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Development and validation of a competitive immunoassay for urinary *S*-phenylmercapturic acid and its application in benzene biological monitoring

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An immunoassay that quantifies urinary *S*-phenylmercapturic acid (PMA), a benzene-specific biomarker, has been developed and its potential usefulness as a screening tool for monitoring occupational exposure to benzene has been demonstrated. Analytical reliability has been confirmed by correlation of results with gas chromatography-mass spectrometry (GC/MS) data ($R = 0.92$). The assay has been configured as a competitive enzyme-linked immunosorbent assay (ELISA) to facilitate rapid throughput of samples. The ELISA has a working range of 40–1200 nmol l^{-1} urinary PMA and appears to be unaffected by the presence of structurally related urinary metabolites. Background levels of 0–1.9 μmol PMA/mol creatinine (mean 0.9 μmol mol^{-1}, $n = 32$) were measured in non-smoking control subjects. Recent exposures to benzene (8 h time-weighted averages-TWA), during diverse industrial processes, over the range 0–4.8 ppm were identified by application of the assay in biological monitoring programmes.

Keywords: benzene, *s*-phenylmercapturic acid, immunoassay, biological monitoring.

Introduction

The serious adverse health effects associated with prolonged exposure to benzene, notably myelotoxicity (Snyder *et al.* 1993), which have been observed both in animal (Ward *et al.* 1985) and human (Fishbeck *et al.* 1978, Yin *et al.* 1989, Travis *et al.* 1994) studies, advocate the implementation of monitoring programmes where occupational exposure to this volatile solvent could occur. Biological monitoring serves to quantify a hazardous chemical, or its metabolic product(s), in biological fluids, e.g. urine, blood and saliva, thereby enabling an estimate of its uptake in an exposed individual (Zielhuis 1978, van Sittert 1984). A principal value of biological monitoring is that it provides a guide to internal dose and body burden of a chemical, irrespective of whether the route of exposure is inhalation, dermal absorption or ingestion.

The major hepatic metabolites of benzene are phenol, catechol and hydroquinone (Snyder and Hedli 1996). The oxidative metabolites of benzene, activated by the cytochrome P450-dependent mixed-function oxidase system, are thought to mediate myelotoxicity and carcinogenicity (Smith *et al.* 1989, Parke 1996). An alternative metabolic route involves conjugation of benzene oxide to glutathione.

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This yields a pre-mercapturic acid, which is further metabolized in the kidney and eliminated as the water-soluble *S*-phenylmercapturic acid (PMA) (Parke and Williams 1951). PMA derives solely from benzene metabolism and this specificity renders the metabolite a biomarker of choice for monitoring occupational uptake of benzene.

In humans, PMA has an elimination half-life of ~9 h (van Sittert *et al.* 1993). Although only up to 1% of absorbed benzene is converted to PMA, its rate of urinary excretion provides an effective measure of benzene exposure dose. Boogaard and van Sittert (1996) reported a linear relationship between low levels of air-borne benzene and urinary PMA concentrations in workers in the petrochemical industry. They argued that superior specificity and a longer elimination half-life render PMA a more reliable biomarker than *trans*-, *trans*-muconic acid for monitoring benzene exposures during 12-h working shifts (Boogaard and van Sittert 1995). Consequently, PMA has biological exposure indices both in the USA (ACGIH 2001) and Germany (DFG 2000) as a marker of benzene exposure.

However, implementation of a routine benzene biological monitoring screening programme, based on the quantification of urinary PMA, appears to have been hindered both by analytical complexity and cost. GC/MS analysis of urinary PMA offers analytical excellence, but is sophisticated, expensive and protracted (Stommel *et al.* 1989). During the last three decades, immuno-analysis has made a significant impact on clinical diagnostics and trace chemical analyses where it has been established as a routine technology (Vanderlaan *et al.* 1991, Aston *et al.* 1992). The production of antibody reagents directed towards clinical analytes has enabled development of a diverse range of clinical immunoassays and has contributed not only to the differential diagnoses of pathological conditions, but also to the widespread implementation of screening programmes.

The aim of the study was to develop and validate a relatively simple, cost-effective assay for urinary PMA that could be deployed as a screening tool for monitoring occupational exposure to benzene.

