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Determination of urinary S-phenylmercapturic acid by liquid chromatography-tandem mass spectrometry

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Abstract

Urinary S-phenylmercapturic acid (S-PMA) is considered a useful biomarker for the measurement of low levels of benzene exposure, related to occupational exposure, smoking habits or environmental pollution. S-PMA quantitative analysis requires highly sensitive and specific techniques and purification procedures, mainly based on liquid–liquid or solid-phase extraction, which result in time expensive analyses. A method was developed for the quantitative determination of S-PMA in urine by using a simple, reproducible and easily automatizable HPLC purification followed by LC/ESI-NI/MS^2 analysis. In order to reduce the cost of the analysis, related to the use of expensive labeled standards, *p*-bromo-S-phenylmercapturic acid (*p*-Br-S-PMA) was synthesized, characterized and used as internal standard.

The feasibility and efficacy of the proposed method were examined by constructing calibration curves in the range from 6.2 to 200 μg/l and data were analyzed in terms of linearity and statistical parameters. The detection limit, related to the purification of 1 ml urine sample is 5 μg/l. The method was applied to the analysis of 12 urine samples from smoker subjects non-occupationally exposed to benzene. S-PMA urinary levels ranged from 13.6 to >200 μg/l, suggesting a high influence of life style in the S-PMA excretion. The proposed analytical method is suitable for the biological monitoring of both smoker and non-smoker workers, occupationally exposed to benzene. By processing at least 2 ml of urine samples, the method appears to be also useful for the evaluation of benzene uptake due to the environmental pollution.

Keywords: Benzene exposure; Biological monitoring; S-Phenylmercapturic acid

1. Introduction

Benzene is a common industrial chemical, a component of tobacco smoke and of gasoline, a constituent of engine emissions and combustion. Data from epidemiological studies indicate that benzene is toxic to

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humans [1]: exposure to benzene is associated with the development of acute non-lymphocytic leukemia [2–4], aplastic anemia [5], chromosomal aberrations [6–9] and a progressive degeneration of the bone marrow [1]. The American Conference of Governmental Industrial Hygenists (ACGIH) classifies benzene in group A1 (carcinogen to human) and defines a threshold limit value-time weighted average (TLV-TWA) of 0.5 ppm [10]. Owing to its volatility and lipophilicity, benzene is rapidly absorbed via inhalation or dermal contact. Following inhalation exposure, a fraction of benzene (about 12%) is exhaled from the human lungs and only 0.1% is excreted unchanged in the urine, the remaining fraction is metabolized and excreted through urine [11].

Benzene urinary metabolites have long been studied, since they are considered useful biomarkers for assessing the occupational and environmental exposure to benzene [12–17]. There are three different metabolic pathways, which end in excretion of different phenols, *trans*,*trans*-muconic acid (*trans*,*trans*-2,4-hexadienedioic acid) and S-phenylmercapturic acid (S-PMA), formed from reaction of benzene epoxide with glutathione. Unlike the first two, urinary S-PMA is considered a sensitive and specific biomarker for the evaluation of low levels of benzene exposure. In presence of low levels of benzene in air, the measurement of phenols in urine does not allow the estimation of individual risk [18], since they are not specific and they may also be formed endogenously from metabolism of various aromatic amino acids [1]. *trans*,*trans*-Muconic acid is a fairly good indicator of benzene exposure at low concentration (0.1–1 ppm). However, urinary muconic acid may not be specific to benzene exposure, since it is also a minor metabolite of sorbic acid (a widely used food preservative) in mice [19] and humans [20]. In 1995, ACGIH proposed S-PMA as biomarker for benzene. The actually accepted biological exposure index (BEI) value is 25 μg S-PMA/g creatinine [10]. The conversion rate of benzene to mercapturic acid in humans is lower than 1% [21], so quantitative analysis of this metabolite requires highly sensitive and specific techniques. Liquid chromatography/negative electrospray ionization/tandem mass spectrometry with selected reaction monitoring (LC/ESI-NI/MS^2-SRM) has been widely used in the determination of mercapturic acids, however a purification procedure, such as liquid–liquid extraction or solid-phase extraction, was always required [1,22,23].

