# Immunoenrichment of urinary *S*-phenylmercapturic acid

Lathan Ball, Alan S. Wright, Nico J. van Sittert and Paul Aston

**A carefully designed hapten–protein conjugate has enabled the generation of a monoclonal antibody reactive with *S*-phenylmercapturic acid (*S*-PMA). The immobilized antibody retains immunoreactivity and can be used to enrich *S*-PMA from the urine of workers exposed to benzene. The performance of the immunoaffinity column has been validated by comparison with data obtained by GC/MS analysis from the urine of benzene-exposed workers (range 12–168 $\mu g l^{-1}$, corr. coeff. 0.98, $n = 23$). Furthermore immunoaffinity chromatography facilitates the quantitative determination of urinary *S*-PMA by reversed phase HPLC. Bioconcentration of *S*-PMA from the urine of benzene-exposed workers has permitted the quantification of *S*-PMA by HPLC at 8 h TWA (time weighted average) exposures of around 1 ppm. The potential application of immunoaffinity enrichment in biomonitoring studies is discussed.**

Keywords: *monoclonal antibody, immunoaffinity, S-phenylmercapturic acid, benzene, biomonitoring.*

Abbreviations: *AUFS, absorbance units full scale deflection; BSA, bovine serum albumin; d5-S-PMA, deuterated S-phenylmercapturic acid; GC, gas chromatography; MEL, maximum exposure limit; MS, mass spectroscopy; NMR, nuclear magnetic resonance; OD, optical density; PBS, phosphate buffered saline; PEL, permissible exposure limit; QC, quality control; S-PMA, S-phenylmercapturic acid;* t,t-*MA, trans,trans-muconic acid; TLC, thin layer chromatography; TWA, time weighted average.*



## Introduction

Benzene is an important industrial chemical, and the current occupational exposure limits (8 h TWA) in the United Kingdom (maximum exposure limit, MEL), European Union and United States (permissible exposure limit, PEL) are 5 ppm, 1 ppm and 1 ppm respectively. In order to comply with a limit value of 1 ppm 8 hr TWA exposure must be, for most of the time, well below this value. Methods sensitive enough to measure below 1 ppm are therefore needed.

Benzene, both in blood and exhaled air, has been used as a specific and sensitive biomarker for measuring occupational and non-occupational exposures (Perbellini *et al.* 1988). For occupational exposure monitoring, however, non-invasive sampling methods are preferred. Phenol and its conjugates are the main urinary metabolites of benzene (Yardley-Jones *et al.* 1991). Urinary phenol, however, is suitable only for determining exposures down to 10 ppm owing to the relatively high background levels (Van Haaften and Sie 1965). Urinary *S*-PMA (Stommel *et al.* 1989) and *trans,trans*-muconic acid (*t-t*-MA) (Inoue *et al.* 1989) have been proposed as two highly specific and sensitive markers of benzene exposure. *S*-PMA and *t,t*-MA allow the determination of exposures down to 0.3 ppm and 1 ppm (8 h TWA) respectively (Boogaard and Van Sittert 1995). The increased sensitivity achieved with *S*-PMA is due to the relatively low background levels in non-occupationally exposed persons. The determination of *S*-PMA by GC/MS, however, requires a relatively complex work-up of samples prior to assay and sensitivity of detection is limited by the purity of the sample. These, and other factors, limit the usefulness of the conventional method, particularly in large scale screening programmes.

The use of immunochemical methods for the detection of biomarkers offers the prospect of an alternative approach (Van Welie *et al.* 1992). Immunoaffinity chromatography is a well documented procedure which utilizes immobilized antibody molecules of pre-determined specificity to capture and enrich analyte (Aston *et al.* 1992, Yarmush *et al.* 1992, Booth *et al.* 1994). Using antibody of appropriate binding affinity and specificity, *S*-PMA may be adsorbed from a complex matrix and the immunopurified mercapturic acid then subjected to quantitative analysis. Thus, effective immunoenrichment may both facilitate analyses by reducing the preparative steps associated with conventional analytical methods and, by improving signal to noise ratios, enhance assay sensitivity.

