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Validation of an HPLC–MS/MS method for the simultaneous determination of phenylmercapturic acid, benzylmercapturic acid and *o*-methylbenzyl mercapturic acid in urine as biomarkers of exposure to benzene, toluene and xylenes

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Abstract

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method was developed and fully validated, according to U.S. Food and Drug Administration guidance, for the simultaneous determination of phenylmercapturic acid, benzylmercapturic acid and *o*-methylbenzyl mercapturic acid in human urine as biomarkers of exposure to benzene, toluene and xylenes (BTX). After solid phase extraction and LC separation, samples were analyzed by a triple–quadrupole mass spectrometer operated in negative ion mode, using isotope-labeled analogs as internal standards (ISs). The method meets all the validation criteria required. The limits of detection of the three analytes, ranging from 0.30 to 0.40 $\mu g\,l^{-1}$, and the high throughput make the method suitable for the routine biological monitoring of co-exposure to BTX both in the occupational and environmental settings. The validated method was applied to assess exposure to BTX in a group of 354 urban traffic wardens.

Keywords: Liquid chromatography–mass spectrometry; Mercapturic acids; Benzene; Toluene; Xylenes

1. Introduction

Aromatic hydrocarbons benzene, toluene and isomeric *ortho*-, *meta*- and *para*-xylene (BTX) are important industrial chemicals widely used, singly and in combination, as organic solvents and in the synthesis of other chemicals. In addition, these compounds are volatile components of gasoline and constituents of tobacco smoke. Due to their formation in many combustion processes, they are widespread environmental pollutants [1]. Thus, the general population undergoes lifelong exposure to these pollutants, and some categories of workers, like gas station attendants and oil refinery workers, are exposed at particularly high levels.

Benzene is classified from the International Agency for Research on Cancer (IARC) [2] as a human carcinogen (group 1). Currently, no evidence exists to suggest that toluene and xylenes are carcinogenic. Nevertheless, exposure to high concentrations of these compounds can induce changes in the central nervous system and other neurotoxic effects [3–5]. The metabolism of these aromatic compounds has been thoroughly investigated [6–8]. At least 90% of the absorbed BTX is excreted through the kidneys as metabolites, including *trans*,*trans*-muconic acid and phenol for benzene, hippuric acid and *o*-cresol for toluene and methylhippuric acids for xylenes. However, the validity of an exposure biomarker mainly relies on its specificity for the toxic compound under consideration. Among the known urinary metabolites, mercapturic acids (MAs) have been recently considered the most specific biomarkers of aromatic compounds, despite their relatively low levels of metabolic production (1% or less of the absorbed BTX) [8,9]. MAs are products of a metabolic detoxification pathway and are excreted in urine after the reaction of electrophilic intermediates

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with endogenous glutathione [10]. At low levels of exposure, phenylmercapturic acid (PMA) has indeed been validated as a more specific biomarker for benzene [7,11]. Furthermore, benzylmercapturic acid (BMA) and *o*-methylbenzyl mercapturic acid (MBMA) have been proposed as reliable biomarkers of exposure to toluene [12] and xylenes [13], respectively. In order to perform biological monitoring of occupationally and/or environmentally exposed individuals, several analytical methods have been developed for the determination of MAs in urine, based on high performance liquid chromatography (HPLC) or gas chromatography–mass spectrometry (GC–MS) [14]. Among published methods, HPLC/tandem mass spectrometry (MS/MS) is currently considered as the first choice for MAs determination in human urine due to its high specificity and sensitivity. Several HPLC–MS/MS methods have been reported for the determination of PMA and BMA in urine [13,15–20] and Gonzalez-Reche et al. [21] described a method for the quantification of dimethylphenyl mercapturic acid (a MA derived from the metabolism of *o*-xylene) in urine as a biomarker of exposure to xylenes. However, the metabolism of alkyl aromatics mainly occurs at the side chain [22–24], producing, in the case of xylene, a higher amount of MBMA. Nevertheless, to our knowledge, no HPLC–MS/MS methods have been developed for the analysis of MBMA in urine. In addition, analytical methods for the simultaneous determination of several MAs would be particularly relevant in cases of co-exposure to several solvents. In fact, many studies have demonstrated that administration of toluene and xylenes to rats causes depletion of glutathione in the liver, influencing the metabolic routes of these and other toxicants [6,25]. Moreover, it is important to note that none of the abovementioned methods was fully and rigorously validated following specific guidelines. As a consequence, little data are available concerning the reliability of previously obtained results.

