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Determination of urinary mercapturic acids of styrene in man by high-performance liquid chromatography with fluorescence detection

Luciano Maestri, Sergio Ghittori*, Marcello Imbriani

Centro Ricerche di Fisiopatologia e Sicurezza del Lavoro, University of Pavia, Clinica "S. Maugeri", IRCCS, Medical Center of Pavia, Via S. Boezio 24, 27100 Pavia, Italy

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Abstract

A method for the determination of urinary N-acetyl-*S*-(1-phenyl-2-hydroxyethyl)-L-cysteine (M1) and N-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-L-cysteine (M2) in man was developed. Clean-up of urine samples was obtained by a chromatographic technique, using a short reversed-phase precolumn; purified samples were then deacetylated with porcine acylase I for 16 h at 37°C and deproteinized by centrifugal ultrafiltration. Derivatization was performed with *o*-phthaldialdehyde and 2-mercaptoethanol and the fluorescent derivatives were separated on a reversed-phase analytical column with a gradient mobile phase consisting of 50 m*M* acetate buffer (pH 6.5) and methanol. The retention times of the diastereoisomers of M1 (M1-"*S*" and M1-"*R*") were 52.8 and 73.7 min, respectively; M2 diastereoisomers eluted as a single peak at 70.5 min. The fluorescence detector was set at 330 nm (excitation) and 440 nm (emission). The detection limit (at a signal-to-noise ratio of three) was about 7 μg/l. The method was applied to 25 urine samples from workers exposed to styrene. A relationship was found between urinary mandelic and phenylglyoxylic acids and mercapturic acids specific for styrene. Urine samples from ten non-exposed subjects showed no detectable amounts of analytes.

Keywords: Mercapturic acids; Styrene

1. Introduction

Styrene, a chemical used extensively in the production of plastics, polyester resins and synthetic rubbers, has been shown to be responsible for neurotoxic effects, including behavioural changes and neuroendocrine disturbances [1–3]. A possible relationship between occupational exposure to styrene and lymphopoietic cancers and leukaemia have been suggested, although at present no conclusive epidemiological data have been collected [2–4]. The initial step of styrene metabolism is conversion by hepatic cytochrome P-450 to styrene-7,8-epoxide [5], which is present in two enantiomeric forms [*R*-(+)-styrene-7,8-epoxide and *S*-(−)-styrene-7,8-epoxide] [6–8]; this electrophilic intermediate is capable of binding covalently with macromolecules [9,10] and is considered directly responsible for most of the toxic effects of styrene. The main subsequent detoxification processes of styrene-7,8-epoxide involve two distinct metabolic pathways (Fig. 1): it may be both hydrolyzed to styrene glycol by epoxide hydrolase [11] or conjugated with glutathione (GSH), spontaneously or by glutathione-S-transferase (GST) [12,13]. The major urinary metabolites derived from the biotransformation of styrene glycol

*Corresponding author.

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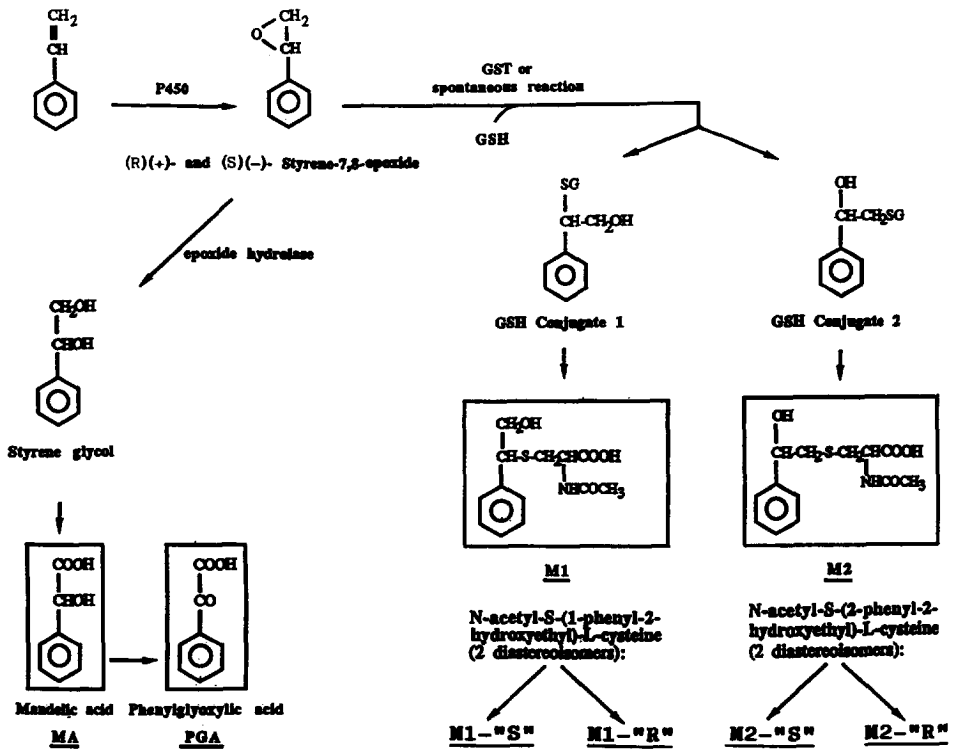