Materials and methods

Materials

Ninety-six-well microtitre plates (maxisorb) were obtained from Life Technologies (Nunc Plasticware, Paisley, UK). *S*-PMA and benzylmercapturic acid were purchased from Janssen Chimica (Cheshire, UK). D_7-benzylmercapturic acid was a custom synthesis from Ultrafine Chemicals (Manchester, UK). Sodium bicarbonate, sodium carbonate, sodium chloride, sodium hydrogen phosphate, disodium hydrogen phosphate, hydrochloric acid, sucrose and EDTA were from BDH (Lab3 Ltd, Bristol, UK). Anti-sheep immunoglobulin G (whole molecule) alkaline phosphatase conjugate (donkey), bovine γ-globulin, bovine serum albumin, hippuric acid, nitrophenyl phosphate, pentafluorobenzyl bromide, Tween-20, creatinine kits and urinary creatinine control samples were from Sigma-Aldrich (Poole, Dorset, UK). Keyhole limpet haemocyanin was from Calbiochem (La Jolla, CA, USA). Bis(sulphosuccinimidyl)suberate was from Pierce Chemicals (Luton, UK. HPLC-grade ethyl acetate and toluene were obtained from Ratherburns (Walkerburn, UK) and potassium hydroxide pellets were from Fisher (Loughborough, UK).

Methods

Immunogen preparation. A PMA-analogue was designed, synthesized and coupled to carrier protein to produce an immunogen capable of generating a PMA-specific antiserum. The method was described by Ball *et al.* (1997). Briefly, PMA was deacetylated to yield phenylcysteine, which was covalently coupled via the methylene group, using the homobifunctional cross-linker bis(sulphosuccinimidyl)suberate, to the carrier proteins bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH).

Immunization protocol. PMA-KLH conjugate (100 μg) was emulsified in adjuvant (1 ml) and administered by subcutaneous injection to sheep ($n = 2$). Booster injections were repeated at intervals of ~4 weeks and test bleeds were obtained 7 days after successive boosts.

Evaluation of anti-PMA immune response. Serum from venous blood (S360) was separated by centrifugation and assayed for binding reactivity as follows:

Microtitre plates (96-well) were coated with PMA-BSA conjugate (1 μg ml^{-1}, carbonate/bicarbonate buffer, pH 8.6) at 4°C for 24 h. The wells were washed with phosphate-buffered saline (PBS)-Tween solution (0.05%) before blocking with a solution of PBS, sucrose (2%), BSA (1%) at 4°C for 24 h (200 μl/well). Serial dilutions of S360 were prepared in assay buffer {PBS (0.2 M) containing 0.025% BSA, 0.002% bovine or γ-globulin and 0.2% EDTA} over a dilution range of 1/1000–1/160 000 and incubated in coated wells for 2 h at room temperature (170 μl/well). After washing, an excess of alkaline phosphatase-labelled anti-sheep immunoglobulin was added (100 μl/well) and the assay plate was incubated for a further 1 h at room temperature. Enzyme substrate solution (*p*-nitrophenyl phosphate (1 mg) in Tris buffer (0.2 M, 1 ml), 100 μl/well) was added to visualize anti-PMA binding and, after 30 min, absorbance (405 nm) was monitored using a Vmax kinetic microplate reader (Molecular Devices).

Optimization of urinary PMA immunoassay. A competitive enzyme-linked immunosorbent assay (ELISA) was optimized in terms of conjugate coat concentration and antiserum dilution. PMA standard solutions were prepared in pooled urine from non-occupationally exposed subjects (range 0-2.8 μmol l^{-1}). The urine was filtered using a 0.2 μm filter and acidified with HCl (75 mM). PMA standard solutions were stored at −20°C.

PMA antiserum specificity was investigated by incubating a limiting concentration of anti-PMA antiserum separately with fixed concentrations of the following metabolites: PMA, phenyl cysteine, benzylmercapturic acid and hippuric acid. Urinary matrix effects were obviated both by sample dilution and the use of assay buffer diluent devised with sufficient buffering capacity to maintain an assay pH of 7.4 and a constant salt concentration.

Analytical repeatability of the ELISA was determined by intra- and interassay variance studies using defined concentrations of urinary PMA.