This work reports a method for the quantitative determination of urinary S-PMA by using an HPLC purification procedure before the LC/ESI-NI/MS^2 analysis. Moreover, an internal standard structurally related to S-PMA, *p*-bromo-S-phenylmercapturic acid (*p*-Br-S-PMA), was synthesized and characterized. The use of *p*-Br-S-PMA as internal standard, which can be easily synthesized in high amounts, reduces the cost of the analysis, avoiding expensive labeled internal standards.

The feasibility and efficacy of the proposed method have been examined and data were analyzed in terms of linearity, sensitivity and statistical parameters. In order to verify the actual applicability of the proposed method to real samples, urine from smoker subjects, non-occupationally exposed to benzene, were analyzed.

2. Experimental

2.1. Materials

S-PMA was kindly supplied from Dr. Luciano Maestri (Fondazione Maugeri, Pavia, Italy). *p*-Bromothiophenol and 2-acetamidoacrylic acid were purchased from Sigma-Aldrich (Milwaukee, WI, USA). HPLC grade solvents were from Carlo Erba (Milan, Italy). A RP Nova-Pak® C18 (20 mm × 3.9 mm, 4 μm, 6 nm) column, equipped with its Sentry Guard Holder (Waters, Milford, MA, USA) was used for the complete characterization of S-PMA and *p*-Br-S-PMA and for the subsequent quantitative determination of S-PMA. LC/ESI-NI/MS^2-SRM analyses were carried out using a Perkin-Elmer 200 series HPLC modular system (Norwalk, CT, USA) and an API 2000 (Applied Biosystems, Foster City, CA, USA) triple quadruple mass spectrometer, equipped with a turbo ion spray source.

2.2. Synthesis of p-Br-S-PMA internal standard

p-Br-S-PMA was synthesized from *p*-bromothiophenol and 2-acetamidoacrylic acid by a Michael reaction [24]. In brief, *p*-bromo-thiophenol (1.6 g; 8.5 mmol) and 2-acetamidoacrylic acid (1.0 g;

7.7 mmol) were added to 20 ml of dioxane, then 0.2 ml of piperidine were added. The suspension was flushed with N_2, heated for 3 h in dark conditions and with reagents reflux. The residue was dried and partitioned between ether and a sodium bicarbonate solution. The aqueous layer was neutralized, extracted with ether and acidified with HCl to pH 2, obtaining crude *p*-Br-S-PMA. The acid was crystallized from aqueous methanol and characterized by nuclear magnetic resonance (data not shown) and mass spectrometry. Data showed the purity of the synthesized acid, which was used as internal standard in all quantitative determinations of S-PMA.

2.3. Solutions and validation samples

Solid S-PMA and *p*-Br-S-PMA were dissolved in methanol obtaining 1000 mg/l stock solutions, which were progressively diluted (1:10) in order to obtain four working solutions at concentrations of 100 and 10 mg/l of S-PMA or *p*-Br-S-PMA. 100 mg/l solutions were used for the mass spectrometric characterization; the others were used for the preparation of urine samples used for the construction of calibration curves.

S-PMA solution of 10 mg/l (=10,000 μg/l) was added to urine from non-smokers and non-occupationally exposed volunteers, obtaining a urinary S-PMA concentration of 200 μg/l. This urinary solution was progressively diluted (1:2) with urine of the same volunteers, in order to have six *spiked* urine samples at decreasing S-PMA concentrations (200, 100, 50, 25, 12.5 and 6.2 μg/l). Each sample was added with 10 mg/l methanolic solution of *p*-Br-S-PMA, in order to have a constant urinary internal standard concentration of 1000 μg/l. pH was adjusted to 2 with formic acid; the urine samples were centrifuged (4000 rpm for 15 min) and kept at −20 °C until the HPLC purification step.

Urinary quality control samples were independently prepared at four levels of concentration (10, 30, 65 and 150 μg S-PMA/g urine), each containing the same quantity of the internal standard (1000 μg *p*-Br-S-PMA/l urine).

