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Determination of monobromobimane derivatives of phenylmercapturic and benzylmercapturic acids in urine by high-performance liquid chromatography and fluorimetry

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Abstract

A method was developed for the determination in human urine of *S*-phenylmercapturic (PMA) and *S*-benzylmercapturic (BMA) acids, metabolites respectively of benzene and toluene. PMA and BMA were determined, after alkaline hydrolysis, to give respectively thiophenol and benzylmercaptan, and coupling of the thiol-containing compounds with monobromobimane (MB), by reversed-phase HPLC on a diphenyl-silica bonded cartridge (100×4.6 mm I.D., 5 μm particle size) with fluorimetric detection. Wavelengths for excitation and emission were 375 and 480 nm, respectively. The recovery of PMA and BMA from spiked urines was >90% in the 10–500 μg/l range; the quantification limits were respectively 1 and 0.5 μg/l; day-to-day precision at 42 μg/l was C.V. <7%. The suitability of the proposed procedure for the biological monitoring of exposure to low-level airborne concentrations of benzene and toluene, was evaluated by analyzing the urinary excretion of PMA and BMA in subjects exposed to different sources of aromatic hydrocarbons, namely occupationally-unexposed referents (non-smokers, $n=15$; moderate smokers, $n=8$; mean number of cigarettes smoked per-day=17 cig/day) and non-smoker workers occupationally exposed to toluene in maintenance operations of rotogravure machines (non-smokers, $n=17$). Among referents, non-smokers showed values of PMA ranging from <1 to 4.6 μg/l and BMA from 1.0 to 10.4 μg/l; in smokers, PMA values ranging from 1.2 to 6.7 μg/l and BMA from 9.3 to 39.9 μg/l, were observed. In occupationally exposed non-smoker subjects, BMA median excretion value (23.6 μg/l) was higher than in non-smoker referents (3.5 μg/l) ($P<0.001$) and individual BMA values (y, μg/l) were associated and increased with airborne toluene concentration (x, mg/m^3) according to the equation $y=6.5+0.65x$ ($r=0.69$, $P<0.01$, $n=17$). The proposed analytical method appears to be a sensitive and specific tool for biological monitoring of low-level exposure to benzene and toluene mixtures in occupational and environmental toxicology laboratory.

Keywords: Exposure biomarkers; Tobacco smoking; Phenylmercapturic acid; Benzylmercapturic acid; Toluene; Benzene

1. Introduction

Benzene, toluene and xylenes (BTX) are members of the large family of aromatic hydrocarbons. They find wide use in many industrial applications, alone or in mixtures, as solvents and starting products for chemical synthesis and as ingredients of gasoline blending. Recently they became ubiquitous pollu-

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tants widespread in the environment principally as a result of motor vehicle emissions. Tobacco smoking habit constitutes a further source of aromatic hydrocarbon exposure, because aromatic hydrocarbons are generated by tobacco pyrolysis processes [1,2].

Along with the ever increasing awareness and knowledge about the toxicity of environmental or industrial chemicals for humans (incidentally, benzene is a recognized leukemogenics in human), there have been an increasing need for reliable biomarkers and sensitive analytical methods for the biomonitoring of populations potentially exposed to occupational or environmental pollutants.

Gluthatione conjugation is one of the most relevant detoxication pathway in human and mercapturic acids were identified as urinary metabolites of aromatic hydrocarbons for many years. Recently their application as biomarkers for occupational and environmental exposure to aromatic hydrocarbons has been investigated [3,4]. *S*-phenylmercapturic acid (*N*-acetyl-*S*-phenylcysteine) has been demonstrated to be a valuable biomarker of human exposure to airborne benzene concentrations as low as 0.3 ppm, and is now proposed in substitution of phenol, the previously preferred but unspecific metabolite of benzene [5,6]. In the case of toluene, the urinary excretion of mercapturic acids has been demonstrated in rats treated with toluene [7] and recently some evidences were given about the excretion in urine of occupationally exposed subjects of specific *S*-containing metabolites, namely *S*-benzylmercapturic and *S*-cresylmercapturic acids [8]. By now, hippuric acid is world-wide used as biomarker of exposure to toluene in occupational settings. However, this metabolite is physiologically excreted in relevant amounts (around 1–2 g/l) in urine of toluene-unexposed subjects, because it derives from endogenous and dietary precursors. Thus the prospect of a more specific biomarker of exposure is worth of interest.