ELISA validation. Urine samples were collected from workers potentially exposed to benzene in the workplace and from a non-occupationally exposed control group. Samples were acidified immediately after collection (7.5 M HCl, 1% v/v) and stored at −70°C. After thawing, urine samples were subaliquoted and PMA analyses were carried out by ELISA and a gas chromatographic-mass spectrometry procedure.

ELISA was carried out essentially as described above incorporating six urinary PMA standards, three quality control samples and 21 urine samples in duplicate for each assay plate. Acidified urine samples (10 μl) were diluted in assay buffer (80 μl) and dispensed to the wells of a coated microtitre plate before addition of anti-PMA antiserum (80 μl) also diluted in assay buffer.

GC/MS procedure. Stock solutions of PMA and D_7-benzylmercapturic acid (1 mmol l^{-1}) were prepared in double-distilled water. From these solutions, working solutions (4 μmol l^{-1}) were also prepared in double-distilled water. Urine aliquots (2 ml) were spiked with PMA working solution to give standards in the range 0-500 nmol l^{-1}, test samples were also analysed in 2-ml aliquots. Hydrochloric acid (200 μl) and D_7-benzylmercapturic acid working solution (150 μl) were added to all tubes. Samples were then extracted into 4 ml ethyl acetate. The organic layer was transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The residue was resuspended in 200 μl methanolic KOH (0.5 g KOH in 50 ml methanol) and the samples were derivatized with 50 μl PFPBr for 1 h at 60°C. Following derivatization, 300 μl toluene and 300 μl water were added to the tubes. After mixing and centrifugation, an aliquot of the organic layer was removed for analysis by GC-MS.

Analysis was performed using an HP5973MSD coupled to an HP6890 gas chromatograph (Agilent, Cheadle, UK). Splitless injections (1 μl) were made at 250°C into a CP-Sil8 MS column (30 m × 0.32 mm i.d., 1 μm film, SGE, Varian, UK). The oven was raised at 8°C min^{-1} from an initial temperature of 150 to 290°C, where it was held for 1 min. The transfer line was held at 280°C. The mass spectrometer was operated in negative-ion chemical ionization mode using methane as a reagent gas. Selected-ion monitoring was used: m/z 238 for PMA and 259 for D_7-benzylmercapturic acid.

The interassay coefficient of variation was 8% ($n = 10$) with a detection limit of 10 nmol l^{-1} (signal : noize ratio > 3).

Biological monitoring database construction. Post-shift urine samples (8 h TWA) were collected from employees in diverse sectors of industry (Europe and North America) including petrochemical refineries, coke oven works, chemical manufacturing plants and from workers involved in a clean-up operation after a chemical spill of material containing up to 40% benzene. Samples were analysed for PMA and creatinine levels and results were compared with data obtained from a control population of non-occupationally exposed subjects.

Creatinine measurement. Urinary creatinine levels were determined by the modified Jaffe reaction, using reagents supplied by Sigma-Aldrich diagnostics, following the manufacturer's instructions.

Results

An antiserum dilution profile of a blood sample (S360) obtained from an immunized sheep is illustrated in figure 1. Comparison with the binding properties of a negative control, hyperimmune antiserum, confirmed induction of an immune response directed towards the conjugate material (PMA-BSA) coupled to the assay wells.

A competitive inhibition study using antiserum S360 (dilution 1/7000) in the presence of exogenous PMA (0–2.8 μmol^{-1}) in human urine demonstrated a dose-dependent effect on antiserum binding (figure 2). An inverse relationship was observed between PMA dose and the signal generated in the immunoassay. Under the ELISA conditions deployed, the dose-response was approximately linear over the range 40-1200 $nmol\,l^{-1}$ PMA (figure 2).

Potentially cross-reactive metabolites, which may be endogenous in human urine, added to pooled human urine in molar excess quantities, did not cause significant antibody displacement in the PMA ELISA (table 1).

The percentage coefficient of variation (CV) resulting from within assay analyses ($n = 30$) of a urine sample containing 130 $nmol\,l^{-1}$ PMA is expressed in table 2. Interassay CVs for analyses of samples ($n = 12$) containing 86 and 448 $mol\,l^{-1}$ PMA were 7.6 and 7.9%, respectively (table 2).

Results obtained on samples analysed by ELISA and GC-MS ($n = 63$, range 3–1644 $nmol\,l^{-1}$) are compared in figure 3. The coefficient of correlation was 0.92.