The aim of our study was to validate an HPLC–MS/MS method to simultaneously determine PMA, BMA and MBMA in human urine for a complete and accurate monitoring of exposure to BTX. For this purpose, a 96-well solid phase extraction (SPE) procedure and a micro(μ)-HPLC separation coupled to electrospray ionization (ESI)-MS/MS were used. This analytical method was fully validated according to the FDA [26], using two deuterated analogs as internal standards (ISs) and different urine lots to assess matrix effect variability. Moreover, the validated method was successfully applied to measure exposure to BTX in a group of 354 traffic wardens.

2. Experimental

2.1. Reagents and chemicals

All chemicals were of analytical reagent grade. Formic acid (98%) was purchased from Fluka (Buchs, Switzerland) and methanol of chromatographic grade was obtained from Merk (Darmstadt, Germany). Deionized water (18.2 MΩ cm) was produced with a Direct-Q Millipore Waters system (Millford, MA, USA). Isolute® ENV+ cartridges (50 mg, 1 ml) and Isolute® C18 cartridges (50 mg, 1 ml) were purchased from IST (Mid Glamorgan, UK); Evolute™ ABN cartridges (25 mg, 1 ml) were purchased from Argonaut Technologies Ltd. (Mid Glamorgan, UK). PMA, BMA and MBMA were supplied by Tokyo Kasei (Prodotti Gianni, Milan, Italy). Deuterated analogs PMAd-5 and BMAd-7 were custom synthesized by Alchemy s.r.l. (Bologna, Italy) to use as internal standards (ISs). All the standard compounds were of the highest available purity (>99%). Stock solutions were prepared by dissolving pure powder of each analyte and ISs in methanol to give a concentration of 200 mg l^{-1}. Stock solutions were stored at −20 °C. Working standard solutions (500 μg l^{-1} for PMAd-5 and BMAd-7; 50–1000 μg l^{-1} for PMA, BMA and MBMA) were prepared weekly by dilution of the stock solutions in MeOH/20 mM formic acid 1:1 (v/v) and were kept at +4 °C.

2.2. Apparatus

HPLC–ESI-MS/MS analysis was performed using a series 1100 μ-HPLC system (Agilent Technologies Inc., Waldbronn, Germany), equipped with a thermostatted well-plate autosampler and thermostatted column compartment modules. The HPLC system was interfaced to an API 2000 triple–quadrupole mass spectrometer from PE Sciex (Concord, ON, Canada) equipped with a TurboIonSpray™ source. A nitrogen generator system 75-72 Whatman (Maidstone, Kent, UK) was employed to produce N_2, used as curtain and auxiliary gas. Instrument control and data acquisition were performed with Analyst Software PE Sciex (rev. 1.3.2). An EZ-2^{plus} Evaporator (GeneVac Ltd., Ipswich, UK) was used for solvent evaporation. SPE was performed on a vacuum system 96-well plated VacMaster (IST).

2.3. Extraction procedure

Urine samples were collected and stored at −20 °C. Prior to analysis, each sample was thawed, vigorously mixed and then centrifuged at 1500 × *g* for 10 min to obtain clear supernatant. The clean-up procedure was optimized by comparing three different types of reversed phase SPE cartridges: Isolute® C_{18}, Isolute® Env$^+$ and Evolute™ ABN.

Evolute™ ABN cartridges were finally chosen for their higher recovery (data not shown). We loaded 1 ml of centrifuged and diluted (1:1 with aqueous formic acid 1%, v/v) urine sample spiked with 5 μl of each IS working solution. The cartridges were previously conditioned with 1 ml of MeOH and 1 ml of aqueous formic acid 0.1% (v/v). The stationary phase was then washed with 1 ml of aqueous MeOH solution (10%, v/v) and the analytes were eluted with two aliquots (250 μl) of methanol. The eluate was evaporated to dryness under vacuum at +40 °C in an EZ-2 Plus concentrator. The residue was redissolved in 50 μl of 20 mM formic acid/MeOH 1:1 (v/v) and a volume of 0.5 μl was injected into the μ-HPLC system.

