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Determination of *S*-phenylmercapturic acid by GC-MS and ELISA: a comparison of the two methods

G. MARRUBINI[1], S. DUGHERI[2], M. PACENTI[2], T. COCCINI[3], G. ARCANGELI[2], V. CUPELLI[2], & L. MANZO[3,4]

[1]*Department of Pharmaceutical Chemistry, University of Pavia, Pavia, Italy,* [2]*Occupational Health Division, Department of Public Health, University of Florence, Florence, Italy,* [3]*Research Centre, Toxicology Division, IRCCS Salvatore Maugeri Foundation, Pavia, Italy,* [4]*Department of Internal Medicine, Clinical Toxicology Division, University of Pavia, Pavia, Italy*

Abstract

S-phenylmercapturic acid (PMA) is a specific urinary biomarker of benzene at exposure levels lower than 1 ppm. However, measuring PMA in urine is an expensive task by either GC or HPLC due to the necessity of extensive sample pretreatment. In the present study, a commercial chemiluminescence enzyme-linked immunosorbent assay (ELISA) test for PMA and GC-MS were used for screening urine samples of 60 workers employed in petrochemical settings. The ELISA results were evaluated by comparison with the GC-MS. Overall, the ELISA test proved sensitive (limit of detection $=0.1\ \mu g\,l^{-1}$), rapid, robust and reliable, affording results in good agreement with the GC-MS (54% of measurements) and no false-negatives. On the other hand, 46% of the ELISA assays were assigned as false-positives (arbitrarily established when ELISA $>5\ \mu g\,l^{-1}$, GC-MS $<5\ \mu g\,l^{-1}$) and a correlation coefficient of 0.687 was calculated between the two methods. It appears that urinary PMA routine biomonitoring on large numbers of samples is carried out in a cost-effective and rapid approach by preliminary screening with the ELISA assay followed by GC-MS confirmation of concentrations exceeding the biological exposure index for PMA.

Keywords: S-*phenylmercapturic acid, chemiluminescence, enzyme-linked immunosorbent assay (ELISA), gas chromatography-mass spectrometry (GC-MS), benzene, biomonitoring*

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Introduction

Benzene is an important by-product of the petrochemical industry. It is a recognized carcinogen for man with current exposure limits in the European Community at 1 ppm (i.e. 3.2 mg m^{-3}, threshold limit value-time weighted average for an 8-h work shift).

Over the past few years, much research has been devoted to the study of reliable biomarkers of benzene exposure in order to monitor the risk for human health represented by the absorption of this solvent. Unmetabolized benzene itself in blood (Perbellini et al. 2003), exhaled air (Weisel et al. 1996) and urine (Waidyanatha et al.

Correspondence: G. Marrubini, Department of Pharmaceutical Chemistry, University of Pavia, via Taramelli 12, I-27100 Pavia, Italy. Fax: 39-0382-422975. E-mail: giorgio.marrubini@unipv.it

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2001, Basilicata et al. 2005) was determined to assess exposure. However, for biomonitoring campaign studies, practical, non-invasive sampling methods and stable, non-volatile target analytes are generally preferred. Therefore, research was mainly dedicated to studies on urinary benzene metabolites such as phenol, *trans*,*trans*-muconic acid (MA) and *S*-phenylmercapturic acid (N-acetyl-*S*-phenyl-(L)-cysteine, PMA).

Phenol and its metabolites hydroquinone, cathecol and 1,2,4-benzenetriol, as well as their sulfate- and betaglucurono-conjugates, have been studied and were found to be suitable for assessing benzene severe exposures, i.e. those occurring at levels exceeding 5–10 ppm (Inoue et al. 1988, 1989, 2001).

MA is a sensitive and specific biomarker for benzene exposure levels above 1 ppm, whereas PMA is considered highly specific and useful for evaluating exposure levels which fall under the 1 ppm threshold (Boogard & Van Sittert 1995). Thus, at the present allowed exposure limits in the European Community, PMA can be regarded as the biomarker of exposure of choice because of both its sensitivity and its specificity. On the other hand, measuring PMA in urine is an expensive task due to the necessity of extensive sample pretreatment involving extraction and derivatization independently of the instrumental technique available for detection. In addition, highly selective chromatographic techniques are required (HPLC and GC) coupled to sensitive detectors such as the fluorimeter (Maestri et al. 1993), mass spectrometer (Angerer et al. 1998) or tandem mass spectrometer (Melikian et al. 1999, Lin et al. 2004a, b). These requirements enhance the costs of analyses and present disadvantages when planning the measurement of PMA in large-scale screening programmes.

