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Comparison of hydrolysis and HPLC/MS/MS procedure with ELISA assay for the determination of S-phenylmercapturic acid as a biomarker of benzene exposure in human urine ☆

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ABSTRACT

The present study compared three methods for the determination of S-phenylmercapturic acid (S-PMA), a metabolite of benzene, in human urine: a HPLC/MS/MS technique with two different sample treatments (strong and partial hydrolysis) and a commercial assay based on anti-S-PMA monoclonal antibodies with chemiluminescence detection. Biological monitoring was done on 126 volunteers and the results were compared for the three methods and also with benzene exposure levels (range <3.0–592.5 $\mu g/m^3$). The correlation between environmental monitoring data and S-PMA levels in non-smokers ($n = 73$) was highly significant ($p < 0.0001$, Student's t-test) for both HPLC/MS/MS methods ($r = 0.65$ both for strong acidic hydrolysis of the urine and hydrolysis at pH 2) but not for the immunoassay, which overestimated the S-PMA levels by about 8 μg/g creatinine (creat.). Therefore the immunoassay is only useful as a semiquantitative screening test, but quantitative results need to be confirmed by a more accurate method like HPLC/MS/MS. The HPLC/MS/MS procedure with strong acid hydrolysis led to a recovery of S-PMA about double that using pH 2 hydrolysis, giving more accurate results. The difference between the results with the two methods makes it difficult to compare the strong acidic hydrolysis data with the ACGIH BEI value of 25 μg/g creat. since the BEI® documentation is based on data collected in pH conditions that were not always controlled, which may underestimate the true S-PMA concentration. Besides, as levels of benzene exposure were high, smoking was not considered a confounding factor. The BEI for S-PMA in end of shift urine samples could be reconsidered when sufficient data are available from studies where the analyses are carried out in comparable conditions of hydrolysis and monitoring only non-smoking subjects.

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1. Introduction

S-phenylmercapturic acid (S-PMA), together with trans–trans-muconic acid (t,t-MA), is one of the most widely used biomarkers of benzene exposure. Unlike t,t-MA, however, S-PMA is considered a highly specific biomarker. Since there are no other substances, exogenous or endogenous, known to be metabolized to S-PMA except benzene, the excretion of this metabolite can only be attributed to benzene exposure. The American Conference of Governmental Industrial Hygienists (ACGIH) recommends a biological exposure index (BEI®) of 25 μg/g creatinine (creat.) in the end-shift urine of workers when assessing occupational exposure to benzene [1].

Sophisticated analytical methods are necessary to ensure sufficiently sensitive S-PMA determination in urine. S-PMA excretion in the urine of exposed and non-exposed subjects has been assessed using liquid and gas chromatography (GC) coupled with mass spectrometry (HPLC/MS/MS and GC/MS) [2–9]. HPLC/MS/MS is preferable to GC/MS because it does not require derivatization. The amount of S-PMA actually measured depends, among other factors, on the degree of hydrolysis of its precursor, pre-S-PMA, whose proposed formula is N-acetyl-S(1,2-dihydro-2 hydroxyphenyl)-L-cysteine [10,11]. The precursor can be turned into S-PMA if the urine sample is acidic and therefore the recovery changes in relation to the pH and to the storage conditions. In the analytical

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methods, most commonly used for S-PMA, the pH is usually not controlled, as it is not considered critical, with a few exceptions. Strong hydrolysis of the precursor was obtained, for instance, using sulphuric acid [10] and this procedure for sample preparation was used by Paci et al. [7] to validate an HPLC/MS/MS method for the determination of S-PMA in urine.

A commercial assay based on anti-S-PMA monoclonal antibodies with chemiluminescence detection has also been developed and has been used to quantify urinary S-PMA for assessing benzene exposure [12–14]. The present study compared the performance of this immunoassay with the HPLC/MS/MS method, using both hydrolysis procedures, i.e. strong hydrolysis and hydrolysis at pH 2, and to find out which results correlate best with environmental benzene exposure.