The analytical procedures available for determination of mercapturic acids derived from aromatic hydrocarbons are often unsensitive, time consuming or have a high level of complexity [9–16]. In this paper, an high-pressure liquid-chromatography method with fluorescence detection for the quantitative determination of *S*-phenylmercapturic (PMA) and *S*-benzylmercapturic (BMA) acids in human urine is described. To this goal, a HPLC method, previously devised for the determination of urinary thiols [15] and then used for PMA analysis [16], has been developed to improve analytical performances and to allow the simultaneous quantification of PMA and BMA. The suitability of the analytical procedure for the biological monitoring of human exposure to low-level concentrations of airborne benzene and toluene mixture was then evaluated by studying the urinary excretion of PMA and BMA in people exposed to aromatic hydrocarbons from different sources (namely, tobacco smoke and occupational exposure to low levels of airborne toluene in maintenance operations of rotogravure machines).

2. Experimental

2.1. Materials

S-phenylmercapturic (PMA) and *S*-benzylmercapturic acids were obtained from Tokyo Kasei Kogyo (Tokyo, Japan); thiophenol was obtained from Aldrich (Milan, Italy); acetonitrile, chloroform, acetone, tetrahydrofuran, methanol, trifluoroacetic acid, phosphoric acid (15 *M*), hydrochloric acid (12 N), anidrous magnesium sulphate, ammonium bicarbonate, sodium hydroxide, ethylendiaminotetracetic acid disodium salt ($EDTANa_2$) were purchased from C. Erba (Milan, Italy); Thiolite® reagent Monobromobimane (MB) was from Calbiochem (Inalco, Milan, Italy); all chemicals were of analytical purity or HPLC grade. Glass screw-capped 4-ml vials with rubber/teflon pierceable gasket were purchased from Supelco (Aldrich, Milan, Italy). Oasis HLB-1cc cartridges were from Waters (Milan, Italy). The phenyl column (100×4.6 mm I.D., 5-μm particle size) Supelcosil DP and the Supelguard DP guard column (20×4.6 mm I.D., 5-μm particle size) were obtained from Supelco.

2.2. Instrumentation

HPLC separation was carried out using a Waters LC Module 1 plus instrument (Waters, Milan, Italy) equipped with a LS-1 fluorimetric detector (Perkin-

Elmer, Monza, Italy). Data acquisition and elaboration were by means of a Millenium-2010 software (Waters).

2.3. Other methods

Aromatic hydrocarbon airborne concentrations in occupational settings, collected by passive diffusive sampling on stainless steel tubes filled with 250 mg Tenax TA (60–80 mesh, Alltech, Milan, Italy), were determined by thermal desorption and gaschromatography with FID detection [17]. Urinary cotinine was determined through a modified HPLC method [18]. Urinary creatinine was determined photometrically through a Hitachi 917 analyzer.

2.4. Statistical elaboration

Statistical analysis of results was done by means of a Statgraphic-plus Version 3 package (Statistical Graphic Co., Rockville, USA). Differences among groups were considered statistically significant when the *P*-value was below 0.05.

2.5. Study group

To establish the suitability of the proposed procedure for monitoring exposure to low-level airbone concentrations of benzene and toluene, urinary excretions of PMA and BMA were studied in 23 reference subjects not occupationally exposed to aromatic hydrocarbons (15 non-smokers; 8 smokers, mean number of smoked cigarettes per-day=17 cig/day). Spot urine samples were collected from each volunteer at 12:30 h, before lunch interval.

For the sake of comparison, BMA and eventually PMA excretion was also evaluated in 15 non-smoker workers occupationally exposed to toluene in maintenance operations of rotogravure machines. Urine spot samples were collected from workers at the end of a 7-h workshift. Environmental monitoring of airborne toluene exposure was done by means of passive diffusion personal samplers worn in the respiratory zone by each subject during the working period.

2.6. Urine sample storage and processing

As soon as possible after collection, 2-ml aliquots were separated and stored into polyethylene disposable tubes at −20°C until analysis.

Before analysis, frozen samples were conditioned at 37°C for 15 m, with frequent stirring.