2.4. HPLC/UV purification and LC/ESI-NI/MS^2-SRM analysis

The purification and the concentration of S-PMA and *p*-Br-S-PMA from urine samples were performed via HPLC by using a Nova-Pak® column equipped with its Sentry Guard Holder. A linear gradient from 100% A (aqueous solution of formic acid 0.1 M, pH 2) to 100% B (methanolic solution of formic acid 0.1 M) over 10 min and an isocratic elution at 100% B for 3 min were employed in the elution program. Both A and B solutions were prepared by adding pure formic acid to bidistilled water or methanol.

Aliquots of 500 μl of urine samples (*spiked* urine or authentic samples) were loaded into the HPLC/UV system, setting a flow of 1 ml/min and a wavelength of 225 nm. Fractions between 5.6 and 6 min (S-PMA) and in the range 6.8–7.2 min (*p*-Br-S-PMA) were collected. The procedure was repeated twice; fractions were gathered and dried under a nitrogen stream.

The LC/ESI-NI/MS^2-SRM analysis was performed by dissolving the residue with 100 μl of bidistilled water. A 20 μl aliquot was injected in the Rheodyne valve and a 200 μl/min flow was used. The same chromatographic conditions (buffers and elution program) were used and the Nova-Pak® column was directly connected to the turbo ion spray source with a heated capillary temperature of 350 °C. The mass spectrometer operated in the negative ion mode (ESI-NI); the spray voltage was −5.5 kV. Data were acquired and processed using the Analyst program (version 1.2, Applied Biosystems). Data from S-PMA and *p*-Br-S-PMA were first acquired in MS^1 mode, in order to register signals corresponding to ions $[M-H]^-$ of both mercapturic acids; these signals were subsequently used as precursor ions for MS^2 experiments. A MS^2-SRM analysis was used for the quantitative determination of S-PMA: the ion at *m*/*z* 237.8 (precursor ion) was isolated in the first quadruple and fragmentation was induced by collision activated dissociation (N_2 as collision gas). Fragment ion at *m*/*z* 109.1 was acquired. *p*-Br-S-PMA was detected by using the signal at *m*/*z* 315.9 as precursor ion and the ion at *m*/*z* 186.8 as product ion.

3. Results

3.1. LC/ESI-NI/MS characterization of S-PMA and p-Br-S-PMA

HLPC separation of mercapturic acids was optimized in order to obtain well resolved peaks and a

complete elution of both acids within few minutes. The above described HPLC method allows an excellent separation of S-PMA and *p*-Br-S-PMA despite their structural analogies. In particular, a RT of 11.3 min was obtained for S-PMA, while *p*-Br-S-PMA elutes at 12.5 min.

Fig. 1 shows the LC/ESI-NI/MS *full scan* mass spectra of S-PMA (panel a) and *p*-Br-S-PMA (panel b). The *full scan* mass spectrum of S-PMA shows a $[M-H]^-$ ion, at *m/z* 237.8, which represents the base peak, and a signal at *m/z* 109.1, which derives from ion at *m/z* 237.8 after the loss of CO_2 and CH_2=CH–NHCOCH$_3$, in agreement with literature data [1]. Such fragmentation hypothesis was confirmed by the MS^2 analysis: ion at *m/z* 237.8 was isolated and further fragmented, obtaining a fragment ion at *m/z* 109.1 (data not shown).

The *full scan* mass spectrum of *p*-Br-S-PMA shows two $[M-H]^-$ ions, at *m/z* 315.9 and 317.9, with a relative intensity of 1:1 (Fig. 1, panel b). These signals reflect the natural isotopic bromide abundance; in fact the ion at *m/z* 315.9 derives from *p*-^{79}Br-S-PMA, while ion at *m/z* 317.9 corresponds to *p*-^{81}Br-S-PMA. A MS^2 analysis was also performed on both isotopic ions (data not shown). The fragmentation pattern of *p*-Br-S-PMA was similar to S-PMA one: ions at *m/z* 315.9 and 317.9 give rise to ions at *m/z* 186.8 and 188.8, respectively.