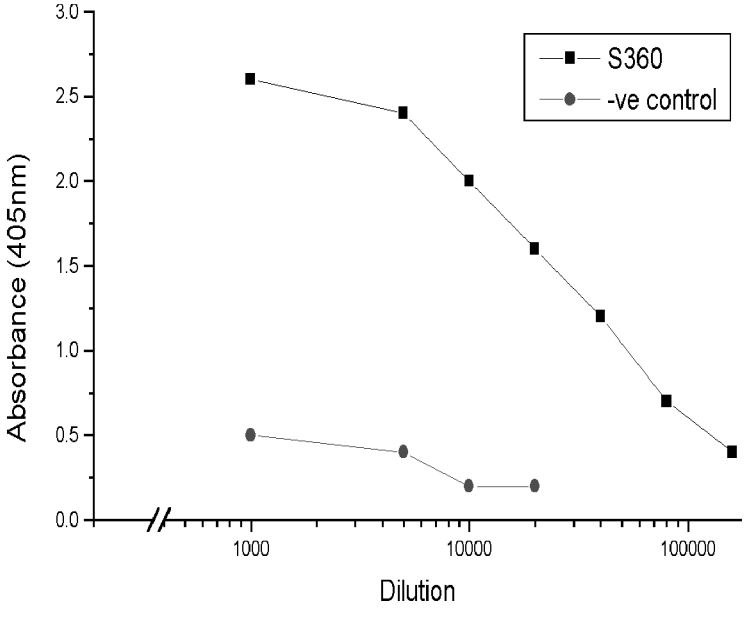

Figure 1. Dilution profile of sheep anti-PMA antiserum. S360, obtained from the immunized donor, and a hyperimmune control antiserum were incubated at various dilutions on a PMA-BSA-coated microtitre plate. Assay signal is expressed as a function of the logarithm of antiserum dilution.

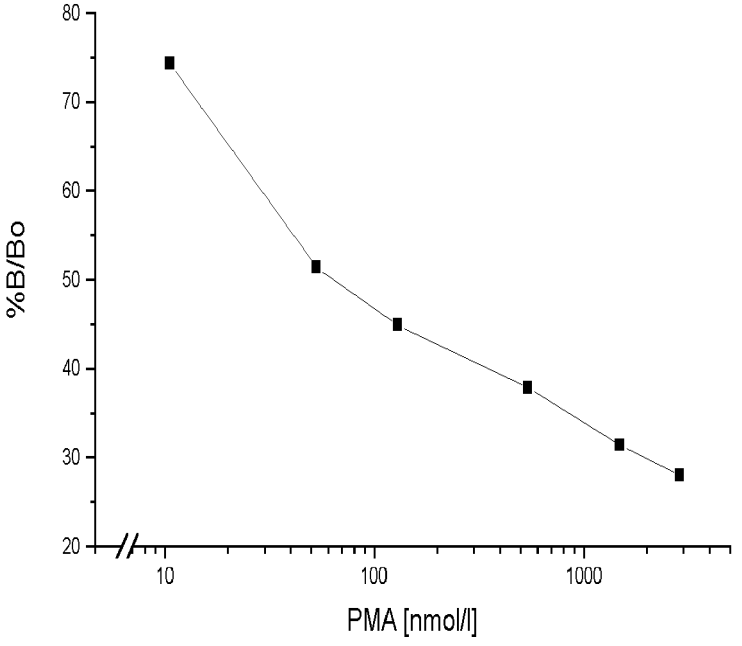

Figure 2. Competitive inhibition of PMA antiserum binding with urinary PMA. Defined concentrations of PMA in pooled human urine were incubated with a limiting concentration of S360 in PMA-BSA coated wells. Percentage binding of S360, relative to the binding of a zero standard, is expressed as a function of the logarithm of PMA dose.

Table 1. S360 cross-reactivity study. Percentage cross-reactivity (%CR) of molar excess concentrations of urinary metabolites with antiserum S360 as determined by the degree of antibody binding displacement, relative to PMA, in a competitive ELISA format.