The aim of this study was to produce an anti-PMA monoclonal antibody and to develop an immunoaffinity column to enrich *S*-PMA from the urine of benzene-exposed workers. Antibody characterization and an evaluation of an immunoenrichment method by GC/MS is described. The potential of immunoaffinity chromatography/HPLC to determine urinary *S*-PMA is investigated.

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## MATERIALS AND METHODS

### *Materials*

#### Reagents

*S*-PMA (Lot nos A0069174, > 98% pure) was obtained from Janssen Chimica (Hyde, Cheshire, UK). The following reagents were purchased from BDH Merck (Merck Ltd, Dagenham, Essex, UK): acetic acid (chromatography grade), butanol (GPR), citric acid (AnalaR), hydrochloric acid (GPR), di-sodium hydrogen phosphate (AnalaR), sodium bicarbonate (AnalaR), sodium carbonate (AnalaR), sodium chloride (AnalaR), sodium di-hydrogen phosphate (AnalaR), sodium hydroxide (AnalaR). The following were purchased from Sigma Chemical Co. (Poole, Dorset, UK): bovine serum albumin (BSA, catalogue no. A7030), Freund's complete and incomplete adjuvant, Keyhole Limpet haemocyanin *Megathura crenulata* (catalogue no. H2133), Tween-20, methanol (HPLC grade), and 3,3′,5,5′-tetramethyl-benzidine liquid substrate system (TMB). Acetonitrile (HPLC grade) was obtained from Rathburns (Walkerburn, UK). Other reagents were: bis(sulfosuccinmidyl)suberate, Coomassie Blue protein assay reagent kit (Pierce Chemicals, Luton, UK), CNBr-activated sepharose (Pharmacia Ltd, Milton Keynes,



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UK), peroxidase-conjugated rabbit immunoglobulins to mouse immunoglobulins (Dakopatts a/c, Denmark), PBS tablets (Unipath, Basingstoke, UK), sodium acetate (GPR, Hopkin and Williams Ltd, Essex, UK), and d5-*S*-PMA (Shell Biomedical Laboratory, Rotterdam, The Netherlands). The myeloma cell line Sp2/0 Ag 1–4 was obtained from Flow Laboratories Ltd (Rickmansworth, Herts, UK), Balb/c mice were supplied by Charles River (Margate, Kent, UK). Nunc Immunoplates maxisorb f96 cert (Gibco Ltd, Paisley, UK) and Amicon Cetriprep 30 (Amicon Ltd, Stonehouse, UK).

Urine samples came from workers in two refineries (Netherlands and Brunei) and a chemical manufacturing plant (Netherlands) where exposure to benzene may occur. End of shift samples were collected in polythene bottles, acidified to pH 1.5–2 with 6 M hydrochloric acid and stored at 4 °C until assay (approx. 1 year after collection). Under these conditions *S*-PMA is known to be stable for at least 6 months (personal communication with H. van der Waal, Shell Biomonitoring Laboratory, Rotterdam).

### Working standards

ELISA standards (0–2.5 mg $l^{-1}$) were prepared in PBS from a 852 mg $l^{-1}$ solution in 1% methanol:PBS. HPLC standards (0–1.0 mg $l^{-1}$) were diluted in mobile phase.

### Buffers

Phosphate (0.05 mol $l^{-1}$, pH 8), coating (sodium carbonate 0.03 mol $l^{-1}$ pH 9.8), coupling (0.1 mol $l^{-1}$ sodium carbonate, pH 8.3, containing 0.5 mol $l^{-1}$ NaCl), glycine (0.2 mol $l^{-1}$, pH 4, containing 0.5 mol $l^{-1}$ NaCl) and acetate (0.1 mol $l^{-1}$, pH 4, containing 0.5 mol $l^{-1}$ NaCl) were prepared on the day of use. Saline/Tween wash solution consisted of 0.9% w/v saline, 0.05 % w/v Tween-20 in distilled water.