2. Experimental

2.1. Chemicals and supplies

The analytical reference standard, DL-S-PMA, was purchased from Tokyo Kasei Cogio Ltd. (Tokyo, Japan). The internal standard, deuterated DL-S-PMA-3,3-d_2, was obtained from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). Personal diffusive samplers Radiello® were purchased from Fondazione Salvatore Maugeri (Pavia, Italy). Glacial acetic acid (100%, Merck, Darmstadt, Germany) was used for preparing the mobile phase, for solid phase extraction (SPE) and, together with 25% NH_3 (Merck, Darmstadt, Germany), to adjust the urine pH, after dilution with purified water obtained from a Milli-Q Plus system (Millipore, Milford, MA, USA). Carbon disulphide, sulphuric acid (95% purity) and NaOH (50:52% (v:v) water solution used for hydrolysis), were purchased from Fluka–Sigma–Aldrich (Munich, Germany). Methanol was purchased from J.Y. Baker (Deventer, Holland). The SPE vacuum manifold and Sep-Pak Plus C18 (360 mg) cartridges for S-PMA were supplied by Waters (Milford, MA, USA). Anotop 10 IC® syringe filter devices (0.2 μm pore size, 10 mm diameter) were purchased from Whatman Inc. (Maidstone, England). A Supelco Discovery C18 HPLC column (150 mm × 4.6 mm, 5 μm film thickness) was purchased from Sigma–Aldrich (Bellafonte, PA, USA). Kits for the S-PMA immunoassay test with chemiluminescence detection were purchased from MLT Research (Cardiff, UK). Urinary creat. was determined with a colorimetric test kit purchased from Sigma Diagnostics Inc. (St. Louis, MO, USA).

2.2. Sample collection

Samples were obtained from 126 healthy males volunteers occupationally exposed to benzene at different levels. Exposure was measured using the Radiello® diffusive sampler, containing an active carbon cartridge, worn by all the subjects at the height of the nose for about 8 h. After this environmental monitoring, each subject provided one fresh urine sample that was stored at −20 °C until analysis which was made within one week of collection. Consent and information about smoking habits were obtained by a self-administered questionnaire; an identification number was assigned to each completed questionnaire.

2.3. Preparation of urine samples

Each 3-mL urine sample was treated with 1 mL of 9 M H_2SO_4 for 10 min for strong hydrolysis of pre-S-PMA, then 0.75 mL of NaOH 50% in water were added to reach a pH around 2. A second 3-mL urine sample was brought to pH 2 with glacial acetic acid. After this step 30 μL of a 0.5 mg/L solution of the internal standard (S-PMA-d_2) in methanol were added to both these samples, to a final concentration of 5 μg/L.

SPE purification was on Sep-pack C18 cartridges preconditioned with 3 mL of methanol followed by 3 mL of 0.1% CH_3COOH. After loading the sample the cartridge was washed with 3 mL of 0.1% CH_3COOH and eluted with 3 mL methanol. The eluate was filtered on a 0.2 μm filter device and 20 μL were injected into the HPLC/MS/MS system.

A third sample, 5 mL, was acidified with 50 μL of HCl 6 M, sealed, and stored at 2–8 °C until analysis. Immunochemiluminescence analysis was done following the instructions supplied with the kit: 200 μL of urine or standard solution were diluted with 1000 μL of a phosphate-buffered saline solution. The dilution tubes were Vortex-shaken for 2 s. A luminescent antibody conjugate reagent (200 μL) was added in an assay tube containing 200 μL of the diluted samples (two tubes for each sample). The tubes were incubated at 22 °C for 60 min, decanted onto tissue paper and washed with 1000 μL of the phosphate-buffered solution, twice. An additional 1 mL of urine was used for determining urinary creatinine.

2.4. Analytical methods

2.4.1. HPLC–MS/MS analysis

HPLC analysis of urine samples and calibration and matrix standards was done using a Series 200 LC quaternary pump (Perkin Elmer, Norwalk, CT, USA). The mobile phase was as follows: 1 min equilibration with 30% methanol (phase A) and 70% 0.1 M acetic acid (phase B), 12 min with a linear gradient up to 90% phase A and 10% phase B, 2 min with a linear gradient back to the starting conditions, flow rate 1.0 mL/min. In these conditions the retention time of S-PMA and the internal standard was 7.7 min.