2.7. Standard solutions and calibration curves

Standard solutions of PMA and BMA were prepared by individually dissolving the acids in methanol: concentration was adjusted to 1 μmol/ml (PMA=239 μg/ml; BMA=253 μg/ml). For calibration purposes, working standard solutions (0.10, 0.20 and 0.40 nmol/ml each of PMA and BMA) were freshly prepared at the time of analysis in 2 *M* NaOH containing 2 m*M* $EDTANa_2$.

2.8. Analytical procedure

PMA and BMA are separated from urine matrix by liquid–liquid extraction (LLE). An aliquot of urine (2.0 ml), in 5-ml polyethylene tube, is added with 100 μl of 12 N HCl and 2 ml of extraction mixture (chloroform–acetone 2:1). The tube is shaken by hand for 1 m and centrifuged at 1200 *g* to improve phase separation. The organic lower layer is transferred to an other test tube, 2 ml of chloroform are added to the aqueous layer in the first test tube and the mixture is extracted again. The aqueous upper layer is discarded. The organic phases are combined together and dehydrated by addiction of anhydrous magnesium sulphate (200 mg); 2 ml of the organic phase are put into a 4-ml glass vial and taken to dryness under nitrogen stream at 50°C; the residue is dissolved with 500 μl of NaOH 2 *M* containing 2 m*M* of $EDTANa_2$. Air is expelled by gently bubbling a stream of nitrogen for 30 s into the solution; vials are immediately closed by means of screw cap equipped with pierceable gasket. Vials are then tranferred into a dry bath and hydrolized at 95°C for 25 min; then they are cooled in iced water for 5 min. Reagents for derivatization purpose are then injected into the vial through the pierceable gasket: 90 μl of 5 *M* phosphoric acid, 200 μl of 0.5 *M* ammonium bicarbonate (to obtain pH values in the range 7.5–8.5) and 50 μl of MB (2 m*M* in

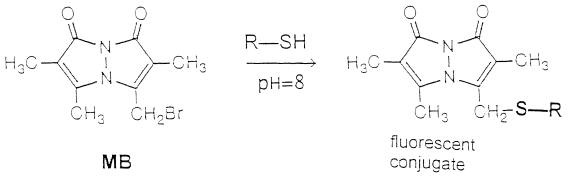

Fig. 1. Schematic description of the reaction between monobrobimane (MB) and thiols (R–SH) to give fluorescent conjugates.

acetonitrile) are added sequentially. Derivatization reaction is driven at room temperature for 15 min. The reaction in shown in Fig. 1. Aryl thiol–MB derivatives are extracted from the reaction mixture by solid-phase extraction (SPE) by means of Oasis HLB-1cc cartridges. Cartridges are conditioned with 1 ml of methanol and 1 ml of water. The reaction mixture is loaded onto the cartridge. Washings consist of 1 ml of water and 8 ml of 50% (v/v) methanol–water solution. MB derivatives are eluted with 200 μl of acetonitrile. The acetonitrile eluates are reduced to dryness by applying a stream of nitrogen at 50°C. Dry residues are resolved in 50 μl of acetonitrile and diluted with water to a final volume of 250 μl. Aliquots of this solution (50 μl) are injected into the HPLC system.

Calibration curves were set up by plotting the metabolite peak height against the respective concentration of each mercapturic acid. Urinary PMA and BMA concentrations were calculated by external standardization, comparing integrated peak height counts of MB derivatives obtained from unknown urine sample with that from aqueous standard calibration curve.

2.9. Chromatographic conditions

Chromatographic separation was obtained by gradient elution at 35°C on a reversed-phase DP column. The eluent system consisted of two components: solution (A), water–tetrahydrofuran–trifluoroacetic acid 85:15:0.1, and solution (B), acetonitrile–water–trifluoroacetic acid 60:40:0.1. The initial mobile phase was 100% solution A, kept at a flow-rate of 2.0 ml/min for 15 min. The back-pressure was approximately 13.1×10^6 Pa (1900 p.s.i.). Then, the flow-rate was incremented to 4 ml/min and 100% solution B was run into system for 3 min. At this stage the back-pressure increased approximately up to 19.3×10^6 Pa (2800 p.s.i.). A 3-min gap was scheduled to restore the initial conditions. This elution gradient permitted a shortening of the time of analysis owing to more rapid elution of additional compounds. The eluate was monitored by fluorescence detection (excitation wavelength, 375 nm; emission wavelength, 480 nm).