Fig. 1. Metabolic pathways of styrene.

in man are mandelic acid (MA) and phenylglyoxylic acid (PGA), which are commonly used in the biological monitoring of occupational exposure to styrene [14]. GSH conjugates of styrene-7,8-epoxide are further metabolized to mercapturic acids [15,16]. As a consequence of the conjugation of two enantiomers of styrene-7,8-epoxide to GSH, the mercapturic acids of styrene excreted in urine consist of two diastereoisomeric forms [17,18] (Fig. 1). The stereochemistry of the conjugation has been described by Hiratsuka et al. [19]. Stereoselectivity of the enzymes involved in the metabolism of styrene has been demonstrated by "in vitro" and "in vivo" studies [6–8]. Specific mercapturic acids have been identified in the urine of rats exposed to styrene [17]; the total excretion amounted to about 10% of the administered dose. A close relationship was found between the dose of styrene and the urinary excretion of N-acetyl-*S*-(1-phenyl-2-hydroxyethyl)-L-cysteine (M1) and N-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-L-cysteine (M2), the major mercapturic acids of styrene in rats [20]. Such results seem to indicate that the determination of mercapturic acids allows a direct estimation of the internal dose of styrene-7,8-epoxide. In comparison with a large number of experiments on animals, very little data has been collected about the excretion of mercapturic acids specific for styrene in man. Norstrom and co-workers [21] failed in their attempt to detect M2 in urine of volunteers exposed to a styrene concentration of 205–220 mg/m^3 for 2 h, by means of HPLC with electrochemical detection: it was not possible to detect any amount of M2 higher than the detection limit of the method, i.e. 10 mg/l. Hallier and co-workers [22] and Gerin et al. [23] found measurable levels of mercapturic acids in the urine of only a few workers occupationally exposed to styrene. These findings emphasize that the conversion of this solvent to mercapturic acids is much lower in man (below 1% of the absorbed dose) than in rats (about 10%). Thus, a method is needed which allows the evaluation of the low levels of mercapturic acids that are expected in the urine of humans exposed to styrene. Methods based on gas chromatography

appear to be unsuitable because of the thermal instability of the methyl esters of these compounds [21,24]; on the other hand, methods reported in the literature for the determination of M1 and M2 in rats by HPLC with UV detection seem to have poor sensitivity [20,25]. This paper describes a method for the analysis of urinary M1 and M2 in man by means of HPLC with fluorescence detection after deacetylation and derivatization with *o*-phthaldialdehyde and 2-mercaptoethanol. The method also makes it possible to separate the diastereoisomers of M1 which arise from *R*-(+)- and *S*-(−)-styrene-7,8-epoxide, respectively, i.e. M1-"*R*" and M1-"*S*". In this study, M1-"*R*" and M1-"*S*" refer exclusively to the compounds which derive from *R*-(+)- and *S*-(−)-styrene-7,8-epoxide, regardless of the absolute stereochemical configuration of the diastereoisomers.