2.4. Liquid chromatography

HPLC analysis was performed at a flow rate of 10 μl min^{-1} using 20 mM formic acid (solvent A) and methanol (solvent B). Separation was accomplished on a Synergi 4u Max-RP capillary column (0.5 mm × 50 mm, 4 μm, 80 Å, Phenomenex® Torrance,

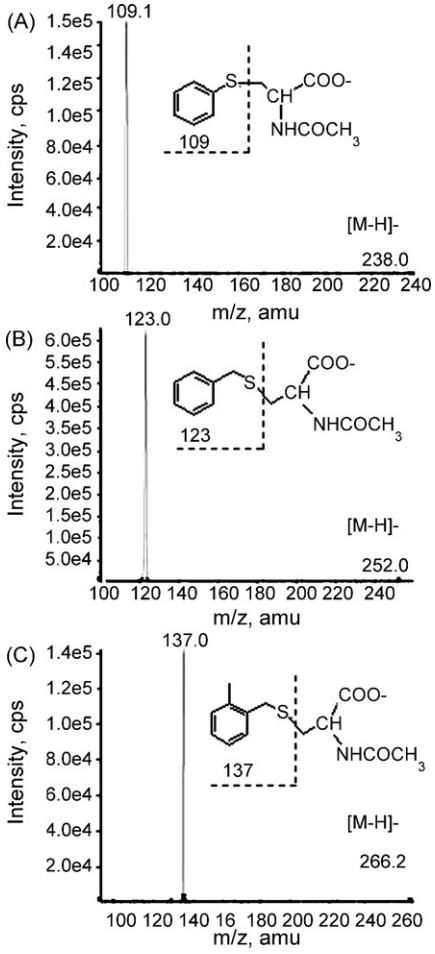

Fig. 1. Product ion mass spectra (ESI$^-$) and ion structures of: (A) PMA (precursor ion $m/z = 238$); (B) BMA (precursor ion $m/z = 252$); (C) MBMA (precursor ion $m/z = 266$).

CA, USA), with an appropriate pre-column (Synergi 4u Max-RP, 0.5 mm × 20 mm, 4 μm, 80 Å, Phenomenex®). The elution gradient was performed as follows: after 1 min of isocratic elution (solvent A 65%, solvent B 35%), solvent B was increased from 35 to 90% in 2 min along a linear gradient curve. The isocratic elution was held for 12 min, after which solvent B was decreased from 90 to 35% in 1 min and column equilibration was conducted isocratically for 11 min (total run time 27 min). The autosampler tray was thermostatted at +15 °C.

2.5. Mass spectrometry

MS/MS analysis was performed on a triple–quadrupole mass spectrometer operated in negative ion mode. Polypropylene glycol standard solution was used to calibrate the instrument over the m/z range 100–900 and to adjust the resolution to 0.7 m/z for MS/MS analysis. Product ion mass spectra from the deprotonated molecular ions $[M-H]^-$ were recorded in the range 100–300 m/z for analytes (PMA, BMA and MBMA) and ISs (PMAd-5 and BMAd-7) in order to characterize the fragmentation behavior of each compound. Fig. 1 shows the product ion mass spectra of PMA, BMA and MBMA. Transitions of the deprotonated molecular ions $[M-H]^-$ for the analytes and ISs were monitored for quantitative analysis in multiple reaction monitoring (MRM) mode (dwell time/channel 600 ms, unitary resolution). Transitions 238 → 109, 252 → 123, 266 → 137, 243 → 114 and 259 → 130 were selected for PMA, BMA, MBMA, PMAd-5 and BMAd-7, respectively. Instrumental parameters were optimized for each analyte by infusion of the corresponding standard solution (0.5 mg l^{-1} in 20 mM formic acid/MeOH 1:1, v/v) at a flow rate of 10 μl min^{-1}, using a syringe pump integrated in the API 2000 mass spectrometer. Nitrogen was used as curtain and auxiliary gas and air was used as nebulizer gas. Electrospray conditions for PMA, BMA, MBMA and ISs were curtain gas, 20 psi; ion-spray voltage, −4500 V; nebulizer and auxiliary gas, 40 and 60 psi, respectively; turbo temperature, 250 °C; collision energy, −18.0 eV; declustering potential, −10 V; focusing and entrance potentials, −350 and −8 V, respectively.

