assays used for the urinary analytes, we conclude that the repeated-measurements protocol reduced exposure measurement errors that can obscure exposure–biomarker relationships (Symanski et al. 2001, Lin et al. 2005).

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