2. Experimental

2.1. Chemicals

o-Phthaldialdehyde (OPA), 2-mercaptoethanol (MCE), porcine acylase I (grade III) (EC 3.5.1.14) and N-acetyl-L-cysteine were from Sigma (Munich, Germany). Racemic and *R*-(+)-styrene-7,8-epoxide were from Aldrich (Steinheim, Germany), methanol and water were of HPLC grade (BDH, Poole, UK), while all other reagents and solvents were of analytical grade. The derivatizing reagent solution was prepared by dissolving 50 mg of *o*-phthaldialdehyde in 300 μl of a 0.2% solution of MCE in methanol, then adding 2 ml of borate buffer (pH 10). The filtered mixture could be stored at −20°C for at least one week without noticeable degradation. Centrifugal ultrafiltration units (Centrisart I, cut-off 10 000) were from Sartorious (Gottingen, Germany). Drierite (anhydrous calcium sulfate) was from Aldrich.

2.2. Apparatus

The HPLC system consisted of a Waters (Milford, MA, USA) 600 E pump, a Jasco (Tokyo, Japan) 820-FP spectrofluorometer, a Waters WISP 715 autosampler and a NEC APC-IV (Boxborough, MA, USA) computer for the acquisition and processing of the data. A Gilson (Villiers-le-Bel, France) 201 fraction collector and a Waters 484 UV detector (set at 225 nm) were used for the isolation of single M1 and M2 isomers and for the clean-up of urine samples. The analytical column was an ODS Hypersil, 250×4.6 mm I.D., 3 μm (Shandon, UK).

2.3. Synthesis of mercapturic acid standards

Standards of M1 and M2 were synthesized by the method of Yagen et al. [26], with some modifications. Briefly, N-acetyl-L-cysteine (2 mmol) was dissolved in 25 ml of water adjusted to pH 7.4 with NaOH, then 2.5 mmol of racemic or *R*-(+)-styrene-7,8-epoxide dissolved in 2 ml of dimethyl sulfoxide were added. The mixture was stirred under nitrogen for 24 h at room temperature and extracted with 5×15 ml of ethyl acetate. The aqueous residue was acidified to pH 1 with HCl, then saturated with NaCl. The filtered solution was extracted with 6×100 ml of chloroform, which was evaporated under vacuum. The crude standard prepared with racemic styrene-7,8-epoxide consisted of a mixture of M1 and M2, each in two diastereoisomeric forms, while the standard prepared from *R*-(+)-styrene-7,8-epoxide consisted of M1-"*R*" and M2-"*R*". Pure standards of M1-"*R*", M1-"*S*", M2-"*R*" and M2-"*S*" were isolated by semipreparative HPLC on a Supelcosil C_{18}, 150×4.6 mm I.D., 3 μm column (Supelco, Bellefonte, PA, USA). The mobile phase consisted of water (adjusted to pH 3.0 with phosphoric acid)–methanol (87:13, v/v) delivered at a flow-rate of 0.8 ml/min. Under these conditions, mercapturic acid isomers were separated enough to allow four fractions to be collected. Each fraction was then acidified to pH 1 with HCl and extracted with two volumes of ethyl acetate. The organic layers were evaporated under nitrogen and the residues were kept in a desiccator filled with Drierite. Purity of the fractions was found to range between 86.8 and 95.6% and experimental data was corrected for, based on the purity of standards. The identity was confirmed by NMR analysis. In order to confirm that the chemical standards did correspond to mercapturic acids excreted "in vivo", the following experiment was performed: rats were exposed i.p. to racemic or *R*-(+)-styrene-7,8-epoxide, urine samples were acidified and extracted with ethyl acetate. The organic layers were evaporated to dryness, reconsti-

tuted with methanol–water (1:1, v/v) and analyzed by the same analytical system described for semi-preparative HPLC. Urine samples taken from rats exposed to racemic styrene-7,8-epoxide showed the same four peaks of crude standard derived from racemic styrene-7,8-epoxide, while samples from rats exposed to *R*-(+)-styrene-7,8-epoxide showed only two peaks, corresponding to M1-"*R*" and M2-"*R*".