2.6. Urine specimens

For method validation purposes, spot urine samples were collected from healthy non-smoker male and female subjects not occupationally exposed to any organic solvents. However, since in urban areas BTX are ubiquitous pollutants, it is extremely difficult to acquire real blank urine samples. Due to the unavailability of a blank urine matrix, a screening procedure was used to determine which urine samples contained the lowest concentrations of PMA, BMA and MBMA. Thirty lots of urine were analyzed by HPLC–MS/MS in order to quantify the background concentrations of the three analytes. The creatinine concentration of each urine sample was also measured using the Jaffé method [27].

Six lots with urinary creatinine ranging between 0.3 and 3.0 g l^{-1} were then chosen for their low levels of MAs (PMA and MBMA: non-detectable; BMA: between 1.0 and 3.2 μg l^{-1}, mean: 1.7 μg l^{-1}). These urine samples were used separately to assess matrix effect and pooled to obtain calibration and quality control (QC) samples.

2.7. Statistics

A skewness–kurtosis test was used to assess the normal distribution of values. In case of normal distributions, continuous variables were tested by the Student's test. For non-normal distributions, the Wilcoxon rank–sum test was used. Stata 8.0 SE software (Stata Corporation, TX, USA) was used for all analyses, with significance set at $p < 0.05$.

2.8. Calibration curves

Calibration samples were prepared in triplicate in urine, in accordance with the FDA requirements. Pooled urine was spiked with working standard solutions of the three analytes, in order to obtain seven concentration levels (0, 0.6, 1, 2, 10, 25, 50 μg l^{-1} for PMA; 0, 0.7, 1, 2, 10, 25, 50 μg l^{-1} for BMA and 0, 0.8, 1, 2, 10, 25, 50 μg l^{-1} for MBMA). The zero sample was a pooled urine sample (matrix) with ISs. Calibration samples were extracted by SPE and analyzed by μ-HPLC–ESI-MS/MS (as

described above) in the same day and every 50 samples. Analyte peak area/IS peak area ratios were plotted against nominal concentrations (PMAd-5 was used as IS for PMA and BMAd-7 was used for BMA and MBMA). Concentrations were back calculated from the corresponding calibration curves.

2.9. Validation procedures

The method was fully validated according to the FDA guidelines. Limit of detection (LOD), limit of quantification (LOQ), linearity, precision and accuracy, recovery, ion suppression, specificity and stability of the HPLC–ESI-MS/MS method were determined.

2.9.1. Limit of detection and limit of quantification

The LOD and LOQ of the method were assessed for each analyte by sextuplicate analysis of the calibration samples. The LOD and LOQ were estimated by calculating the standard error of the intercept (*Sb*) on the calibration curves ($y = mx + b$). For BMA, the calibration curve was constructed by subtracting the background level peak area from the values determined for the spiked specimens. The LOD and LOQ were expressed as two and four times the *Sb*/*m*, respectively. Precision and accuracy at the LOQ level should meet the FDA acceptance criteria (R.S.D. $\leq 20\%$ and bias $\leq 20\%$).

2.9.2. Precision and accuracy

The precision and accuracy of the entire method were assessed for each analyte at three QC concentration levels in pooled urine samples spiked at 2, 20 and 50 $\mu g\,l^{-1}$. QCs were extracted and analyzed in six replicates on the same day (intra-day precision and accuracy) and on six different days within 2 months (inter-day precision and accuracy). Precision was expressed as the relative standard deviation (R.S.D.) and accuracy was calculated as the relative difference between measured and nominal concentration of the QC samples (bias%).

2.9.3. Recovery and ion suppression

SPE recovery was calculated for the three analytes by comparing the area responses of extracted and non-extracted standard solutions containing PMA, BMA and MBMA at three concentrations (2, 20 and 50 $\mu g\,l^{-1}$), each analyzed in triplicate.

Moreover, ion suppression was evaluated according to the FDA guidelines using two different procedures. Six different human urine samples were processed (as described above) and dry extracts were dissolved with 50 μl of working solution at 10 $\mu g\,l^{-1}$ for each analyte. The analytical responses of these samples were compared with those of the working solutions.