The physiological levels of PMA in the urine of people not exposed to benzene are generally well below the 5 $\mu g\,l^{-1}$ level in non-smokers and exceed the 10 $\mu g\,l^{-1}$ level in heavy smokers exposed to benzene with concentrations that span a wide range of values (e.g. from 13 to $>200\ \mu g\,l^{-1}$ according to Pieri et al. 2003). In Italy, workers by law cannot be exposed to benzene concentrations exceeding 1 ppm (i.e. 3.2 $mg\,m^{-3}$), and the American Conference of Governmental Industrial Hygienists (ACGIH) Biological Exposure Index (BEI) which is at 25 $\mu g\,g^{-1}$ creatinine (i.e. broadly about 25 $\mu g\,l^{-1}$) is accepted as a reference value.

Therefore, the requirement for a method for monitoring PMA in urine is primarily to be as sensitive as possible in order to determine concentrations equal to or lower than 25 $\mu g\,l^{-1}$ of the target analyte with sufficient accuracy and precision.

At present, PMA is determined in urine by HPLC methods requiring SPE extraction, derivatization and fluorimetric detection (Maestri et al. 1993, Inoue et al. 2001). Determination of PMA by GC-MS (Van Sittert et al. 1993, Einig et al. 1996, Angerer et al. 1998, Waidyanatha et al. 2004), GC-MS-MS (Van den Berg 2003), LC-MS (Maestri et al. 2005) and LC-MS-MS (Melikian et al. 1999, Liao et al. 2002, Pieri et al. 2003, Barbieri et al. 2004, Lin et al. 2004a, b) were also reported.

Ball et al. (1997) and Aston et al. (2002) reported the development and validation of a competitive enzyme-linked immunosorbent assay (ELISA) specific for PMA which was designed to simplify the procedures used until recently for routine biomonitoring of low levels of benzene exposure. In the work of Aston et al., an enzyme substrate solution (*p*-nitrophenyl phosphate in Tris buffer) was added to the

urine samples in microplates in order to visualize anti-PMA binding. After 30 min, the absorbance at 405 nm was measured by an ultraviolet light-visible photometer.

After reviewing the current knowledge on PMA, it was decided to carry out its routine determination in urine of exposed workers by using the chemiluminescence-ELISA test. The ELISA screening results were then compared with data obtained by GC-MS according to the method of Van Sittert et al. (1993) which was adapted because of its apparent simplicity.

This paper presents and discusses the results obtained by the comparison of the measurements of PMA in urine obtained by the two methods.

Materials and methods

Reagents

S-phenylmercapturic acid (PMA, purity 99%) was purchased from Tokyo Kasei (Tokyo, Japan), and [$^{13}C_6$]-PMA (purity assayed $>95\%$) was supplied by Dr Emanuele Attolino, University of Pisa, who synthesized the molecule according to the procedure detailed by Melikian et al. (1999).

Ampoules of 5 ml each containing 0.5 N hydrochloric acid in methanol were purchased from Supelco (Supelco-Sigma Aldrich group, Milan, Italy). Ethyl acetate and toluene were analytical-grade reagents obtained from VWR-Merck (Milan, Italy).

The kit for urinary PMA chemiluminescence-ELISA assay and detection reagents to measure flash chemiluminescence were purchased from Molecular Light Technology Research Ltd. (Cardiff, UK). Each kit provides sufficient materials for 40 specimen tests in duplicate. All kit components are ready for use, except the wash buffer, which is a 25-ml concentrated reagent, that must be diluted to 250 ml with deionized/distilled water just before use.

Urine samples collection

Pre- and post-shift urine samples were collected from 60 subjects employed in refineries and fuel deposits located near the urban areas of Milan, Rome and Palermo. All workers were potentially exposed to benzene in the workplace at levels ranging from 0.006 to 0.321 mg m^{-3} (mean concentration $\pm$ standard deviation 0.2 $\pm$ 0.1 mg m^{-3}, median 0.27 mg m^{-3}, $n=60$). For method testing, 'blank' urine was collected from three healthy non-occupationally exposed to benzene, non-smoking volunteers.

Immediately after collection, the urine samples were subdivided into two aliquots. For GC-MS analysis 10 ml of each urine sample were frozen at -20°C, stored in the dark and dispatched to the University of Pavia. The second aliquot of 5 ml of each urine sample was acidified with 50 µl HCl 6 M and stored in a refrigerator at $+2-8^{\circ}$C, as recommended by the producer of the ELISA test kits (MLT urinary PMA assay instructions supplied with Kit Catalogue No. 3-001 'Benzene Exposure Biomonitoring'; overview of the assay principle; available at: http://www.mltresearch.com/pma.htm). These samples were then sent to the University of Florence for chemiluminescence-ELISA testing.