2.4. Sample collection and storage

Urine samples (10 ml) were frozen at −20°C immediately after collection and thawed just before analysis. Biological samples from 25 workers exposed to styrene were collected at the end of the workshift; the control group consisted of ten non-exposed non-smoker subjects.

2.5. Pretreatment of samples

Sample clean-up was performed as follows: a 100-μl volume of filtered urine (acidified to pH 1–2 with H_3PO_4) was injected onto the chromatographic system equipped with a Water Resolve C_{18} pre-column; the mobile phase consisted of a water (adjusted to pH 3.0 with H_3PO_4)–methanol (82:18, v/v) mixture delivered at a flow-rate of 2 ml/min. The fraction which eluted between 0.7 and 1.15 min was collected (previous studies have shown that, under these conditions, M1 and M2 eluted in this fraction). The precolumn was washed with 100% methanol for 3 min and reconditioned with mobile phase for 5 min prior to each injection. To each purified sample, 200 μl of a solution containing 42 000 U/ml of acylase I in 0.5 *M* phosphate buffer (pH 7.0) were added. Samples were taken at 37°C for 16 h and deproteinized by centrifugal ultrafiltration (15 min at 1000 *g*).

2.6. Derivatization

Aliquots of the filtered samples (90 μl) were derivatized for 5 min at room temperature with 10 μl of the fluorescent reagent (see Section 2.1). The mixture was than injected onto the HPLC apparatus (sample derivatization and injection were automatically performed by the autosampler).

2.7. Chromatographic analysis

Separation was performed by gradient elution with solvent A (acetate buffer, 0.05 *M*, pH 6.5), solvent B (methanol), delivered according to the scheme shown in Table 1. The temperature of the analytical column (35°C) was controlled by means of a column heater. Solvents were constantly degassed with helium during analysis. The retention times of M1-"*S*", M2 and M1-"*R*" derivatives were 52.8, 70.5 and 73.7 min, respectively. The excitation and emission wavelengths of the detector were set at 330 and 440 nm, respectively.

2.8. Determination of MA and PGA

Urinary MA and PGA were determined by an HPLC method as described in literature [27].

Table 1
Chromatographic conditions for the determination of the mercapturic acids of styrene

Time (min)	Curve	Flow-rate (ml/min)	Mobile phase % A	Mobile phase % B
67	Isocratic	0.65	60	40
9	Isocratic	0.65	10	90
6	Increasing flow-rate up to	1.00	10	90
18	Isocratic	1.00	10	90
5	Isocratic	1.00	60	40
4	Decreasing flow-rate down to	0.65	60	40
15	Isocratic	0.65	60	40

Column: ODS Hypersil, 250×4.6 mm I.D., 3 μm (Shandon, UK). Detector: fluorescence spectrophotometer; excitation 330 nm, emission 440 nm. Sample volume: 100 μl. Mobile phase: A=0.05 *M* acetate buffer (pH 6.5), B=methanol. Solvents were constantly degassed with helium to avoid bubble formation.