3.2. Quantitative analysis of S-PMA

Quantitative determination of S-PMA, using *p*-Br-S-PMA as internal standard, was performed by the LC/ESI-NI/MS^2-SRM analysis of urinary samples. The ion suppression effect was studied by comparing the peak areas from purified urinary samples with those from methanolic solutions containing the corresponding amount of pure S-PMA. Three concentration levels were investigated (100, 50, 25 μg/l S-PMA) and purified urinary samples showed an average decrease in the peak areas of 40%. Such decrement is attributable both to extraction losses and to ion suppression effects, so we can state that signal decreases through ion suppression is less than 40%.

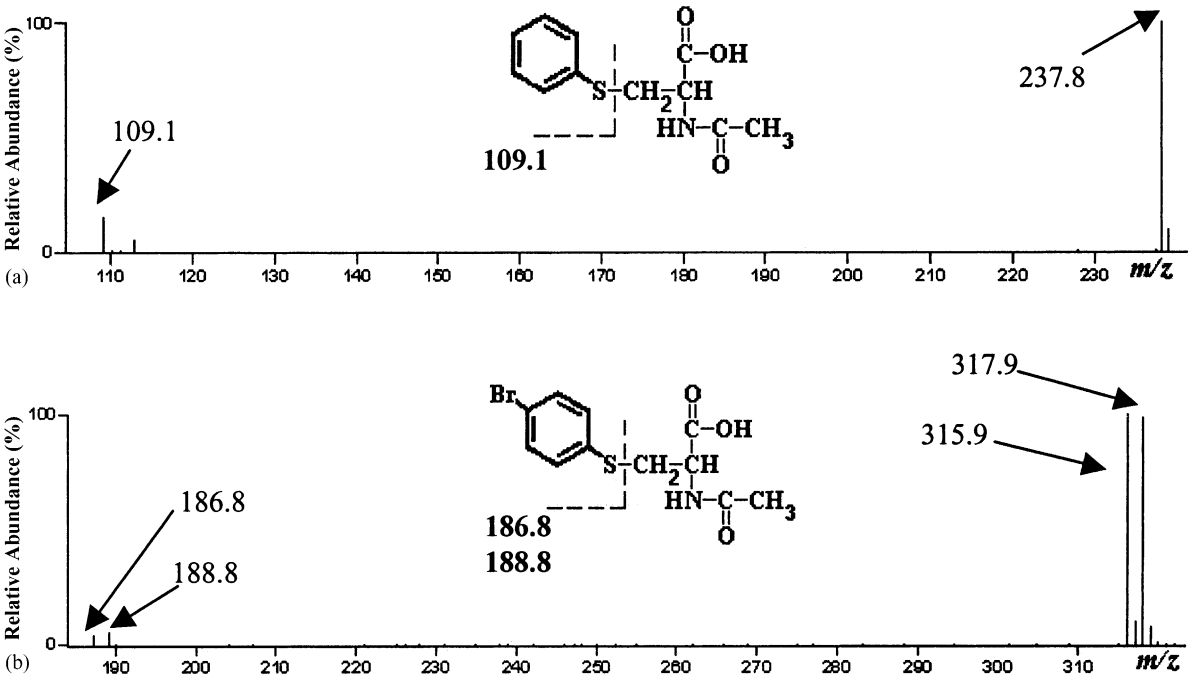

Fig. 1. ESI-NI/MS analysis of a 100 mg/l methanolic solution: *full scan* mass spectra of S-PMA (panel a) and of *p*-Br-S-PMA (panel b).

Table 1
Calibration curve equations and coefficients of determination of four different calibration curves

Time of analysis	Calibration curve equation	Coefficient of determination (R^2)
1st day	$y = 0.0112x + 0.115$	0.993
2nd day	$y = 0.0100x + 0.165$	0.999
24th day	$y = 0.0066x + 0.0479$	0.997
25th day	$y = 0.0072x + 0.0515$	0.996

Each calibration curve equation was obtained from quadruplicate analysis at each of six concentration levels of S-PMA in urine.

The detection limit, measured in signal-to-noise ratio scale, was 5 μg S-PMA/l urine (S/N = 3/1).