GC-MS analysis of urinary PMA

Stock solutions of 1 mg ml^{-1} each of PMA and [$^{13}C_6$]-PMA were prepared in methanol and stored at −20°C in the dark. From these solutions, working solutions (10 μg ml^{-1} for PMA and 20 μg ml^{-1} for [$^{13}C_6$]-PMA) were prepared in water just before every session of analyses.

Urine aliquots of 3 ml were spiked with PMA working solution to give standards in the range 5–100 μg l^{-1}, and with [$^{13}C_6$]-PMA working solution to give a final concentration of internal standard of 200 μg l^{-1}.

Sample pretreatment was carried out basically according to the method of Van Sittert et al. (1993), although with some slight modifications. Briefly, aliquots of 3 ml urine after being thawed were added with [$^{13}C_6$]-PMA (30 μl internal standard solution 20 ng μl^{-1} in water). Samples were homogenized by vortex mixing and acidified to pH <2 with 50 μl concentrated HCl in polythene tubes with plastic screw caps. Ethyl acetate (12 ml) was added and the tubes vortex-shaken for 2 min. Centrifugation (15 min at 4000 rpm) followed and the supernatant was transferred into glass tubes for evaporation under nitrogen stream at 45°C. The dry residue was reconstituted in 0.5 ml 0.5 N HCl/MeOH, the tube capped and the reaction left at room temperature for 30 min. The end-reaction content of the tubes was evaporated to dryness under nitrogen stream at 45°C and reconstituted in 0.1 ml toluene. A total of 1 μl toluene was injected into the GC-MS.

Mass spectra of standard pure PMA methyl ester (PMA-Me) and [$^{13}C_6$]-PMA methyl ester ([$^{13}C_6$]-PMA-Me) were obtained after derivatizing in glass tubes the dry residue of methanolic standard solutions of PMA and [$^{13}C_6$]-PMA by the same procedure described above. For comparison purposes, mass spectra of the target analyte and internal standard were also collected after derivatizing the pure standards extracted from aqueous solutions. The aqueous solutions were acidified, extracted and derivatized as described for urines.

All analyses were performed on bench-top standard equipment from Agilent Technologies consisting of model 6890N gas chromatograph connected to a model 5973*Network* mass selective detector, and equipped with a model 7683 Series injector.

Injections were performed at 250°C in pulsed-splitless mode (injection pulse pressure 20 psi until 1 min, purge flow 50 ml min^{-1} at 1 min) in a glass liner (conventional Agilent split-splitless Part. No. 5183-4693) containing a plug of deactivated glass-wool at the bottom.

High-purity He (99.999% or better) was used as a carrier gas at about 10 psi (constant flow of 1 ml min^{-1} or around 37 cm s^{-1}).

An Agilent Technologies-type HP-5 capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness) was used under the following conditions: 100°C × 2.25 min, 10°C min^{-1} to 260°C, 30°C min^{-1} to 290°C, 290°C for 5 min.

The GC-MS interface temperature was at 280°C. The ion source was at 230°C, the electron impact ionization energy was 70 eV, and the quadrupole was heated at 150°C.

Full-scan of masses from *m*/*z* 50 to 300 was carried out to obtain mass spectra of PMA-Me and of [$^{13}C_6$]-PMA-Me, whereas selected ion monitoring (SIM) was performed on the ions *m*/*z* 253 and 194 (qualifier and quantifier, molecular mass and base peak of PMA-Me, respectively), and on the ions *m*/*z* 259 and 200 (qualifier and quantifier, molecular mass and base peak of [$^{13}C_6$]-PMA-Me, respectively).

Chemiluminescence-ELISA analysis of urinary PMA

Five standards were employed for assay, each containing pure solid PMA in a urine-like matrix within the range 0–125 nmol l^{-1} (0–23.45 μg l^{-1}) supplied in the ELISA kit.

The precision and accuracy of the ELISA method were studied using a urine pool prepared from samples obtained from three non-smoking volunteers not occupationally exposed to benzene. The PMA concentration pre-screened with the ELISA method on a urine pool aliquot was <0.1 μg l^{-1}. Known PMA quantities were thus added to the urine pool aliquots to realize test samples in the range between 1.98 and 23.45 μg l^{-1}.