As further tests to assess ion suppression, post-column infusion experiments were done. By using an infusion pump (Model 11 plus, Harvard Apparatus, Holliston, MA), a continuous post-column infusion (at 5 $\mu l\,min^{-1}$) of standard solution (PMA, BMA and MBMA 500 $\mu g\,l^{-1}$ in 20 mM formic acid/MeOH 1:1, v/v) was introduced into the analytical LC system through a T-connector, during injection of an extract of urine (spiked with ISs). Ion suppression of PMA, BMA and MBMA signal by the urinary matrix was examined as "negative" chromatographic peaks from the elevated baseline using six different urinary samples.

2.9.4. Specificity

The specificity of the method was assessed by analysis of six individual batches of control urine, each analyzed both unspiked and spiked at the LOQ level. The peak heights for blank matrix samples should not exceed 20% of peak heights at the LOQ level and accuracy at the LOQ should be within 80–120% of the nominal value.

2.9.5. Stability

Stability of the analytes and the ISs was investigated in standard solutions and in urinary matrix before and after sample extraction, according to the FDA guidelines. Stability of the stock and working solutions was evaluated for 8 h at room temperature and under storage conditions (−20 °C for 1 year and +4 °C for 1 week for stock solutions and working solutions, respectively).

Stability in human urine was assessed in triplicate on three QC samples (2, 20 and 50 $\mu g\,l^{-1}$) after long-term storage (2 months at −20 °C), short-term storage (24 h at room temperature) and after three freeze/thaw cycles, by comparison of the results with those obtained from freshly prepared samples. Furthermore, post-preparative stability was assessed in the final extract by testing reproducibility in autosampler tray over a single batch period (+15 °C for 48 h).

3. Results and discussion

3.1. Analytical characteristics

3.1.1. Mass spectrometry

Fig. 1 shows the product ion mass spectra of the three analytes. For PMA, the product ion mass spectrum was recorded in negative ion mode from the precursor ion *m*/*z* 238; the most intensive fragment was detected at *m*/*z* 109 (Fig. 1a). Fig. 1b shows the product ion mass spectrum of BMA: the signal at *m*/*z* 123 was monitored as the larger fragment of the deprotonated molecular ion (*m*/*z* 252). For MBMA, the most abundant fragment obtained from the precursor ion *m*/*z* 266 was detected at *m*/*z* 137. PMAd-5 and BMAd-7 gave ions at *m*/*z* 243 and 114 and at *m*/*z* 259 and 130, respectively (data not shown). The main fragments detected for all these compounds resulted from the deprotonated ions by loss of CO_2 and $CH_2{=}CH{-}NHCOCH_3$ (proposed fragmentations for PMA, BMA and MBMA are shown in Fig. 1a–c, respectively). Our results on the negative ionization of PMA and BMA are consistent with those previously reported [7,28]. Thus, for HPLC–MS/MS analysis in MRM mode, *m*/*z* 238 → 109, 252 → 123, and 266 → 137 were selected as the most sensitive transitions for PMA, BMA and MBMA, respectively. Transitions *m*/*z* 243 → 114 and *m*/*z* 259 → 130 were monitored for PMAd-5 and BMAd-7, respectively.

3.1.2. Liquid chromatography

The elution gradient and influence of mobile phase were investigated in order to optimize the analytical performances. As reported in previous studies on the ESI$^-$ behavior of compounds containing carboxylic acid groups, a mobile phase containing formic acid in water/methanol gradient resulted to be optimal [29]. We found that the response of acids increased when the concentration of formic acid decreased. However, formic acid increased analytes retention on the column and allowed to resolve analyte peaks from interfering co-eluting matrix components. A short analytical column and elution gradient with 20 mM formic acid and methanol were chosen as best compromise between retention time and ionization of the three analytes (data not shown). Under these chromatographic conditions, analytes and ISs were eluted in 10 min. Fig. 2 shows the MRM chromatogram of a urinary calibration sample spiked with LOQ concentrations of each analyte.

3.2. Method validation

3.2.1. Linearity, LOD and LOQ

The linearity of the calibration curves was determined over the ranges 0.6–50.0 μg l^{-1}, 0.7–50.0 μg l^{-1} and 0.8–50.0 μg l^{-1} for PMA, BMA and MBMA, respectively. Each calibration equation was fitted by the linear regression equation $y = ax + b$, where y is the signal peak area ratio between the analyte and its IS and x is the concentration of the spiked analyte. Although isotopically labeled analogs would be the ideal ISs in HPLC–ESI-MS analysis, isotopically labeled MAs are not commercially available. Therefore, PMAd-5 and BMAd-7 were synthesized to use as ISs for PMA and BMA, respectively. BMAd-7 was also used to correct MBMA. In fact, we verified that, as compared to analysis without IS (data not shown), the correction of MBMA signal by that of BMAd-7 increased both the correlation coefficient of the calibration curve and the accuracy of back-calculated concentrations, while guaranteeing to meet the FDA requirements (see MBMA, Table 2).