The MS ion source of the PE Sciex API 4000 triple quadrupole mass spectrometer can work without split so the HPLC column eluate was fed directly into the Turbo Ion Spray (TIS) probe. The instrument was calibrated using polypropylene glycol and resolution was adjusted to a peak width (FWHM) of 0.7 Th over the m/z 100–1000 range. Detection was in the negative ion MRM mode, and parameters were optimized for the analytes by the automated "infusion quantitative optimization" procedure and subsequently refined by FIA (flow injection analysis) using the pure standards. The following m/z ion combinations (precursor → product) were monitored and the SRM transitions were as follows: respectively −238.1 → −109.1 and −240.1 → −109.1 for S-PMA and deuterated internal standard. All values are summarized in Table 1.

The 1.4 version of Analyst® software was employed for instrument control, using the section "Quant Method Editor" to process quantitative data. The performance of this method, determined in our laboratories, was as follows [7]:

- Sensitivity: limit of detection was 0.05 μg/L, and lowest limit of quantitation 0.1 μg/L;
- intra-assay variation (CV %): max. 6.4% on 5 replicates at 5 μg/L;
- inter-assay variation (CV %): max. 8.6% on 5 replicates at 5 μg/L.

2.4.2. Immunochemiluminescence analysis

The concentration of S-PMA in urine was determined by reading the assay tubes in a luminometer (Berthold model D 75173) equipped with dual on-line reagent injection. Two 300-μL samples, one the detection reagent 1 (0.5% hydrogen peroxide and 0.1 M nitric acid) and the other detection reagent 2 (0.025 M sodium hydroxide and 0.2% surfactant), were added to the tubes before the measurement [15,16]. The performance characteristics of this method, determined by the manufacturer, were as follows:

- sensitivity (estimated as two standard deviations from the mean of 20 replicates of the zero standard): 2.0 nmol/L;
- intra-assay variation (CV%): max. 12% on 6 replicates;

Table 1
Environmental and biological monitoring results for all subjects.

Analyte	Method	Mean	Median	Range
Benzene (μg/m^3)	GC-FID	33.8	9.6	<3.0–592.5
S-PMA (μg/L)	HPLC/MS/MS after strong acid hydrolysis	3.59	1.05	0.10–33.32
	HPLC/MS/MS after hydrolysis at pH 2	1.65	0.61	<0.10–13.79
	Immunoassay	10.03	9.07	<0.50–29.55
S-PMA (μg/g creat.)	HPLC/MS/MS after strong acid hydrolysis	2.78	0.79	0.08–38.59
	HPLC/MS/MS after hydrolysis at pH 2	1.26	0.51	<0.08–13.03
	Immunoassay	7.34	6.54	0.34–67.35

- inter-assay variation (CV%): max. 18% on 6 replicates;
- measured values were normalized for the concentration of urinary creatinine, to give an S-PMA/creatinine ratio for each sample.

2.4.3. GC-FID analysis

Radiello analysis was done with a GC Autosystem XL equipped with FID (Perkin Elmer, Norwalk, CT, USA), after desorption of benzene from the active carbon with low-benzene-content carbon disulphide, according to a modified NIOSH method [17]. The detection limit of the procedure was 3.0 μg/m^3.

2.5. Statistical analysis

The StatsDirect statistical software was used, on $\log_e$-transformed values. Parametric statistical tests were applied to $\log_e$-transformed values, in order to obtain normal distribution, which was assessed by the Kolmogorov–Smirnov test. Differences between groups were assessed using the *t*-test for independent samples. Differences were also confirmed by the Mann–Whitney non-parametric *U*-test. Correlations between variables were assessed by the Pearson's *r* coefficient. In all tests, a *p* value lower than 0.05 (two-tailed) was considered significant.

3. Results and discussion

The environmental and biological monitoring data obtained with the three methods are shown as mean, median and range in Table 1 for all subjects and in Table 2 for smokers (42) and non-smokers (84) separately. The benzene levels were in the range <3.0–592.5 μg/m^3, corresponding to the airborne benzene concentrations to which the general population or workers in controlled settings could be exposed. The mean S-PMA obtained with hydrolysis at pH 2 was about 46% of that obtained with strong hydrolysis. These results confirm that at pH 2 more than 50% of urinary S-PMA is still in the form of pre-S-PMA and the precursor can be further hydrolyzed only with a more acidic condition [7,10]. The mean S-PMA levels in the immunoassay were respectively about three and six times that detected at pH 2 and with strong hydrolysis.