Urine samples and standard aliquots of 200 μl were dispensed into dilution tubes containing the sample diluents (1000 μl phosphate-buffered saline solution) according to the sequence: standards 1–5, urine specimens. The dilution tubes were vortex-shaken for 2 s. A luminescent antibody conjugate reagent (200 μl) was added into each assay tube (coated with PMA–protein conjugate) containing 200 μl of the mixed prediluted samples (standard/urine specimen). The assay tubes were vortex-shaken for 2 s then incubated in a water bath at 22°C for 60 min. At the end of the incubation, the contents of the tubes were decanted onto tissue paper, then phosphate-buffered aliquots (1000 μl) were spiked into each tube and the process repeated twice. To measure flash chemiluminescence, the tubes were transferred into the luminometer (Berthold model LB 952) equipped with dual on-line reagent injection. The aliquots of 300 μl detection reagent 1 (0.5% hydrogen peroxide, 0.1 M nitric acid) and 300 μl detection reagent 2 (0.25 M sodium hydroxide, 0.2% surfactant) were dispensed automatically into the assay tubes for measuring. The luminometer settings were: 2 s for delay injections 1 to 2, zero seconds for delay injection 2 to measure and integrate the number of counts over 2 s of measurement time.

Results

Figure 1 shows typical SIM chromatograms obtained from pooled blank urine (Figure 1A), from the same blank sample spiked with 20 μg l^{-1} PMA (Figure 1B), and from an authentic urine sample of a benzene-exposed worker (Figure 1C). Selectivity was sufficient, and no interfering peaks due to co-eluting components were observed at the retention times of PMA-Me and internal standard [$^{13}C_6$]-PMA-Me.

The GC-MS method was evaluated for the following parameters: linearity, precision, accuracy, limit of quantitation (LOQ) and limit of detection (LOD). A summary of the results is given in Table I. Calibration curves were linear in the range 5–100 μg l^{-1}, and correlation coefficients >0.997 were obtained during the experiments. The LOD was at the 1 μg l^{-1} level and a theoretical LOQ of 2 μg l^{-1} could be calculated. However, in routine applications, the practical method LOQ chosen was at the 5 μg l^{-1} level to ensure optimal precision.

Precision was studied both as within session and as intersession repeatability. It was expressed by calculating the relative standard deviation (RSD%) on replicate measurements on pooled blank urine spiked with PMA. The results demonstrated that RSD% was in the range 2–6% for within session, and 0.4–15% for intersession precision.

Accuracy was evaluated by recoveries (%) (calculated from the ratio per cent between the measured and the nominal concentrations in spiked blank urines) at all