Metabolite	Concentration (mM)	%CR
Hippuric acid	25	0.2
Phenyl	10	0.2
Benzylmercapturic acid	15	0.5

Twenty-one of 63 samples were reported as $<10\,\text{nmol}\,\text{l}^{-1}$ by GC-MS, but gave results ranging from 3 to $75\,\text{nmol}\,\text{l}^{-1}$ (mean $31.5\,\text{nmol}\,\text{l}^{-1}$) by ELISA. A further 12 of 63 samples yielded results that were outside the working range defined for the ELISA. These samples were excluded from the calculation of the coefficient of correlation (R), which was based on data points ($n = 30$) within the working range of the immunoassay (figure 3).

Urinary PMA levels, determined by ELISA, in post-shift samples obtained from workers potentially exposed to benzene and expressed as urinary PMA/creatinine ratios, to account for the hydration status of the subjects, are presented in figure 4. Results of random urine samples obtained from a control group of non-occupationally exposed subjects ($n = 32$, 17/15 male/female), all of whom were non-smokers, are included. The mean ratio of this latter cohort was 0.9 μmol PMA/mol creatinine (range 0–$1.9\,\mu\text{mol}^{-1}$). In the samples obtained for occupa-

Table 2. Urinary PMA ELISA—repeatability study. Percentage coefficient of variation (%CV) and standard deviations (SD) of 'within batch' and 'between batch' ELISA analyses at defined urinary PMA concentrations.

Variance	Urinary [PMA] (nmol/l)	%CV	SD	*n*
Intra-assay	130	5.0	+/− 6.5	30
Interassay	195	7.6	+/− 14.9	12
	448	7.9	+/− 35.5	12

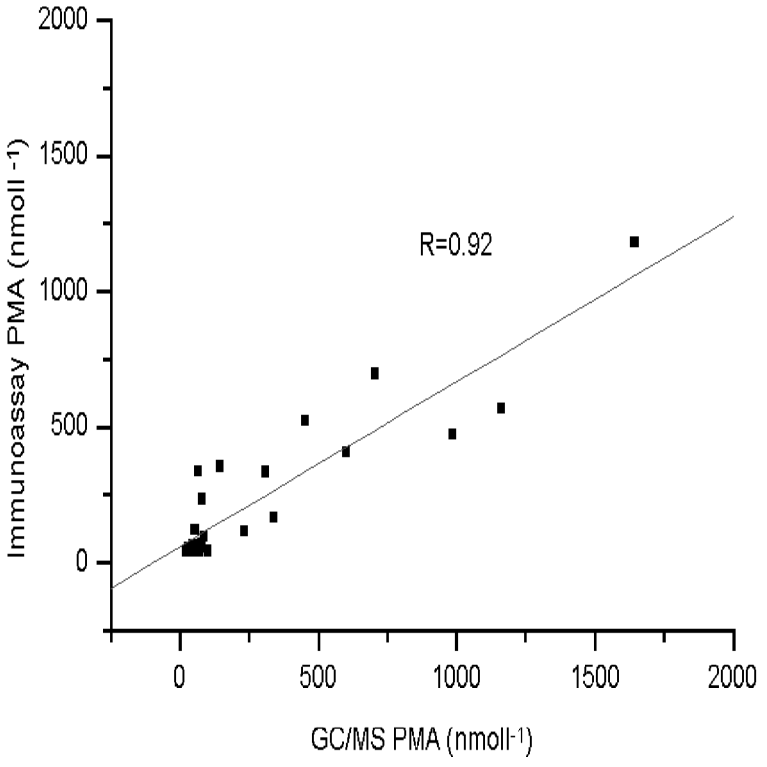

Figure 3. GC/MS-immunoassay urinary PMA scatter diagram. Results of urine samples ($n = 30$) analysed using the PMA ELISA and GC/MS, as described in the Materials and methods. Comparison of the data yielded a coefficient of correlation (R) of 0.92.

tional biological monitoring, ratios ranged from zero to >90 µmol PMA/mol creatinine.