3. Results and discussion

In order to eliminate most interfering substances which may dramatically decrease the sensitivity of the analytical method [21] a clean-up of the urine sample is needed prior to the chromatographic separation. Attempts to purify samples by simple liquid–liquid or solid-phase extraction methods gave poor results, while the method of sample clean-up described in this paper allowed us to obtain highly purified samples with quantitative recovery (>99%) of analytes. To achieve adequate sensitivity, mercapturic acids were then deacetylated [28] and the resulting *S*-(1-phenyl-2-hydroxyethyl)-L-cysteine and *S*-(2-phenyl-2-hydroxyethyl)-L-cysteine were derivatized with the OPA–MCE reagent, a well-known fluorogenic reagent that is specific for primary amines [29]. An enzymatic reaction was necessary because chemical deacetylation of M1 and M2 with concentrated HCl at high temperature gave poor reproducibility, probably due to the partial decomposition of compounds. Preliminary studies of the enzymatic reaction performed with acylase I have revealed a different affinity of the enzyme for each mercapturic acid of styrene, but the reaction was complete for each compound after 16 h at 37°C (Fig. 2). It was not possible to determine the actual extent of M1 and M2 deacetylation because suitable standards of *S*-(1-phenyl-2-hydroxyethyl)-L-cysteine and *S*-(2-phenyl-2-hydroxyethyl)-L-cysteine were not available. Analyses of the reaction mixture by HPLC–UV after 16 h showed only traces of the peaks relative to the mercapturic acids, but this did not demonstrate that all the compounds (M1 and M2) had been deacetylated: the production of unknown products other than *S*-(1-phenyl-2-hydroxyethyl)-L-cysteine and *S*-(2-phenyl-2- hydroxyethyl)-L-cysteine could not be excluded. In our opinion, however, it must be stressed that for practical purposes it was important to verify whether a "plateau" was reached after an appropriate reaction time, and to demonstrate that the conversion rate of the analytes was not influenced by the urinary matrix (Fig. 2). Like most OPA–MCE derivatives [29], the fluorescent derivatives of styrene mercapturic acids also showed long-term instability, thus a careful control of the reaction time was necessary, in order to obtain adequate reproducibility. The fluorescence response (peak areas) after a reaction time of 45 min was one-half of the signal after a reaction time of 5 min: this is in agreement with our previous reports about the OPA–MCE derivatives of specific mercapturic acids of benzene and toluene [30]. When using the OPA–MCE reagent for a compound contained in a complex matrix, attention must be given to eliminate most of amines and amino acids which could cause depletion of reagent, leading to non-linear responses. In our experiments, when the above-described clean-up procedure was used, a close linearity was found between the amounts of the mercapturic acids standard added to urine samples and the peak areas, up to a concentration of 20 mg/l. This demonstrated that the OPA–MCE reagent was in excess with respect to the total amount of derivatizable groups contained in the purified samples. Fig. 3 shows some chromatograms of standards and of urine samples. The analytical conditions described in Table 1 allowed us to separate the peaks of M1-"*R*" and M1-"*S*" derivatives, while M2-"*R*" and M2-"*S*" eluted as a single peak. Further attempts to reduce the analytical time showed that the use of organic modifiers, other than methanol, such as acetonitrile or tetrahydrofurane, gave poorer resolution and that the addition of counter-ions (tetraethylammonium- or tetrabutylammonium chloride) to the mobile phase caused peak broadening which

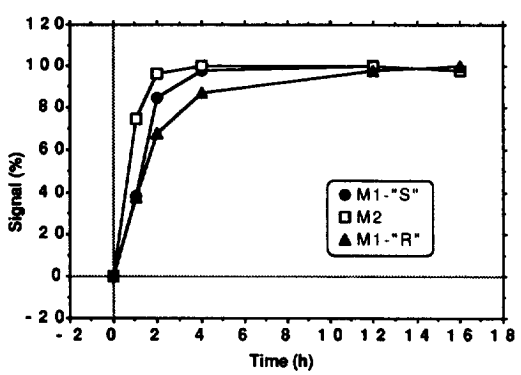

Fig. 2. Kinetic curves of the enzymatic deacetylation of styrene mercapturic acids. The reaction was performed at 37°C on a standard solution of M1 and M2 after the addition of acylase I. Aliquots were analyzed at fixed times by the method described in the text. Fluorescence response (percent of the highest one) was plotted against reaction time for each peak. Kinetic curves obtained for spiked urine were identical to those obtained for the standard solution.