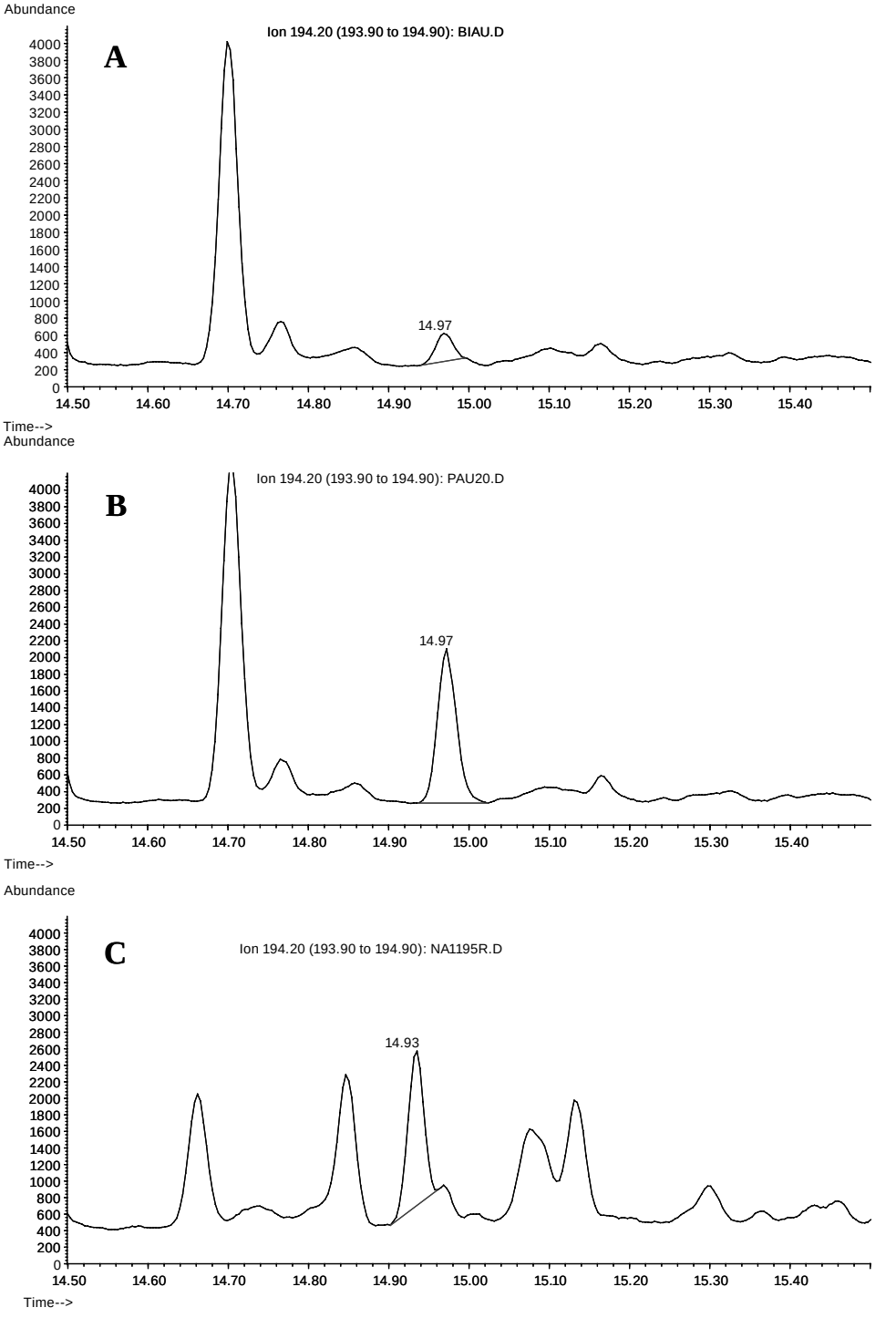

Figure 1. Selected ion monitoring (SIM) chromatograms of ions m/z 194.2, quantifier for PMA-Me. (A) blank pooled urine, (B) blank urine spiked with 20 μg/l of PMA, (C) real sample of post-shift urine obtained from a refinery worker.

Table I. Summary of calibration data ($\mu g\,l^{-1}$) for the GC/MS assay and chemiluminescence-ELISA test.

GC/MS					ELISA test				
Nominal concentration	Measured mean concentration ± SD	Precision (RSD %)	Accuracy (Rec %)	n^b	Nominal concentration	Measured mean concentration ± SD	Precision (RSD %)	Accuracy (Rec %)	n^b
Within session:									
5	4.4 ± 0.2	3	88	3	1.98	1.65 ± 0.09	5.6	83	3
10	10.6 ± 0.6	6	106	3	5.91	6.2 ± 0.16	2.6	105	3
50	49.9 ± 0.9	2	100	3	13.21	13.8 ± 0.94	6.8	105	3
100	100 ± 5	5	99.8	3	23.45	28.86 ± 2.08	7.2	123	3
Intersession:									
5	4.7 ± 0.7	15	94	11	1.98	1.55 ± 0.08	5.3	78	4
10	10.1 ± 0.9	8	101	11	5.91	5.62 ± 0.48	8.5	95	4
50	49.9 ± 0.2	0.4	99.8	10	13.21	12.93 ± 0.83	6.4	98	4
					23.45	29.3 ± 3.63	12.4	125	4
Linearity:		*Sensitivity:*					*Sensitivity:*		
Slope[a] ± SD	intercept[a] ± SD	LOQ		5			LOD		0.1
1.2 ± 0.2	0.02 ± 0.02	LOD		1					

[a]Mean ($n = 11$); RSD %, relative standard deviation (%); Rec %, recovery (%), calculated as the ratio (%) between the measured and the nominal contraction.
[b]Number of experiments; SD, standard deviation; LOQ, limit of quantitation; LOD, limit of detection.

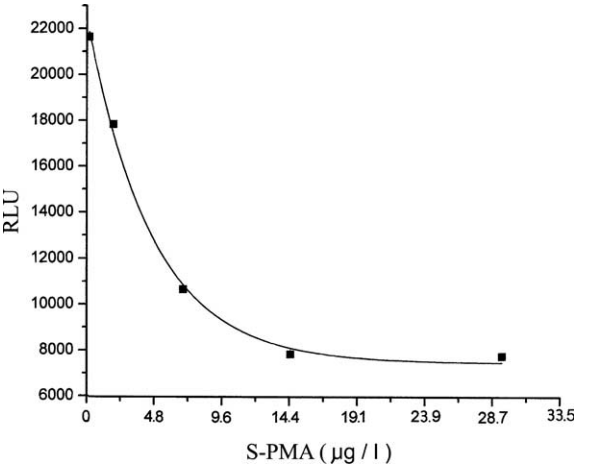

Figure 2. Example calibration curve for the ELISA test. See Table I for nominal and measured concentrations.

concentrations used for the calibration plot. Recoveries were high and ranged from 88 to 106%.