For each analyte, the calibration curve showed a coefficient of determination (r^2) greater than 0.999. Concentrations were back calculated from calibration curves for each calibration sample (including LOQ): deviations from the nominal concentrations (bias%) were between 0.1 and 12.1% for PMA, between 1.4 and 14.3% for BMA, and between 0.04 and 4.9% for MBMA. The R.S.D. values resulted to be <5.1, <2.7 and <21.0% for PMA, BMA and MBMA, respectively. Calibration parameters, LOD and LOQ of the three analytes are summarized in Table 1. The LOQ of the method was determined for each analyte by dividing four times the S.D. of the 'blank' determination by the slope

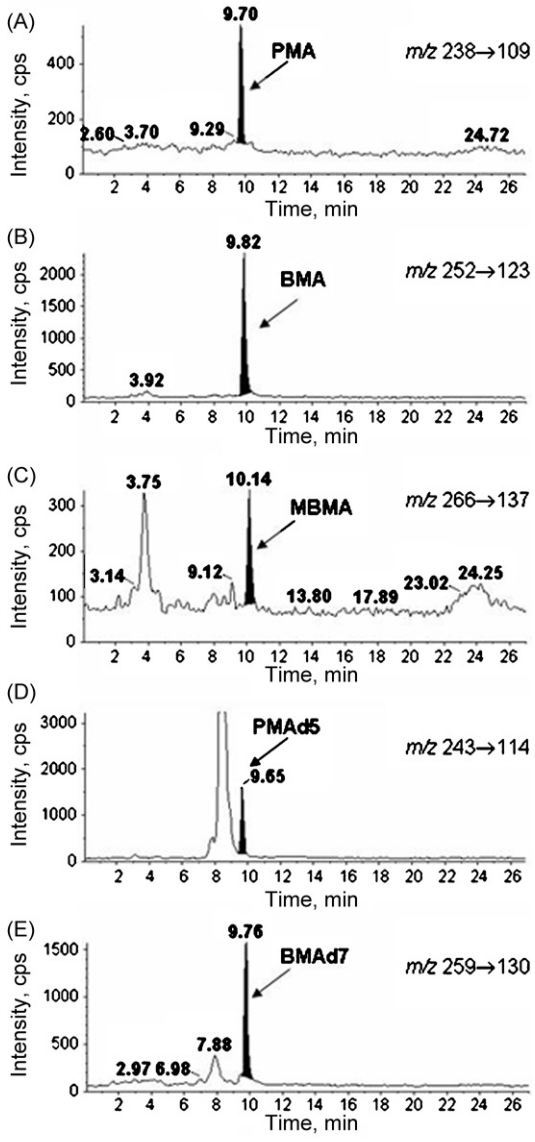

Fig. 2. Representative chromatogram of a calibration sample spiked with 0.6 μg l^{-1} of PMA (A), 0.7 μg l^{-1} of BMA (B), 0.8 μg l^{-1} of MBMA (C) and 5 μg l^{-1} of each IS (D and E).

Table 1
Calibration parameters obtained for PMA, BMA and MBMA against ISs from three different calibration curves prepared in triplicate and analyzed in different days, within 2 weeks

	PMA	BMA	MBMA
Range of calibration (μg l^{-1})[a]	0.6–50.0[a]	0.7–50.0[a]	0.8–50.0[a]
Slope (±S.D.)	0.283 (±0.016)	0.256 (±0.019)	0.200 (±0.015)
Intercept (±S.D.)	0.020 (±0.029)	0.027 (±0.042)	0.037 (±0.048)
Regression coefficient (r^2)	0.9999	0.9998	0.9995
Limit of detection (μg l^{-1})[b]	0.30	0.35	0.40
Limit of quantification (μg l^{-1})[b]	0.60	0.70	0.80

[a] Assessed on seven concentration levels.
[b] Assessed by analysis of calibration samples ($n = 6$).