Four different urine samples, from non-smoker and non-occupationally exposed subjects, were *spiked* with the internal standard and two aliquots (500 μl each) were purified and concentrated via HPLC and then analyzed by LC/ESI-NI/MS^2-SRM as previously described. In all cases S-PMA was not detectable (data not shown). These urine samples were used for the preparation of four independent urine pools at known S-PMA concentrations for the construction of calibration curves.

Intra- and inter-day precision was evaluated by analyzing both calibration urine solutions and quality control samples. Samples from a single pool were analyzed in the same day, while different pools were analyzed in different and not subsequent days. In the same day, together with calibration urine solutions, 16 independent quality control samples at four different levels of nominal concentrations of S-PMA were analyzed.

Calibration curves were constructed by reporting S-PMA concentrations in *spiked* calibration samples on the *x*-axis and on the *y*-axis the peak area ratio between S-PMA and the internal standard. They showed good linearity in the range 6.2–200 μg/l. Calibration curves constructed in two subsequent days gave rise to comparable results, both in curves equation and coefficients of determination, while data obtained within the span of a month were slightly different in the curve equations and analogous in the coefficients (Table 1).

For the estimation of the percentage inaccuracy (%Diff), intra- and inter-assay precision, QC samples at four concentration levels were analyzed each in quadruplicate on each of four different days. One-way ANOVA was used to calculate intra- and inter-assay precision (as percentage coefficients of variation, CV%). The results are given in Table 2.

3.3. Analysis of smokers' urine samples

Urine from smoker volunteers, non-occupationally exposed to benzene, were collected. Information regarding smoking habits and occupation were recorded. Subjects 1–3, besides being hard smokers, were exposed to city traffic 8 h per day, since they were taxi drivers. In these cases, we found high levels of S-PMA exceeding the highest point of the calibration curve concentration range. Other subjects were male nurses or administrative staffs. For these subjects calculated urinary S-PMA concentration varied from 13.6 to 56 μg/l. Fig. 2 reports the LC/ESI-NI/MS^2-SRM chromatographic profile of real sample 1 (panel a), real sample 12 (panel b) and of a urinary 200 μg/l sample (panel c). All chromatograms refer to 1 ml of urine purified by the above described procedure.

S-PMA concentrations were corrected for the creatinine level, obtaining values in the range 18.3–35 μg S-PMA/g creatinine. Results are schematized in Table 3.

Table 2
Inaccuracy, intra- and inter-assay precision data of the LC/ESI-NI/MS^2-SRM assay for the determination of S-PMA in urine ($n = 16$; 4 days with 4 replicates each at each level)

Nominal concentration (μg/l)	Mean calculated concentration (μg/l)	Inaccuracy %Diff[a]	Intra-assay precision CV%	Inter-assay precision CV%
150	150.4	0.3	0.7	0.7
65	65.2	0.3	1.2	1.5
30	30.1	0.3	2.6	2.7
10	10.1	1.1	7.0	6.8

[a] %Diff = [(mean calculated concentration − nominal concentration)/nominal concentration] × 100.