Table I also summarizes calibration data for the ELISA test, and one example calibration curve is illustrated in Figure 2. As shown, the response curve describes the experimental data with good reliability within the range 0–60 nmol l^{-1} (0–14.4 µg l^{-1}). The LOD was 0.1 µg l^{-1}, according to the specifications of the kit producer. At this level of sensitivity, the RSD% for three replicate measurements on four different PMA concentrations (0–23.45 µg l^{-1}) ranged from 2.6 to 7.2% for within session and from 5.3 to 12.4% for intersession precision (Table I).

Using these two methods, we determined the concentration of PMA in pre- and post-work shift urine samples obtained from 60 workers potentially exposed to benzene at levels ranging from 0.006 to 0.321 mg m^{-3} on workplace. The results obtained from the 120 determinations are shown in Table II. Most samples had concentrations measurable by the ELISA test (97% data are above the assay LOD of 0.1 µg l^{-1}), whereas only 13% of the samples had concentrations above the GC-MS method LOQ of 5 µg l^{-1} (Table II).

The medians of PMA concentrations measured by the two methods are similar (8 and 7 µg l^{-1} for pre-shift, and 7 and 9 µg l^{-1} for post-shift urines), but the means obtained by the ELISA test are lower than those calculated by the GC-MS data both on the pre- and post-shift urines.

The overall correlation between the two sets of data is poor considering all data, but the correlation coefficient calculated on the subset of data represented by measurable concentrations (i.e. ELISA data >0.1 µg l^{-1}, GC-MS >5 µg l^{-1}) is 0.687.

Discussion

Biological monitoring of occupational exposures to benzene is a complex task: PMA is recognized as the most sensitive and specific benzene biomarker, even at exposure levels <1 ppm, though it is still unclear how much lower. Recent studies performed in China and in Taiwan on benzene-exposed workers showed that there are still workers

Table II. Urinary PMA concentrations ($\mu g\,l^{-1}$) measured with ELISA and GC/MS.

Subject	ELISA	GC/MS	ELISA	GC/MS
	Pre-shift		Post-shift	
1	10.3	6.4	12.8	9.5
2	0.5	<LOD	2.0	<5
3	14.9	6.7	8.2	15.0
4	18.2	<5	11.8	<5
5	10.8	5.4	<0.1	<LOD
6	8.0	<5	9.3	<5
7	10.4	7.4	12.0	5.1
8	10.5	<5	9.4	<5
9	25.1	22.8	32.1	35.0
10	10.5	13.9	10.9	19.4
11	5.0	<5	2.3	<5
12	8.9	<5	7.2	<5
13	10.5	<5	7.5	<5
14	2.2	<5	8.6	<5
15	12.5	9.43	10.5	<5
16	1.5	<5	1.4	<5
17	4.0	<5	1.7	<5
18	7.9	<5	9.0	8.6
19	11.7	<5	10.7	<5
20	6.7	<5	2.2	<5
21	0.7	<LOD	1.7	<5
22	8.2	<5	1.9	<5
23	1.5	<5	1.7	<5
24	1.6	<5	0.7	<LOD
25	1.0	<5	1.6	<5
26	73.0	<5	21.3	6.0
27	<0.1	<LOD	<0.1	<LOD
28	<0.1	<5	1.4	<5
29	5.7	<5	4.7	<5
30	0.4	<LOD	4.0	<5
31	8.8	<5	9.0	<5
32	3.4	<5	8.6	<5
33	16.5	<5	8.2	<5
34	17.4	<5	16.2	<5
35	12.5	<5	9.9	<5
36	5.7	<5	11.5	<5
37	8.6	<5	8.9	<5
38	12.5	<5	0.9	<LOD
39	1.8	<5	14.0	5.5
40	10.3	<5	2.0	<5
41	7.6	<5	10.7	<5
42	11.0	<5	7.0	<5
43	1.5	<5	11.0	8.4
44	1.9	<5	1.7	<5
45	7.5	<5	8.1	<5
46	8.1	<5	0.5	<LOD
47	14.8	<5	8.2	<5
48	12.8	<5	11.8	<5
49	9.3	<5	5.7	<5
50	7.6	<5	8.2	<5
51	1.9	<5	7.5	<5
52	0.8	<LOD	1.0	<5
53	10.0	<5	1.6	<5

Table II (*Continued*)

Subject	ELISA	GC/MS	ELISA	GC/MS
54	9.5	<5	7.0	<5
55	1.8	<5	0.7	<LOD
56	2.1	<5	1.5	<5
57	5.7	<5	1.6	<5
58	1.8	<5	1.8	<5
59	1.8	<5	1.9	<5
60	8.1	<5	0.8	<LOD
Minimum	0.4	5	0.5	5
Maximum	73	23	32	35
Median	8	7	7	9
Mean ± SD	9 ± 10	10 ± 6	7 ± 6	13 ± 10
Data >LOQ (%)	97	12	97	15