3. Results

3.1. Chromatographic separation

Chromatographic profiles obtained from an aqueous standard solution and urine samples are shown in Fig. 2: Fig. 2A is referred to a calibration standard solution corresponding to urinary concentrations of PMA=20 μg/l and BMA=15 μg/l; in Fig. 2B the elution pattern obtained from a smoker is shown; and in Fig. 2C is reported the chromatogram from a rotogravure worker occupationally exposed to toluene (toluene airborne concentration=35 mg/m^3). PMA and BMA eluted as sharp and symmetrical peaks, sufficiently separated from main contaminants, and other MB derivatives. Retention times of PMA and BMA were respectively of 11.8 and 12.8 min.The entire run, before next sample could be injected, required 20 min. The presence of other natural sulfohydrils did not interfere with the detection of the metabolites of interest.

3.2. Calibration, recovery and reproducibility

Calibration curves were linear in the interval 10–250 μg/l for the two studied metabolites. When concentration of aryl thiol–MB derivatives exceeded the linearity range, samples were adequately diluted with mobile phase and reinjected. The limits of detection (LOD) were obtained from calibration curves (four different concentrations in the range 12–250 μg/l, 5 determinations for each point), by use of the intercept (a) and standard error of its estimate S.E. (a) of the regression line for PMA or BMA concentrations versus signal [19]. The limit of detection, calculated from $y=a+3$ S.E. (a), resulted

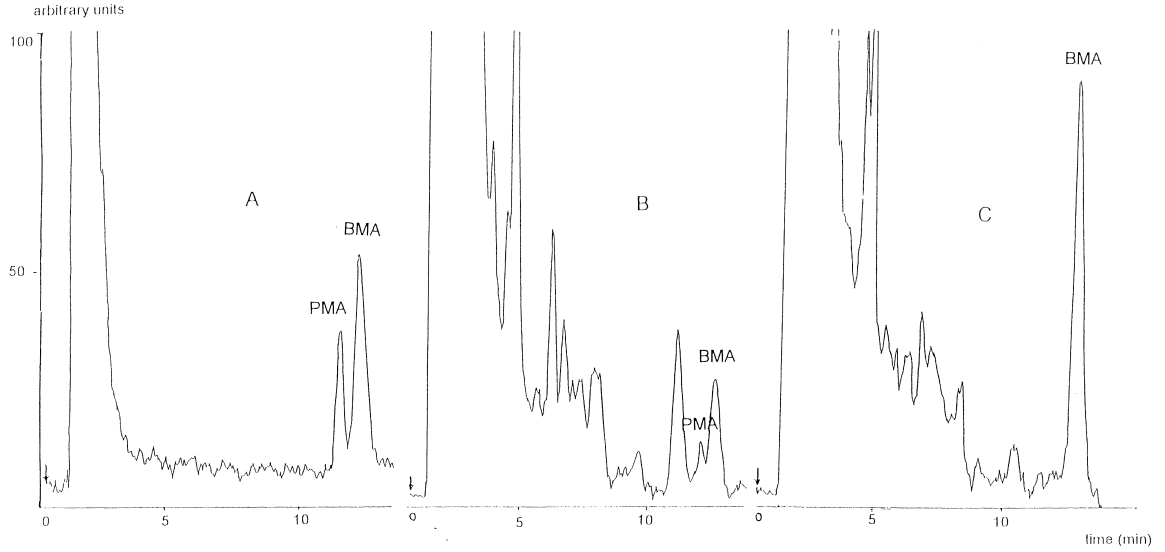

Fig. 2. Representative chromatograms of phenylmercapturic (PMA) and benzylmercapturic (BMA) acids. (A) Calibration solution (PMA=20 μg/l, BMA=15 μg/l). (B) Urine from a smoker referent (PMA=4.6 g/l, BMA=8.1 μg/l). (C) Urine from a toluene-exposed rotogravure worker (BMA=30.3 μg/l).