Cigarette smoke is a known source of benzene exposure so S-PMA levels are expected to be significantly higher in smokers than non-smokers. All three methods distinguished these two groups ($p < 0.0001$; $p < 0.0001$ and $p < 0.01$ respectively for strong hydrolysis, pH 2 and immunoassay).

All variables followed a log-normal distribution; parametric statistical tests were applied on $\log_e$-transformed data and the results were confirmed by non-parametric tests.

Comparison of the S-PMA levels with strong acid hydrolysis and at pH 2 (Fig. 1) showed a very good, significant correlation ($r = 0.97$; $p < 0.0001$). The correlation was also significant between S-PMA levels obtained with strong hydrolysis and immunoassay (Fig. 2), but the correlation coefficient was lower ($r = 0.51$; $p < 0.0001$). Similar results were found for hydrolysis at pH 2 and immunoassay ($y = 0.399x + 2.1235$; $r = 0.57$; $p < 0.0001$, results not shown).

The agreement between the results with HPLC/MS/MS and the immunoassay was also investigated using the differences between results on the same subjects according to the test of Bland and Altman [18]. In this test the 95% limits of agreement, estimated from the mean difference ±1.96 standard deviations, provide the interval in which 95% of the differences between measurements with the two methods are expected to lie. Accordance between the methods was good: only five and four values, respectively for strong hydrolysis and pH 2, were outside the range of agreement defined by the test (Fig. 3A and B). The immunoassay, however, overestimated the S-PMA levels compared to the HPLC–MS/MS findings, the average difference being negative. The overestimation also emerged from the simple correlation of data with the two methods, in which the intercept line was at 6–8 μg/L. This is consistent with the reports by Aston et al. and Marrubini et al. [19,20] who noted that the chemiluminescence method overestimated the results by about 10 μg/L. In the present study overestimation was particularly evident at low metabolite concentrations, where the dispersion of data is greater. However, the binding reactivity of the anti-S-PMA antiserum used in the immunoassay with the pre-S-PMA has not been investigated

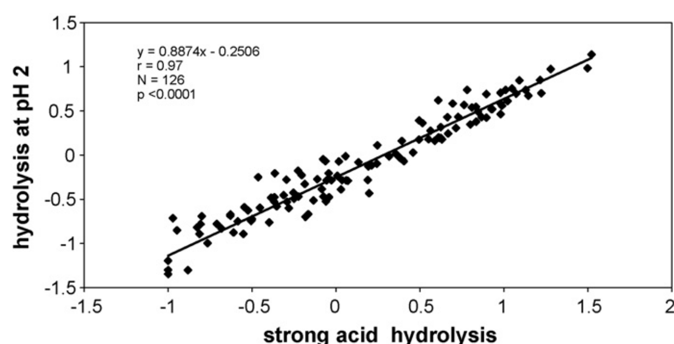

Fig. 1. Correlation between levels of S-PMA in urine (μg/L) obtained with strong acid hydrolysis and those obtained with hydrolysis at pH 2 ($\log_e$-transformed data).

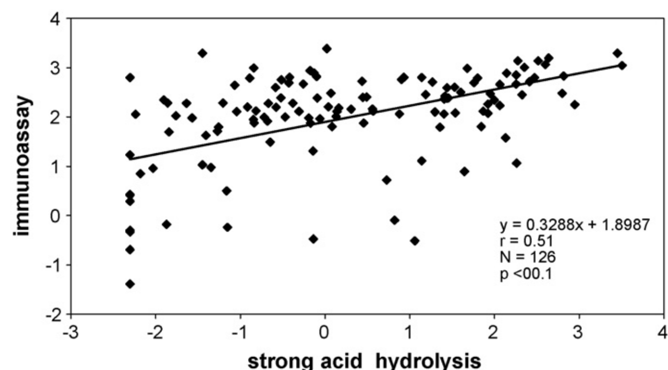

Fig. 2. Correlation between the S-PMA levels in urine (μg/L) obtained with strong acid hydrolysis and immunoassay ($\log_e$-transformed data).