SD, standard deviation; LOD, limit of detection (1 $\mu g\,l^{-1}$); LOQ, limit of quantitation (5 $\mu g\,l^{-1}$).

with urinary levels exceeding the ACGIH BEI in those countries (Melikian et al. 2002, Qu et al. 2003, Lin et al. 2004b), and that urinary PMA is a reliable biomarker of benzene exposure at levels as low as 0.25 ppm (Melikian et al. 2002). Conversely, in most European countries exposures decreased up to levels that are below such a threshold. Consequently, although the use of PMA cannot always distinguish between exposed and unexposed subjects, much effort is spent on improving the performances of analytical systems able to detect reliably very low concentrations of PMA.

Such analytical systems should be at the same time very sensitive and of practical use (e.g. applicable in on-field laboratories) in order to process rapidly large numbers of samples at low cost.

The present study considered the present state of the art. The ELISA assay was compared with GC-MS to verify to what extent it could be used reliably in a biomonitoring campaign.

Based on the results, it appears that most samples that had measurable concentrations by using the ELISA test were below the LOQ of the GC-MS procedure. Consequently, the means of PMA concentrations measured by the ELISA test are lower than those calculated from the GC-MS data. In addition, the medians and standard deviations of the two sets of data are about the same, but the spread of the ELISA data is wider than that of the GC-MS data.

Going more into the data presented in Table II, it appears that the ELISA test never failed to detect PMA at levels lower than the GC-MS LOQ (Figure 3, no false-negatives were assayed). Moreover, the ELISA test also never failed to detect those concentrations lower than the GC-MS LOD (at 1 $\mu g\,l^{-1}$), which actually were not detectable by GC-MS (Table II, 12 of 120 samples).

The overall score for the negative samples was of 49/120 without errors (Figure 3), accounting for the 41% of the set of measurements.

On the contrary, the ELISA provided some high concentrations of PMA that were not confirmed by GC-MS. The urines of subject 26, a non-smoker employee of a refinery, were found by ELISA to have concentrations of PMA respectively exceeding the BEI and close to it (73.0 $\mu g\,l^{-1}$ pre-shift and 21.3 $\mu g\,l^{-1}$ post-shift; Table II), with the highest concentration found in the urine sample collected before starting the work shift. In this case, the GC-MS determined PMA concentrations lower than 5 $\mu g\,l^{-1}$

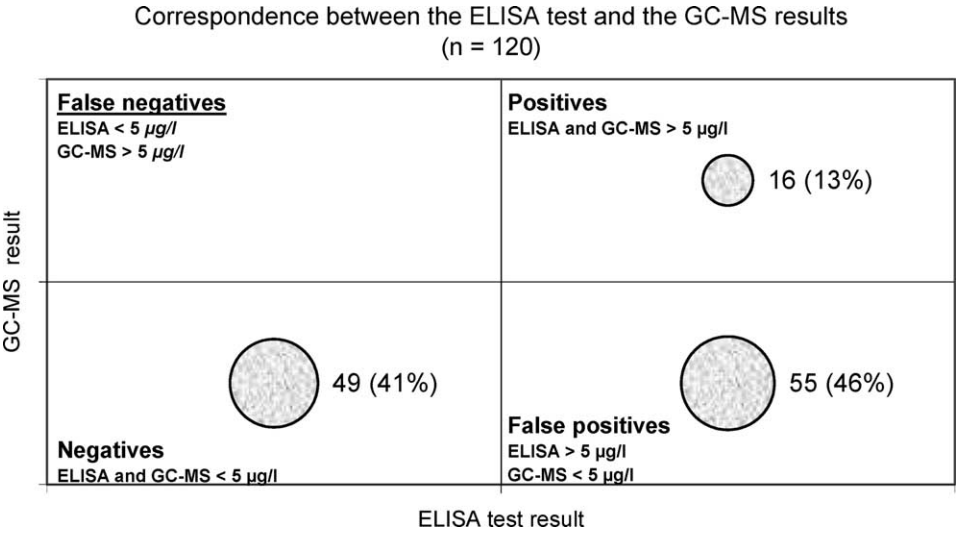

Figure 3. Paired measurements, irrespectively of the shift, were represented in the graph considering the correspondence of the ELISA test with the GC-MS result on each sample submitted to PMA determination. No ELISA data below 5 µg/l was found to be above the 5 µg/l LOQ by the GC-MS. This is interpreted considering that the ELISA test never fails to detect PMA, and therefore no "false negatives" were observed. On 65 samples out of 120 the two techniques gave consistent results, either above 5 µg/l ("Positives" box, 16 samples) or below the GC-MS LOQ ("Negatives" box, 49 samples).
On the other hand, 46% of the ELISA test measurements found to be >5 µg/l when compared with the GC-MS data were not confirmed ("false positives" box, 55 samples out of 120).