respectively LOD PMA=1 μg/l and LOD BMA= 0.5 μg/l, corresponding to 0.2 ng of PMA and 0.1 ng of BMA in a 50-μl injection. The recovery of the overall method was investigated by working up ten urine samples spiked with known concentrations of PMA and BMA (added amounts: 40 μg/l). The absolute recovery was evaluated as the percentual ratio between the amount of metabolite found in spiked urines and the corresponding standard solution in NaOH. Overall percent recoveries higher than 90% were always obtained. Independent confirmation of the satisfying recovery of added mercapturic acids was also acquired from an other experiment. Good results (recovery >90%) were obtained when the recovery of PMA from enriched urine was evaluated by quantifying concentrations by means of calibration curves prepared from equimolar aqueous solutions of thiophenol, directly made to react with MB to give the aryl thiol–MB derivative actually determined by the proposed procedure. The repeatability (precision within a run of ca. 5 h, expressed as coefficient of variation C.V.%) of the method, determined by analysis of 8 aliquots of a urine containing metabolism-derived PMA (23 μg/l) and BMA (19 μg/l), was C.V.<7%. The reproducibility (between-day precision) among different assays on the same samples during a period of 5 months was C.V.<13% (n=9). During the development of the procedure, more than 400 injections have been made on the same HPLC column without any observed column aberrations. The DP pre-column was changed every 100 injections, as a general rule to insure adequate protection and improve lifespan of the analytical column.

Various storage conditions were examined to minimize the loss of urinary metabolites before instrumental analysis. Untreated urine samples were stored at −18°C for 6 months, without significant modifications of metabolite concentrations. The hydrolized mixture appears to be relatively stable for 2 h in sealed vials: delay of 2 and 8 h between hydrolysis and derivatization, decreased the yields of PMA–MB derivative by about 10 and 50% respectively; the ABM–MB derivative was less sensitive to variations in time-lag between hydrolysis and derivatization, showing a decrease of 20–30% of initial fluorescence after 8 h. Derivatized samples, eluates after SPE extraction, or dissolved residues could be stored at 2–8°C, in the dark, for up to 10 days without appreciable modifications of signal intensity.

3.3. Field evaluation of the analytical method

With the purpose of a initial trial to test the suitability of the analytical method for the biological monitoring of exposure to low-level airborne concentrations of benzene and toluene, urinary excretion values of PMA and BMA were assessed in people exposed to different sources of aromatic hydrocarbons, namely 23 occupationally-unexposed reference subjects (15 non-smokers; 8 moderate smokers) and 17 non-smoker workers occupationally exposed to toluene in maintenance operations of rotogravure machines. The self-reported smoking status of studied subjects was verified through determination of urinary cotinine excretion. All subjects with cotinine values below 50 μg/l were considered non-smokers. Preliminary results obtained in the studied subjects are summarized in Table 1. In non-smoker referents, values of PMA ranging from <1 to 4.5 μg/l and BMA from 1.0 to 10.4 μg/l were observed. None of self-reported non-smokers showed cotinine values higher than 50 μg/l. In smoker referents, PMA values ranging from 1.2 to 6.7 μg/l and BMA from 9.3 to 39.9 μg/l, were observed. Smoker cotinine values ranged from 428 to 2552 μg/l (mean and standard deviation value= 1433±678 μg/l). We have chosen not to adjust PMA/BMA values by urinary creatinine concentration since this had negligible influence on the results of statistical elaboration of data or our conclusions. Comparison of PMA and BMA median values between non-smoker and smoker referents, showed a statistical difference in BMA excretion between groups ($P<0.001$); no difference was found

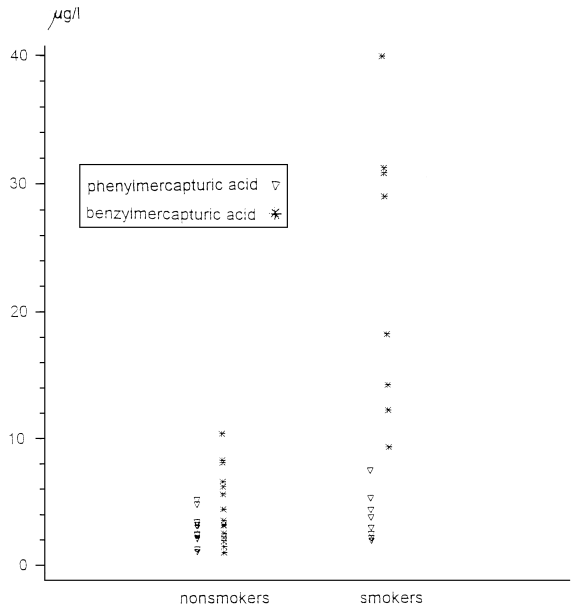

Fig. 3. Distribution of urinary excretion values of phenylmercapturic (▽) and benzylmercapturic acids (✳) in non-smoker referents ($n=15$), and smoker referents ($n=8$).