Table 2
Precision and accuracy for PMA, BMA and MBMA in urinary QC samples

Nominal concentration ($\mu g\,l^{-1}$)	Intra-day		Inter-day	
	R.S.D.	Bias%	R.S.D.	Bias%
PMA				
2	9.0	−8.9	5.6	−5.7
20	3.9	0.1	6.2	2.9
50	5.0	−4.0	2.8	−2.0
Average	6.0	−4.3	4.9	−1.6
BMA				
2	2.8	−4.4	14.0	2.0
20	4.7	8.8	6.0	8.2
50	2.2	10.8	2.7	5.8
Average	3.2	5.1	7.6	5.3
MBMA				
2	8.2	7.9	5.3	9.4
20	4.1	14.2	4.1	14.2
50	1.7	14.9	3.8	11.6
Average	4.7	12.3	4.4	11.7

Number of replicates = 6.

of the calibration equation. Moreover, the accuracy of quantification at the LOQ level should be tested in six different urine lots and should be between 80 and 120% for all urines. Several LC–MS published methods reported slightly lower LOQ values for PMA or BMA in urine. However, these values were obtained by analysis of a pool of urine and not from different urine samples [13,15–19]. Due to the unavailability of a urine sample completely free of BMA, the determination of the LOQ for BMA presented some problems. An estimated LOQ value for BMA in urine was thus calculated to be 0.7 $\mu g\,l^{-1}$. Nevertheless, the actual LOQ of BMA in urine may be lower than this estimate and could be determined only in urine samples with non-detectable amounts of BMA.

3.2.2. Precision, accuracy and recovery

Table 2 shows the intra-day and inter-day accuracy and precision values of the entire procedure for PMA, BMA and MBMA. For all analytes, accuracy was within 91.1 and 114.9% and R.S.D. did not exceed 14.0%. This showed that each analyte met the generally accepted criteria for bioanalytical method validation at all QC concentration levels.

As regards recovery obtained from the SPE procedure, the mean values were 82 (±4.4) % for PMA, 71.2 (±7.8) % for BMA and 78.3 (±11.8) % for MBMA.

3.2.3. Matrix effect and ion suppression

Although matrix-induced alterations (suppression or enhancement) of the ESI-MS/MS signals may critically impair the reliability of a method, little attention is often paid to this topic during method validation. In particular, previously published methods for the determination of MAs in urine did not test matrix effect and ion suppression [13,15–20]. Due to the complexity of biological fluids and to the high inter-subject variability of urine in particular, assessment of matrix effects

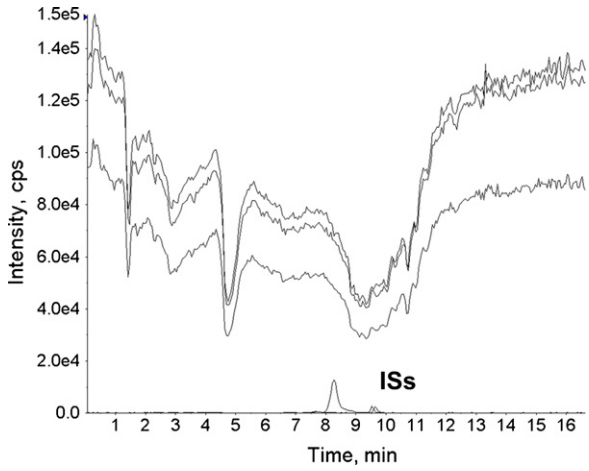

Fig. 3. Chromatogram of an extracted urine sample (spiked with ISs) with post-column infusion of 500 $\mu g\,l^{-1}$ of PMA, BMA and MBMA.

in different batches of urine has been included as part of the validation procedure.

The experiments performed on six different urine samples spiked after extraction showed a high-ion suppression (mean analytes signal was 25.3% as compared to standard solutions). These results are confirmed by post-column infusion experiments, which indicated that the analytes peaks fell into an ion suppression region (see Fig. 3).

In the attempt to decrease ion suppression by increasing analytes retention in column, several elution gradients were tested. However, when the analytes retention time changed, the region of maximum ion suppression moved accordingly (data not shown). This may be due to the fact that ion suppression of the urinary matrix in this region is caused by other MAs, which are present in urine at high concentration levels. For the same reason, different extraction procedures performed using different SPE cartridges (Isolute® C_{18} and Isolute® Env+) did not decrease matrix effect (data not shown).