and of 6 $\mu g\,l^{-1}$ in the pre- and post-shift urines, respectively. Nonetheless, the ELISA measurements on the urines of subject 9 were of PMA levels exceeding the BEI both before and after the work-shift (25.1 $\mu g\,l^{-1}$ pre-shift and 32.1 $\mu g\,l^{-1}$ post-shift; Table II), and were in very good agreement with the GC-MS data (22.8 $\mu g\,l^{-1}$ pre-shift and 35.0 $\mu g\,l^{-1}$ post-shift; Table II).

Similar conflicting findings, though less remarkable, were observed in several other samples as documented by the data presented in Table II.

When the criteria to discriminate between positives was set at the 5 $\mu g\,l^{-1}$ level, a level equal to the LOQ of the GC-MS method corresponding to one-fifth of the BEI, the overall score for the positives was poor and that of the false-positives was bad (Figure 3, ELISA and GC-MS $>5\ \mu g\,l^{-1}$, positives, 16 of 120 samples, or 13% of the data set; ELISA $>5\ \mu g\,l^{-1}$ but GC-MS $<5\ \mu g\,l^{-1}$, false-positives, 55 of 120 samples, or 46% of the data set).

The individual paired data evidence shows that above the 5 $\mu g\,l^{-1}$ level the ELISA test measures invariably a higher concentration than the GC-MS method. This can be attributed to cross-reactivity and matrix interferences that enhance the test reading of the immunoassay and not to losses occurring in the sample preparation for GC-MS analysis. Actually, if any loss of PMA occurred this would also affect the internal standard [$^{13}C_6$]-PMA, thus balancing the final result of the determination.

These conclusions are in agreement with those reported by Aston et al. (2002), who observed that the ELISA assay overestimated PMA concentrations of about 10 $\mu g\,l^{-1}$. However, Aston et al. showed that the ELISA kit is robust and precise for PMA urinary concentrations ranging from 40 to 1200 $nmol\,l^{-1}$ (i.e. from about 10 to over

300 $\mu g\,l^{-1}$). In this interval, the comparison between ELISA and GC-MS allowed a correlation coefficient of $R = 0.92$.

The present study confirms that the ELISA assay overestimates urinary PMA at the levels studied by Aston et al., but also puts in evidence that the test is specific and reliable at PMA levels in urine lower than 5 $\mu g\,l^{-1}$. In addition, the assay never fails to detect PMA, and thus it guarantees to differentiate negative from potential positive samples. The ELISA assay for PMA therefore is, in our opinion, perfectly suitable for rapid semiquantitative screening. Its use drastically reduces the number of the samples needed to be processed by complex and expensive techniques such as those based on MS and MS-MS detection like those recently reported, which require a full method validation, trained personnel and expensive laboratory facilities. Hyphenated chromatographic-mass spectrometric techniques adopted for PMA measurements like GC-MS, LC-MS (Maestri et al. 2005), LC-MS-MS (Melikian et al. 1999, 2002, Liao et al. 2002, Pieri et al. 2003, Barbieri et al. 2004, Lin et al., 2004a, b) and GC-MS-MS (Van den Berg 2003) in the selected reaction monitoring afford undoubtedly outstanding sensitivity and selectivity as compared with chemiluminescence-ELISA and conventional GC-MS. However, the former methods appear more suitable for research purposes than for routine measurements on large numbers of samples. Actually, GC-MS, GC-MS-MS, LC-MS and LC-MS-MS still have limitations such as the high cost of the equipment and the complexity of their operation. Such features prevent their use, for instance, in on-field campaigns of biomonitoring.

In the present study, both the ELISA test and GC-MS method proved to be convenient techniques for analysing PMA in urine. The first especially is suitable for rapid, low-cost, semiquantitative screening of large number of samples. The latter procedure, on the contrary, affords high selectivity, good precision, accuracy and sufficient sensitivity, but at the cost of tedious sample preparation and long turnaround times. Therefore, it appears that one cost-effective approach to routine biomonitoring is that of using the ELISA test for rapid screening and a second reference technique, such as, for example, GC-MS with isotopic-labelled internal standard, for confirmation of concentrations assayed by the immunoassay which exceed the BEI for PMA.

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