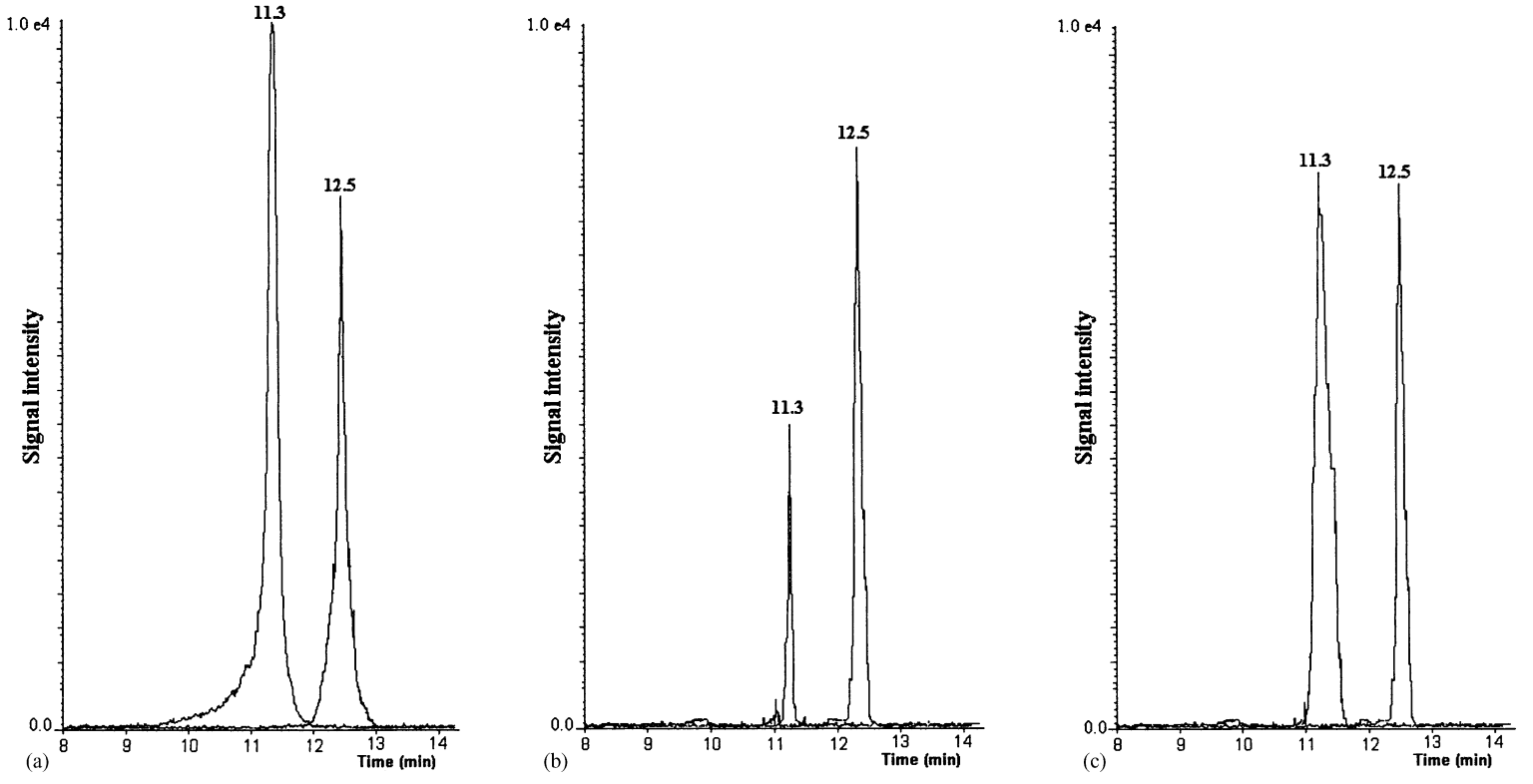

Fig. 2. LC/ESI-NI/MS^2-SRM chromatographic profiles of authentic samples 1 and 12 (panels a, b) and of a *spiked* urinary sample, 200 μg S-PMA/l (panel c). RT: 11.3 min, S-PMA; RT: 12.5 min, *p*-Br-S-PMA.

Table 3
S-PMA levels in urine samples of smoker subjects

Sample	Cigarettes per day	[S-PMA] (μg S-PMA/l creatinine)	[S-PMA] (μg S-PMA/g creatinine)
1	40	>200	–
2	40	>200	–
3	40	>200	–
4	40	19.3 ± 0.4	18.3 ± 0.4
5	40–30	43.6 ± 0.5	27.3 ± 0.5
6	40–30	37.1 ± 0.8	28.6 ± 0.8
7	40–30	56.0 ± 0.4	35.0 ± 0.4
8	35	43.2 ± 1.0	22.1 ± 1.0
9	30	27.5 ± 0.8	29.9 ± 0.8
10	20	15.1 ± 0.7	33.7 ± 0.7
11	20–15	14.3 ± 0.9	29.5 ± 0.9
12	15	13.6 ± 0.6	31.3 ± 0.6

4. Discussion

Urinary S-PMA is a sensitive and specific biomarker of exposure to benzene. Once absorbed, benzene is transformed into benzene oxide by the cytochrome P450 2E1 (CYP 2E1) system.