Several LC–MS analytical methods for the determination of PMA or BMA have been published, but only few showed ion suppression data. Although obtained using a different clean-up procedure, our results are consistent with those previously published [7].

The repeatability of the total procedure was evaluated analyzing six calibration curves constructed in six different lots of urine, instead that in a single (pooled) urine sample. Despite the high suppression due to urinary matrix, the slopes of calibration curves measured with six different lots of urine were highly precise (R.S.D. < 8%), and an identical susceptibility of analytes and ISs to matrix effect may be inferred. This, together with satisfying inter-day precision and accuracy values obtained for LOQ concentration levels from the six lots of urine was indicative for the absence of matrix-caused quantification errors.

3.2.4. Specificity

The method showed good specificity, meeting the acceptance criteria of the FDA. No interfering peaks were detected in the 'blank' samples at the mass transitions chosen for PMA, MBMA and the ISs. The signal detected for BMA in all unspiked

Table 3
Results of HPLC–MS/MS analysis in urine samples of 354 traffic wardens

	% of quantifiable samples	Urinary concentration mean ± S.D. ($\mu g/g_{creatinine}$)
PMA	27	1.76 ± 1.58
BMA	100	12.84 ± 25.71
MBMA	23	3.98 ± 7.19

tested lots (signal to noise ratio larger than 3:1) corresponds to background levels of BMA in urine. The accuracy of the six individual urinary samples spiked at LOQ resulted within the parameters indicated in the guidelines (80–120% of the nominal value).

3.2.5. Stability

All the stability experiments performed met the FDA requirements: the deviation from the initial concentrations of the analytes and ISs was <6% in the standard solutions. No significant changes in concentrations (≤14%) were observed in urinary QCs.

3.3. Application of the method: MAs determination in a group of traffic wardens

The validated analytical LC–MS/MS method was applied to measure the urinary levels of PMA, BMA and MBMA in 354 subjects (225 men, 129 women; 105 smokers, 249 non-smokers) involved in traffic control in Bologna (Italy). Spot urine samples were collected at the end of an 8 h work shift during the period April–November 2006. Since the concentrations of these metabolites, which are excreted by diffusion, are dependent on urine output, correction for creatinine concentration is necessary. Urinary creatinine was determined by the Jaffé method. As shown in Table 3, all samples showed a measurable concentration of BMA, while PMA and MBMA resulted quantifiable only in 27% and 23% of the analyzed urines, respectively. No sample exceeded the BEI value proposed for PMA (25 $\mu g/g_{creatinine}$) by the American Conference of Governmental Industrial Hygienists (ACGIH) [11].

No statistically significant differences were found between male and female subjects in the urinary excretion of these metabolites (all $p > 0.05$). The difference in urinary concentration between smokers and non-smokers was not statistically significant for BMA and MBMA, whereas it was statistically significant ($p < 0.05$) for PMA (smokers: 1.97 ± 1.67 $\mu g/g_{creatinine}$; non-smokers: 0.98 ± 0.87 $\mu g/g_{creatinine}$). Moreover, it is interesting to note that 80% of the samples showing measurable amounts of PMA were urines obtained from smokers. This is not surprising, since it is common knowledge that tobacco smoke represents an important source of benzene. In fact, several studies reported higher concentration of PMA in smokers than in non-smokers [7,30]. Furthermore, previous studies demonstrated that traffic wardens are exposed to relatively low levels of benzene and identified tobacco smoke as the main source of benzene among these workers [30,31]. Our results are in line with these data. Moreover, the PMA levels reported in our study are very similar to those obtained by Bono et al. [30] in a group of 206 traffic wardens.

4. Conclusions

To our knowledge, this is the first fully validated HPLC–MS/MS method for the simultaneous determination of PMA, BMA and MBMA in human urine. The method meets all the FDA acceptance criteria over the following ranges: 0.6–50.0 $\mu g\,l^{-1}$, 0.7–50.0 $\mu g\,l^{-1}$ and 0.8–50.0 $\mu g\,l^{-1}$ for PMA, BMA and MBMA, respectively. Moreover, the high sensitivity and sample throughput make the method suitable for the biological monitoring both of occupational and environmental co-exposure to BTX.

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