in PMA excretion. In Fig. 3, individual excretion values of PMA and BMA observed in non-smoker and smoker referents are depicted. In occupationally exposed workers, toluene airborne concentrations in the range 6–157 mg/m^3 were found. In this group, BMA excretion ranged from 8.0 to 111.3 μg/l and PMA from <1 to 48.4 μg/l. Comparison of BMA median values between non-smoker referents and non-smoker workers showed a statistically significant difference between groups ($P<0.001$). Moreover in occupationally-exposed workers, a statistically sig-

Table 1
Urinary concentration of phenylmercapturic and benzylmercapturic acids, expressed in μg/l, observed in spot urine samples obtained from occupationally-unexposed referents (non-smokers and smokers) at 12:30 h, and from toluene occupationally-exposed rotogravure non-smoker workers (toluene aiborne concentrations in the range 6–100 mg/m^3) at the end of a 7-h workshift

Metabolite (μg/l)	Toluene occupationally-unexposed referents						Occupationally-exposed rotogravure workers		
	Non-smokers ($n=15$)			Smokers ($n=8$)			Non-smoker workers ($n=17$)		
	median	min	max	median	min	max	median	min	max
Phenylmercapturic acid	1.6	<1[a]	4.6	2.6	1.2	6.7	3.0	<1[a]	48.4
Benzylmercapturic acid	3.5	1.0	10.4	23.6	9.3	39.9	31.5	8.0	111.3

[a] Lower than 1 μg/l, analytical detection limit.

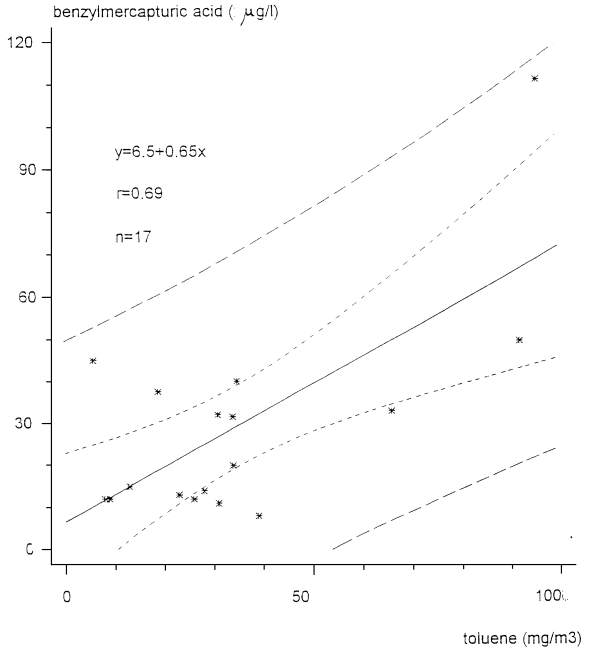

Fig. 4. Relationship observed between airborne toluene concentration (x, mg/m^3) and benzylmercapturic acid (y, µg/l) in toluene occupationally-exposed rotogravure workers.

nificant correlation was evidenced between urinary excretion of BMA (y, µg/l) and airborne toluene concentration (x, mg/m^3) ($y=6.5+0.65\ x$; $r=0.69$, $P<0.01$, $n=17$) (Fig. 4).

4. Discussion

Biomonitoring of low level exposures to benzene and toluene by suitable biomarkers is of both occupational and environmental importance, because the toxicological relevance of low-dose exposure is so far unclear. One of the most common and important biotransformation reactions of potentially toxic xenobiotic chemicals possessing an electrophilic center is the conjugation with glutathione. The glutathione conjugates then are metabolized to mercapturic acids, i.e. *S*-alkylated derivatives of *N*-acetylcysteine and other thioethers, which are excreted in the urine and/or bile [20]. The analytical procedures available for determination of aromatic hydrocarbon-derived mercapturic acids are somewhat unsensitive or are sophisticated, having a high level of complexity [9–16].