Main sources of benzene exposure can be attributed to occupational exposure (chemical synthesis industries, petroleum processing, fuel preparation and distribution) as well as to smoking habits and environmental pollution due to motor vehicle emissions. Owing to benzene carcinogenic properties, the biological monitoring of subjects exposed to this toxic has become of particular relevance. This work was focused on the development of an analytical method for the determination of urinary S-PMA as biomarker of benzene exposure. The procedure is based on an HPLC purification followed by a LC/ESI-NI/MS^2-SRM analysis. The purification of S-PMA from urinary matrix can be carried out by liquid–liquid or solid-phase extraction. Here we used a simple, reproducible and, above all, automatizable extraction procedure via HPLC, before the mass spectrometric analysis.

The use of mass spectrometry as detection system requires an appropriate internal standard. Literature reports are based on the use of labeled internal standards, such as [$^{13}C_6$]S-PMA [1], or on mercapturic acids deriving from other electrophilic agents, such as S-benzylmercapturic acid (S-BMA) [22,25,26]. The use of S-BMA as internal standard does not allow the development of a method with a general applicability: S-BMA is the toluene mercapturic acid, so in case of co-exposure to benzene and toluene the use of this molecule as internal standard is not acceptable since results will be altered. In this paper, an internal standard, *p*-Br-S-PMA, structurally related to S-PMA, was synthesized and characterized. The preparation was simple, rapid and quantitative. *p*-Br-S-PMA was obtained in large amounts with a non-expensive procedure. In this way, additional costs involved by the regular use of synthetic labeled molecules as internal standard are avoided. Moreover, *p*-Br-S-PMA could be applicable as internal standard also in case of simultaneous determination of different classes of mercapturic acids.

The mass spectrometric analysis showed best results in negative ionization mode, firstly because S-PMA and *p*-Br-S-PMA give rise to few signals in the MS *full scan* mass spectra. Among these, the $[M-H]^-$ ion was the most abundant, thus avoiding the loss of signal when operating in MS^2-SRM mode, because almost the total ion current generated from the analyte is selected for the MS^2 experiment. Secondly, in negative ionization mode the chemical noise is generally reduced. Both this features contributes to the reaching of sensitivity levels suitable for achieving the aim of this work, i.e. the quantification of mercapturic acids.

The feasibility and efficacy of the proposed assay have been examined by constructing calibration curves. Linear plots in the explored range were obtained with high correlation coefficients. The reliability of the proposed method was also tested by performing quality control analyses on urine samples *spiked* with known amount of S-PMA. The detection limit, related to the purification of 1 ml urine sample, is 5 μg/l.

Calibration curves were constructed by using urine from four non-smoker volunteers, non-occupationally exposed to benzene. The purification and subsequent analysis of 1 ml aliquot of such samples did not show the presence of signals attributable to S-PMA, as expected considering literature data for non-smoker and non-occupationally exposed subjects (1–4.6 μg S-PMA/l urine [23]). For this reason, we used urine from these subjects in the preparation of urinary S-PMA solutions. At the same time the proposed procedure does not allow the evaluation of S-PMA levels in the general, non-smoking, population unless at least 2 ml aliquot of the urine sample is purified. The proposed procedure is, on the contrary,

suitable for the biological monitoring of smoker subjects and of workers occupationally exposed to benzene (both non-smokers and smokers). In this case, literature data [23,26–29] vary in a wide range (non-smokers, 8–111.3 μg S-PMA/l urine [23] and smokers, 0.31–1126 μg S-PMA/g creatinine [26]) and are comparable with the S-PMA levels here found in the analyses of authentic samples from smokers, occupationally and non-occupationally exposed subjects (from 18.3 to >200 μg S-PMA/l urine).

The obtained results, in sensitivity, specificity and accuracy, show the actual applicability of the proposed method in the biomonitoring of smoker subjects and/or occupationally exposed to benzene.

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