Table 2
environmental and biological monitoring results for smokers and non-smokers.

Analyte	Method	Smokers			Non-smokers		
		Mean	Median	Range	Mean	Median	Range
Benzene (μg/m^3)	GC-FID	22.68	8.90	<3.00–383.48	39.28	10.56	<3.00–592.54
S-PMA (μg/g creat.)	HPLC/MS/MS after strong acid hydrolysis	6.15	3.67[a]	0.13–38.59	1.10	0.40[a]	0.08–18.63
	HPLC/MS/MS after hydrolysis at pH 2	2.62	1.88[a]	0.16–13.03	0.57	0.25[a]	<0.08–9.23
	Immunoassay	9.55	8.22[b]	1.46–67.35	6.23	5.90[b]	0.34–21.56

[a] $p < 0.0001$ (two-tailed t-test for independent samples, smokers versus non-smokers).
[b] $p < 0.01$ (two-tailed t-test for independent samples, smokers versus non-smokers).

[21] and the pH used in the sample preparation does not ensure strong hydrolysis of the precursor.

There was a significant correlation between airborne benzene concentrations and levels of S-PMA in urine, with strong hydrolysis and at pH 2 ($p < 0.01$ for both) in all subjects (results not shown), although the correlation coefficients were low ($r = 0.24$ for both). No correlation was found between airborne benzene concentrations and S-PMA levels measured with the immunoassay. In the 73 non-smokers (11 out of 84 subjects were excluded as airborne benzene concentration was below the LOD) the statistical significance of the correlation and the correlation coefficient were better for both HPLC/MS/MS methods ($r = 0.65$ and $p < 0.0001$) but not for the immunoassay (Fig. 4).

The ACGIH first proposed a BEI® for S-PMA in 1996 and in 1997 adopted the value of 25 μg/g creat. on the basis of field studies which had found significant correlations between benzene in air and S-PMA in post-shift urine. Popp et al., in a study on automobile mechanics, found that a TLV-TWA of 0.5 ppm for benzene corresponded to S-PMA urinary excretion of 22 μg/L. van Sittert et al. found 23–25 μg/g creat. S-PMA in urine for exposures to benzene at the TLV-TWA level; Ghittori et al. found a mean S-PMA level of 22.5 μg/g creat. for the same airborne benzene concentration. All these data were obtained from general groups of workers, smokers and non-smokers together, and suffered some limitations: the urine samples were not always acidified before analysis, or the pH was not controlled [3,22–24].

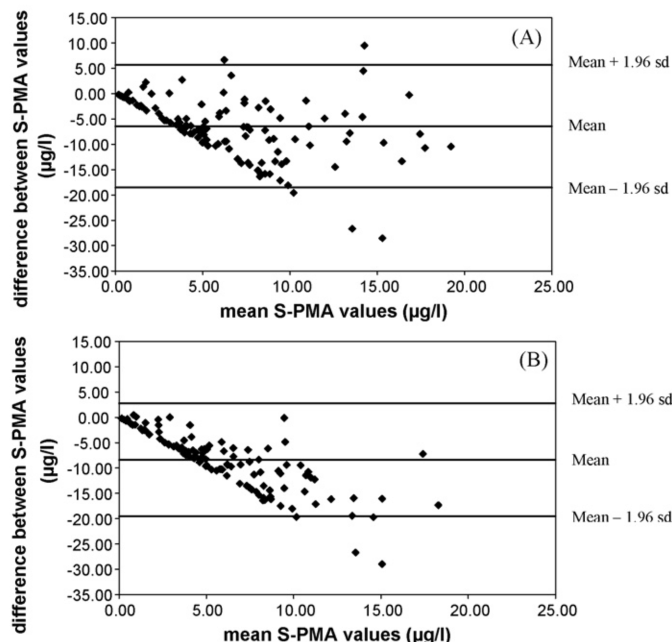

Fig. 3. Bland and Altman test on S-PMA values obtained with strong acid hydrolysis versus immunoassay (A) and hydrolysis at pH 2 versus immunoassay (B).