## *Methods*

### Immunogen preparation

*S*-PMA (225 mg) was deacetylated by heating with 6 mol $l^{-1}$ HCl (20 ml) at 90 °C for 3 h. Acetic acid, a product of the acid hydrolysis, was removed by rotary evaporation at 35 °C. The reaction was monitored by TLC using butanol, acetic acid and water 3:1:1. The product was analysed by proton NMR on a GE QE300 operating at 300 mHz referenced to TMA at 0 ppm with dueterated methanol ($CD_3OD$) as the solvent.

The *S*-phenylcysteine was dissolved in di-sodium hydrogen phosphate (60 ml) and adjusted to pH 8 by the addition of 1 mol $l^{-1}$ sodium dihydrogen phosphate (12 ml). To bovine serum albumin (BSA, 50 mg in 5 ml) and Keyhole Limpet haemocyanin (KLH, 50 mg in 8.3 ml in 0.05 mol $l^{-1}$ phosphate buffer, pH 8) was added *S*-phenylcysteine in phosphate buffer (9 mg in 3.6 ml) with mixing. The homobifunctional cross-linker bis(sulphosuccinimidyl)suberate (70 mg) was dissolved in distilled water (1 ml) and immediately added to the protein solutions (21.35 mg, 152.5 μl) with rapid mixing. The mixtures were reacted overnight with continuous stirring then dialysed extensively, first against distilled water, then against PBS. The protein concentration was determined using Coomassie blue protein assay reagent according to the manufacturer's instructions. The average hapten incorporation ratio was estimated spectrophotometrically from the increase in absorbance at 270 nm after coupling.

### Antibody production

Antibodies were produced using hybridoma technology (Goding 1986). Briefly, mice (Balb/c) were immunized by subcutaneous injection (days 1 and 20) with 100 mg of *S*-PMA–KLH in 200 ml of adjuvant emulsion (50:50 v/v). Primary and secondary immunizations were administered in Freund's complete and incomplete adjuvant, respectively. Test bleeds were obtained on day 30.

Antibody titre was determined by the preparation of antibody titration curves using microtitre plates coated with 1 mg $l^{-1}$ of BSA–hapten conjugate (in coating buffer, overnight at 4 °C). Relative antibody affinities and cross-reactivity were determined by competitive ELISA.

Two consecutive days prior to fusion, mice were immunized intraperitoneally with 100 μg of *S*-PMA–KLH conjugate in PBS. Two days later the splenocytes were fused (day 1) with the myeloma cell line Sp2/0 Ag 1-4. On day 13, cell supernatants were screened for the presence of antibody by ELISA. Wells were selected for cloning to monoclonality by competitive inhibition studies using free *S*-PMA (100 mg $l^{-1}$).

Cell line 3G9 secreted antibody with the highest relative affinity. Antibody 3G9 was therefore selected for the development of an ELISA and an immunoaffinity chromatography column.

The ELISA was carried out by adding 50 μl of standard and 50 μl of cell supernatant (diluted 1: 200 in PBS/tw) to the wells of an antigen-coated plate (coated with 0.1 mg $l^{-1}$ *S*-PMA–BSA conjugate overnight at 4 °C in coating buffer). The plate was sealed with plastic film and incubated overnight at room temperature. The contents of the wells were then emptied and washed five times with saline/Tween wash solution and shaken dry. To each well was added 100 ml of rabbit anti-mouse IgG peroxidase conjugate diluted 1:1000 in phosphate buffered saline. The plate was incubated for a further 2 h at room temperature then washed again. After addition of TMB substrate (100 μl per well), the plate was incubated for a further 30 min and the absorbance measured (650 nm) using a microtitre plate reader.

Inter- and intra-assay reproducibility were determined by the repeat analysis of a low, medium and high QC. QCs were prepared by the dilution of *S*-PMA in PBS at 25, 100 and 500 μg $l^{-1}$ respectively.

### Preparation of immunoaffinity column

Monoclonal antibody was purified from cell supernatant by protein-G affinity chromatography. Purified antibody was desalted and concentrated using an Amicon Centriprep-30 concentrator, according to the manufacturer's instructions.