Discussion

The systematic acquisition of biological monitoring data has a value of enabling the identification of trends of exposure. However, analytical complexity and cost invariably are constraints that may hinder the implementation of routine screening programmes. The availability of testing methods that prove analytically reliable

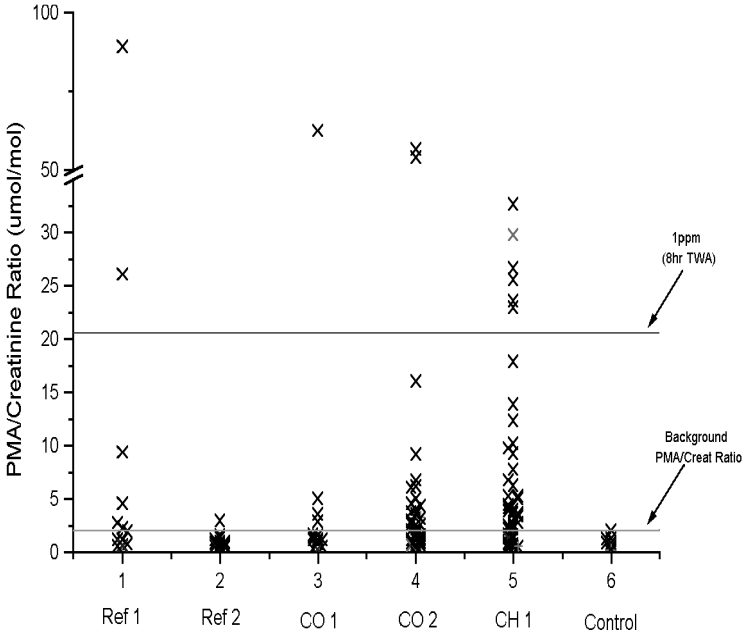

Figure 4. Benzene biological monitoring database. Urinary PMA concentrations, determined by ELISA, and expressed as a function of urinary creatinine, in samples obtained from cohorts of workers in industry. Refs 1 and 2, petroleum refinery workers; CO1 and 2, coke oven workers; CH1, chemical hazard clean-up operation; control, non-occupationally exposed subjects (non-smokers). The lower horizontal line represents the top of the range obtained in the control group. The upper horizontal line indicates, approximately, the PMA/creatinine ratio equivalent to an exposure of 1 ppm benzene (8 TWA) as explained in the Discussion.

and robust, and which are not prohibitive in terms of cost and sophistication, offers the prospect of constructing databases of exposure that may prove helpful in risk assessment exercises (Aston 2001).

Immuno-analytical methods are established firmly as effective screening tools in the field of clinical diagnostics. An objective of this study was to determine the applicability of the methodology for quantifying biomarkers of exposure in human urine. The analytical performance of an immunoassay depends critically on the properties of the antibody reagent which comprises that assay (Aston *et al*. 1992). In particular, antibody-binding affinity and specificity are crucial parameters for defining the limitations of analytical performance.

The merits of measuring urinary PMA as a biomarker of occupational exposure to benzene have been documented (Boogaard and van Sittert 1996). It is important to recognize, however, that the metabolic conversion of benzene to PMA is mediated by the cytochrome P450, CYP2E1 and glutathione-*S*-transferase (GST) enzyme groups (Commandeur *et al*. 1995). Altered enzymic activities due, for example, to genetic polymorphisms, which have been documented in certain human populations, are likely to result in perturbed levels of urinary PMA measured in these subjects (Maestri *et al*. 1997). Thus, whereas urinary PMA is a useful biomarker for the biological monitoring of benzene on a workplace population basis, it is essential to exercise caution in the interpretation of urinary PMA levels of individual subjects.

This paper describes the development of an immunoassay, configured as a competitive ELISA, based on a polyclonal antiserum directed towards PMA. Previous investigations have confirmed that the design of the immunogen, which in this case incorporates a six-carbon spacer, and the chemistry of conjugation to carrier protein, are critical for generating an antiserum with requisite specificity for the free hapten (Ball *et al.* 1997). Invariably, hapten-protein conjugates induce antibodies that exhibit high binding affinities for the conjugate, but relatively poor binding affinities for the hapten itself. Figures 1 and 2, respectively, confirm the induction of an immune response, which is directed towards free PMA. Moreover, the immunoassay is sufficiently sensitive to quantify low levels of PMA (40 nmoll^{-1}) dosed in pooled human urine. An assay signal is generated at low concentrations of urinary PMA and a detection limit of ~2–3 nmoll^{-1} is achievable using the assay conditions described. However, best assay precision is observed in the linear region of the dose-response curve (40–1200 nmoll^{-1} PMA), which is selected, therefore, to define the working range of the immunoassay. The presence of urinary metabolites exhibiting structural complementarities with PMA may show binding cross-reactivity with the antiserum, thereby contributing to potential assay interference phenomena. These interferences could confound interpretation of assay data. The PMA immunoassay is unaffected by the presence of benzylmercapturic acid, which is a urinary product of toluene metabolism (Takahashi *et al.* 1994). This is an important observation especially as co-exposures to benzene and toluene are known to occur. Hippuric acid is a non-specific metabolite of toluene and derives also from the food preservative sodium benzoate. Although it has low structural complementarity compared with PMA, relatively high concentrations of hippuric acid are found in urine and, therefore, low cross-reactivity could manifest as assay interference. The data in table 1 suggest that these metabolites are unlikely sources of interference in the PMA assay.