In the present study the linear correlation between airborne benzene exposure and S-PMA determined by HPLC/MS/MS after strong acid hydrolysis in non-smokers ($r = 0.79$) leads to the following equation:

$$\text{S-PMA}\,(\mu\text{g/g creat.}) = 0.189\ \text{benzene}\,(\mu\text{g/m}^3) + 0.29$$

According to this, the concentration of S-PMA corresponding to an airborne benzene exposure of 1600 μg/m^3 (0.5 ppm, i.e. the current TLV-TWA) is 30.53 μg/g creat.

With reference to the sample preparation, both the HPLC/MS/MS methods compared here showed a good correlation with airborne

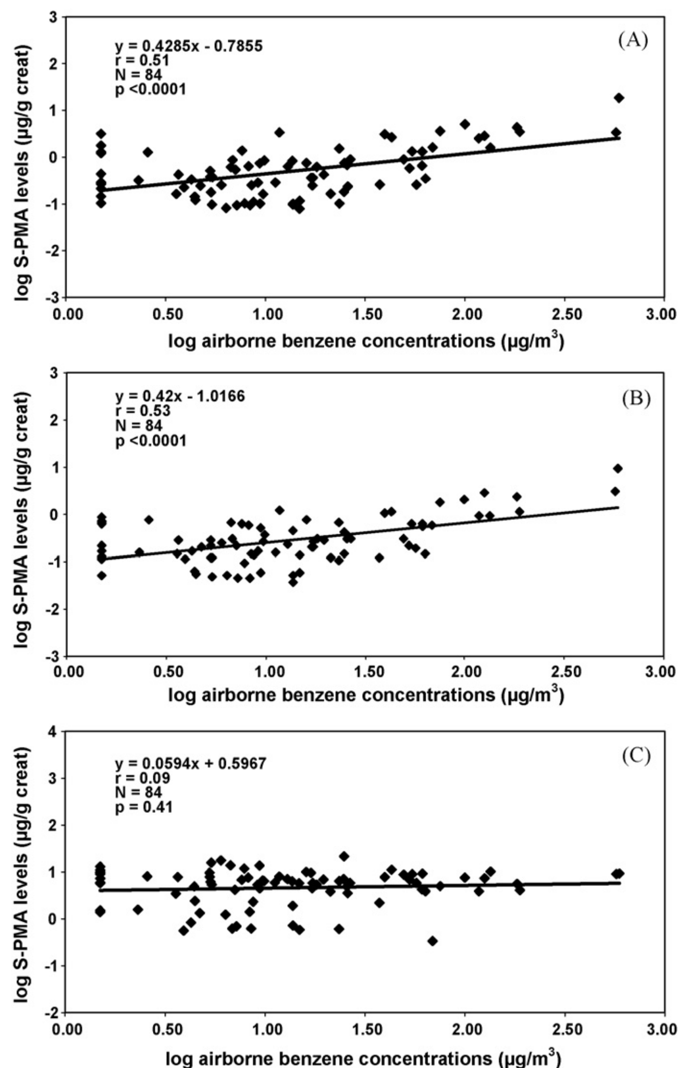

Fig. 4. Correlation between airborne benzene concentrations and S-PMA levels in non-smokers after strong acid hydrolysis (A), hydrolysis at pH 2 (B) and by immunoassay (C).

benzene concentration, but strong hydrolysis offers the advantage of a larger amount of the analyte to be measured.

4. Conclusion

Both the HPLC/MS/MS methods described showed good correlations with airborne benzene concentrations, but strong acid hydrolysis offers the advantage of giving a larger amount of analyte to be measured, consequently reducing the error associated with the measure.

The possibility of using an immunoassay to determine urinary S-PMA needs to be further investigated, particularly as regards crossreactivity with the urine matrix and the correlation with airborne benzene exposure. Presently this assay cannot be recommended to monitor low exposure to benzene.

Finally, the current ACGIH BEI for S-PMA in end-shift urine of 25 μg/g creat. is not appropriate when using the strong acid hydrolysis method. Results from studies where the analysis is done in comparable conditions of hydrolysis should be used for extrapolation, and only on non-smokers, since the ACGIH BEI is not recommended for heavy smokers, as the Conference itself reports in the ACGIH Documentation [24].

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