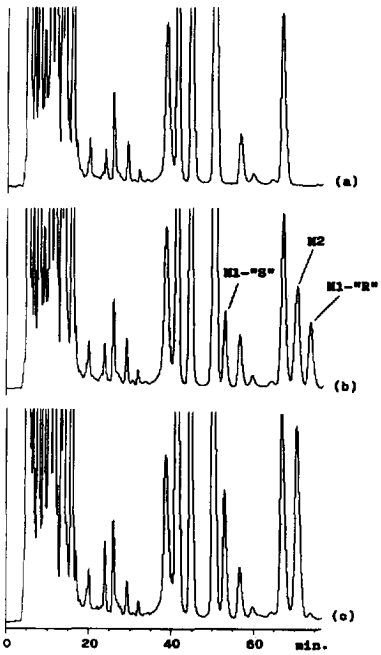

Fig. 3. Examples of chromatograms obtained from the following samples: (a) urine from an unexposed subject; (b) the same as (a) after the addition of authentic mercapturic acid standard; (c) urine sample of a subject that was occupationally exposed to styrene. The concentrations of the mercapturic acids were M1-"*S*"=5728 μg/l, M2=5260 μg/l and M1-"*R*"=280 μg/l.

resulted in higher limits of detection. Some erosion of the stationary phase occurred at pH values >7.3; on the other hand, the stability of OPA derivatives decreased dramatically at pH values <6.4. A chiral inversion of either M2-"*R*" or M2-"*S*" during derivatization (either at the benzylic or at the alpha carbon of cysteine) cannot be excluded; in this case, the same product can be formed as a result of the derivatization of both compounds. A chiral inversion of both M1"*S*" and M1-"*R*" may also occur, but each of them gives a single peak, thus the identity of the conjugation reaction products "in vivo" may be confirmed by simple comparison of the peaks found in urine with those found in authentic purified standards. It was difficult to obtain data about the actual stereochemical configuration of OPA–MCE derivatives because of their instability.

Authentic mercapturic acid standard (from 20 to 20 000 μg/l) was added to the urine used as a blank from a non-exposed subject; samples were then

Table 2
Reproducibility of the method

Sample	Compound	Concentration[a] (μg/l)	Intra-assay C.V. (%)[b]	Inter-assay C.V. (%)[b]
1	M1-"*S*"	339	3.19	4.23
	M1-"*R*"	317	5.62	6.35
	M2	312	4.12	5.71
2	M1-"*S*"	1639	2.37	3.12
	M1-"*R*"	1585	3.30	4.81
	M2	1512	2.98	3.58
3	M1-"*S*"	8460	1.15	2.01
	M1-"*R*"	7932	2.07	3.32
	M2	7553	1.40	2.97

Reproducibility of the retention time C.V.[c] (%)=0.90%
[a] Mean of five determinations for each sample.
[b] Calculated on the basis of five determinations for each sample.
[c] Calculated on thirty determinations.

purified and analyzed to obtain the calibration curves. The reproducibility and the accuracy of the method is summarized in Table 2 and Table 3, respectively. The equation of the regression lines is shown in Table 4. In order to verify whether *S*-(1-phenyl-2-hydroxyethyl)-L-cysteine or *S*-(2-phenyl-2-hydroxyethyl)-L-cysteine (deacetylated M1 and M2, respectively) were present in urine, samples taken from exposed subjects were also analyzed without enzymatic hydrolysis; no appreciable amounts of these compounds were found. M1 and M2 concentrations in unexposed subjects were below the detection limit of the method (about 7 μg/l). Values in exposed workers ranged from 71 to 14 760 μg/l for M1-"*S*", from 7 to 742 μg/l for M1-"*R*" and from 312 to 13 726 μg/l for M2. Six samples of urine, previously analyzed by the method of Truchon et al. [20,25], from rats exposed to styrene and racemic styrene-7,8-epoxide and from unexposed rats were analyzed by the method described above and the results obtained were found to be in good agreement with those previously obtained. Seven samples from humans (selected among exposed workers and from the control group) were further analyzed by the method of Truchon et al. [20,25]: due to the presence of interferents and to the lack of sensitivity (the detection limit was about 3 mg/l), only M1-"*S*" could be quantified in four subjects; also in these cases, results were in agreement with data obtained by the method described in this paper.