The intra- and interassay variance data (table 2) indicate that the repeatability of the ELISA, under the assay conditions deployed, is robust over a working range of 40 nmoll^{-1} to ~1.2 moll^{-1} urinary PMA. Using one coated microtitre plate, the ELISA generates results on 21 urine specimens and three quality control samples, in duplicate, within 4 h. Several assays may be carried out readily, in parallel, thus enabling relatively rapid throughput of large batches of samples such as may be encountered in screening programmes.

A comparison of quantitative results generated by ELISA and GC/MS indicates that the methods correlate ($R = 0.92$). In quantifying occupational exposure to benzene, it is confidently expected that the immunoassay will produce results that are largely consistent with data generated by GC/MS. However, there is evidence of bias in the immunoassay results at very low PMA concentrations, typically encountered with background exposures. Investigations are ongoing to explore this observation further. Possible explanations are that the PMA ELISA overestimates low PMA concentrations, or the GC/MS procedure underestimates these concentrations. Over estimations of low concentrations of PMA by the immunoassay may be due to: (1) unreliability of assay calibration at low dose, (2) the presence of urinary matrix effects, or (3) interferences due to cross-reacting species. Moreover, a relative loss of linearity in immunoassay signal at low dose, under the assay conditions described, may render the assay prone to overestimate low concentrations of PMA. No significant correlation was observed between reported PMA concentrations (immunoassay) and creatinine levels in the samples

identified by GC/MS as containing negligible quantities of PMA (data not shown). Alternatively, the GC/MS procedure may underestimate low level PMA concentrations because of: (1) assay imprecision at PMA levels close to the limit of detection or (2) low PMA recovery rates in these samples. Thus, although the immunoassay may overestimate very low urinary PMA concentrations, it proves fit for purpose in determining PMA levels over a range of concentrations which is relevant to occupational exposure.

The potential usefulness of the PMA ELISA as a tool for benzene biological monitoring is illustrated in figure 4. Large numbers of samples obtained from workers in diverse segments of industry were analysed and results reported within 24 h of sample receipt. The results identify, efficiently and effectively, instances of recent exposure to benzene and serve to quantify the extent of individual exposures. The ACGIH and DFG have published biological exposure indices (BEI) for urinary PMA as a marker of benzene exposure at levels of 25 and 45 PMA/creatinine ($\mu g\,g^{-1}$), respectively. The airborne benzene/urinary PMA regression analysis conducted by Boogaard and van Sittert (1995) indicates that these urinary PMA levels are equivalent to 8-h time-weighted average exposures of ~0.5 and 1 ppm benzene, respectively. The control group of subjects not occupationally exposed to benzene were all non-smokers. The presence of trace quantities of benzene in cigarette smoke may impact on the levels of PMA detected in smokers and Boogaard and van Sittert (1996) reported a mean background ratio of 1.7 μmol PMA/mol creatinine in this group compared with a mean of $0.94\,mol\,mol^{-1}$ in a population of non-smokers. Thus, a urinary PMA level that may be encountered typically in a non-occupationally exposed heavy smoker would be ~5-fold lower than the BEI for a 0.5-ppm exposure.

The construction of a database enables comparisons of the degrees of exposure to benzene during different industrial processes and practices. Moreover, timely reporting of results on employees addressing, for example, the hazards associated with the clean-up of a benzene spill, reassures individuals that safety procedures and personal protective equipment designed to minimize exposure are effective.

In conclusion, this paper suggests that a carefully characterized and validated urinary PMA immunoassay in which analytical limitations are defined contributes usefully to benzene biological monitoring. The quantification of other biomarkers of exposure using immunoassay screening tools could assist the transition of biological monitoring from the research laboratory to a routine screening service.

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