A purpose of the present study was to devise a easy and reliable method to be adopted for routinary determination of PMA and BMA in human urine. HPLC combined with fluorescence detection is one of the most effective tools for the sensitive and specific determination of organic compounds in biological fluids. The high polarity of the metabolites of interest in conjunction with the complex urine matrix represents a difficult analytical challenge. In addition the absence of high-absorbance chromophores or electrochemical-reactive groups prevents the sensitive detection of native analyte. To this instance, we have notably improved an analytical method previously devised for the determination of urinary thiols and PMA [15,16]. The original analytical protocol has been modified both in the purification procedure and the chromatographic conditions, to be applied routinely to the determination of either PMA or BMA in large numbers of samples. The proposed method combines simple sample treatment and derivatization, high sample throughput and reduced analysis time together with sufficient sensibility and acceptable reproducibility in comparison to other published methods. Characteristic features of the present method are: (i) separation by liquid–liquid extraction (LLE) of acidic metabolites from biological matrix; (ii) alkaline hydrolysis to form thiophenol and benzylmercaptan; (iii) direct reaction with monobromobimane reagent; (iv) purification of MB-derivatives; and (v) analysis by reversed-phase HPLC on standard HPLC equipment. This last point is of special interest, because it allows the proposed procedure to be applied on a routine basis, while other available analytical procedures for PMA detection are much more demanding, requiring GC–MS or HPLC equipped either with a programmable sample processor or a post-column derivatization system [10,16]. In the course of the analytical procedure optimization, different approaches to sample clean-up were evaluated, before resolving to adopt the procedure here described. For preliminary assays, a SPE procedure on C_{18} cartridges was adopted to separate PMA and BMA from urine matrix (16). The low percent recovery obtained (<50%) prompted us to assess LLE. LLE was eventually preferred because it offered sufficient

percent recovery (>90%) and acceptable precision. Work-up of samples in sealed vials under nitrogen atmosphere greatly improved reproducibility of the method. Thiophenol and benzylmercaptan were in fact found to be unstable compounds in presence of oxygen. Moreover, they were highly volatile and quickly lost from reaction mixture, when neutralization of hydrolyzed samples was done in open vials. Derivatization of thiols with bimane is easily afforded at room temperature. The MB-derivatized thiol compounds are sufficiently stable and could be stored in the dark in a refrigerator for at least 10 days without appreciable changes in fluorescence intensity. The adoption of aqueous calibration curves for quantitation of PMA and BMA urinary concentrations represents a further procedure simplification. This decision was founded on the observation that calibration curve slopes obtained from different enriched urines or from aqueous solutions were very consistent and appeared to be independent from matrix influence. The lack of use of internal standard for peak identification and quantitation could perhaps appear questionable. Having considered and tried an extended selection of chemicals, we were not able to get a suitable compound, and thus, supported by the acceptable reproducibility (between-day precision) of the procedure, we resolved to use external standardisation.

The HPLC method here established is able to determine *S*-glutathione conjugates of benzene and toluene simultaneously. For these reasons, the method is well suited for studies of the metabolism of benzene and toluene environmental mixtures through the glutathione pathway. The limit of detection is comparable with those obtained by other authors, and is adequate for biological monitoring of low-level exposure to aromatic hydrocarbons, such as that occurring after cigarette consumption, as confirmed by results obtained in non-smoker and smoker referents [21–24].

At present, only few data concerning urinary ABM excretion in occupational exposure are available [8]. By now, the preferred biomarker of occupational exposure to toluene is its unspecific metabolite hippuric acid. The regular and abundant presence of hippuric acid in human urine makes the biomarker unable to reveal exposure to airborne toluene at concentrations below 100 ppm. Preliminary results here reported in non-smoker workers occupationally exposed to toluene seem interesting and worth of deeper consideration, and support the suitability of BMA as biomarker of exposure to low airborne toluene concentrations, where hippuric acid fails to give adequate informations. The proposed analytical procedure may, in principle, also be used to determine other mercapturic acids, such as toluylmercapturic acids, which are actually considered better toxicological markers of the intermediate formation of reactive epoxide-derivatives of toluene. However, it must be underlined that, in toluene exposure, toluylmercapturic acids are excreted in amounts one order of magnitude lower than BMA [8]. This fact, together with the absence from the market of reference parent compounds, contribute to difficulty to perform their determination routinary. In conclusion, the proposed analytical method appears to be sensitive and specific tool for routine biological monitoring of low-level exposure to benzene and toluene mixtures in occupational and environmental toxicology laboratory.

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