Table 3
Accuracy of the assay procedure

Compound	Theoretical concentration (μg/l)	n	Observed concentration (μg/l)	C.V. (%)	Error (%)
M1-"*S*"	50	4	48	3.21	−4.0
M1-"*R*"	50	4	47	4.12	−6.0
M2	50	4	52	2.83	+4.0
M1-"*S*"	10000	4	9830	1.43	−1.7
M1-"*R*"	10000	4	9903	2.18	−1.0
M2	10000	4	9952	1.81	−0.5

Table 4
Calibration curves[a]

Compound	Equation of the regression line[b]	Coefficient of correlation (*r*)
M1-"*S*"	$y=1013x+1488$	0.99997
M1-"*R*"	$y=1329x-1145$	0.9998
M2	$y=2375x+3259$	0.9997

[a] Obtained by addition of standard (20-20 000 μg/l) to the same sample prior to the purification step. Each sample was analyzed three times.
[b] y=peak area; x=concentration (μg/l).

These preliminary results seem to confirm that the conversion of styrene to mercapturic acids is much lower in man than in rats. This may be partially due to the approx. 100-fold lower activity of hepatic GST in humans compared to rats. To calculate the exact conversion of styrene to mercapturic acids, further information is needed about the relationship between the environmental concentration of styrene and excretion of M1 and M2. A correlation was found between mercapturic acids and MA and PGA concentrations in urine (Table 5, Fig. 3). This finding suggests that the determination of specific mercapturic acids in man could allow an estimation of exposure to styrene. Previous studies in exposed rats have shown an enzymic preference for one diastereoisomer of M1, the M1-"*S*" to M1-"*R*" ratio being about three [17,31]. The present study suggests that an even higher stereoselectivity of the enzymes involved in the metabolism of styrene may occur in man, in fact the mean M1-"*S*" to M1-"*R*" ratio was about twenty. It is not clear whether an enzymatic preference occurs only during oxidation of styrene to styrene-7,8-epoxide by cytochrome P-450 [6–8] or also during conjugation of styrene-7,8-epoxide to GSH, or eventually to the subsequent reactions leading to the excretion of mercapturic acids. In any case, such results, if confirmed by further studies, could have some toxicological consequences: for example, *R*-(+)-styrene-7,8-epoxide was found to be more mutagenic to *Salmonella typhimurium* strain TA100 than *S*-(−)-styrene-7,8-epoxide [32]. A slight increase in GST activity was demonstrated in rats after repeated exposure to styrene [25]. Recently, our studies on rats exposed to styrene have suggested

Table 5
Correlation between biological levels of exposure indicators ($y=a+bx$; $n=25$)

y	*x*	*a* (intercept)	*b* (slope)	*r*	*P*
Mandelic acid (mg/l)	M1-"*S*" (μg/l)	600.79	0.117	0.73	<0.0001
Phenylglyoxylic acid (mg/l)	M1-"*S*" (μg/l)	196.84	0.036	0.73	<0.0001
Mandelic acid (mg/l)	M1-"*R*" (μg/l)	702.01	2.289	0.55	0.0037
Phenylglyoxylic acid (mg/l)	M1-"*R*" (μg/l)	216.29	0.780	0.63	0.0007
Mandelic acid (mg/l)	M2 (μg/l)	507.29	0.163	0.71	<0.0001
Phenylglyoxylic acid (mg/l)	M2 (μg/l)	152.99	0.055	0.79	<0.0001

that the M1-"*S*" to M1-"*R*" ratio may be affected by the chronic administration of ethanol [31]. At present, no data is available in the literature on the metabolism of styrene leading to mercapturic acids and on the influence of ethanol or other chemicals on the excretion of M1 and M2 in man. The method described in this paper could be useful for obtaining such information.

4. Conclusion

The present paper describes a method for the analysis of urinary mercapturic acids specific for styrene by HPLC with fluorescence detection, after enzymatic deacetylation and derivatization with OPA–MCE reagent. The method allows the separation of the two diasteroisomers of N-acetyl-*S*-(1-phenyl-2-hydroxyethyl)-L-cysteine (M1), while the diastereoisomers of N-acetyl-*S*-(2-phenyl-2-hydroxyethyl)-L-cysteine (M2) cannot be separated by this method. The procedure is sensitive and specific, thus it seems suitable for research purposes concerning the metabolism of styrene in man. In our opinion, however, the method is quite time-consuming and cannot be used for routine analysis of these metabolites in the urine of workers occupationally exposed to styrene.

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