Antibody was covalently attached to a solid support (cyanogen bromide-activated sepharose) by a modification of a method described by Pharmacia. Freeze-dried CNBr-activated sepharose 4B (0.21 g) was gently mixed with 1 mmol $l^{-1}$ HCl (15 ml for 5 min at room temperature). The gel which formed was sedimented by centrifugation (2000 *g* for 1 min) and the supernatant discarded. The rehydration step was repeated three times. The swollen sepharose was washed with coupling buffer (15 ml) to remove excess acid.

Immunoglobulin solution (1.51 g $l^{-1}$) was added (3 ml) to the sepharose and the suspension gently mixed for 2 h at room temperature. The sepharose was washed with coupling buffer (15 ml), resuspended in glycine buffer (25 ml) and gently mixed overnight at 4 °C to block any remaining active groups on the solid support. The sepharose was washed with coupling buffer (15 ml) and acetate buffer (15 ml), alternately, three times to remove any protein bound non-covalently to the matrix. The gel was washed twice with PBS and a column of approximately 0.5 ml settled bed volume was prepared. Finally, the PBS was replaced with PBS containing sodium azide (0.02% w/v) and the column stored at 4 °C until use. Antibody uptake was monitored spectrophotometrically.

### Immunoaffinity chromatography

Before use, the immunoaffinity column was allowed to reach room temperature and equilibrated with PBS. Samples were chromatographed at a flow rate of approximately 0.5 ml $min^{-1}$. *S*-PMA diluted in PBS (1 mg $l^{-1}$) was loaded (1–20 ml) onto the immunoaffinity column. The column was washed with PBS (5 ml), and the retentate eluted with methyl alcohol (2 ml). Elution fractions, load (1 ml), wash (1 ml) and retentate (1 ml) were collected. The fractions were analysed for the presence of *S*-PMA by ELISA.

Mid-stream urines (5, 10, and 20 ml) dosed with *S*-PMA (50, 25 and 5 μg $l^{-1}$ respectively) were applied to the column for immunoextraction. The retentates were analysed by ELISA and HPLC.

HPLC analyses were performed using C18 reversed phase chromatography (Hichrom column 10 cm × 4.9 mm: mobile phase; 17% acetonitrile, 0.03 mol $l^{-1}$ phosphate buffer, pH 2.1: flow rate 1 ml $min^{-1}$). *S*-PMA was detected at 256 nm (Gilson model 116). A linear calibration plot (0.125–1.0 mg $l^{-1}$) was obtained with *S*-PMA standards prepared in mobile phase. The HPLC limit of detection was 0.05 mg $l^{-1}$.

Prior to analyses, the immunoaffinity chromatography retentates were concentrated by evaporation and diluted in mobile phase.

The performance of the immunoaffinity column was validated against the solvent extraction GC/MS method described by Van Sittert *et al.* (1993). Samples of urine containing 12–168 μg $l^{-1}$ determined by GC/MS were determined after immunoextraction using a modification of the procedure described by Boogaard and Van Sittert (1995). GC/MS analysis was performed at the Shell Biomedical Laboratory, Rotterdam.

Briefly, to 5 ml of urine was added 0.112 μg of d5-*S*-PMA internal standard. The pH of the samples (pH 2) was adjusted to between pH 6.3 and 6.9 by the addition of two drops of 6 mol $l^{-1}$ NaOH and 4–12 drops of 1 mol $l^{-1}$ NaOH solution. Samples ($n$ = 23) were applied to the column and chromatographed as described above. Methanol : water was evaporated from the retentate under a gentle stream of nitrogen at 45 °C and the residue dried by warming above an infra-red lamp for 1 h. After methylation, the concentration of derivatized *S*-PMA in each sample was determined by GC/MS with electron impact ionization. The GC injection protocol was modified as follows. Injector temperature was initially 150 °C, after the sample was loaded this temperature was held for 1 s then increased to 250 °C at 12 °C per second. The injection volume was 1 μl splitless. For quantification, ions at $m/z$ 194 and 199 (*S*-PMA and d5-*S*-PMA fragments respectively) were monitored. Qualification ions at $m/z$ 253 and 258 were recorded.

Finally, the concentration of *S*-PMA in urine samples collected from benzene-exposed workers was determined by immunoaffinity chromatography/HPLC. Samples were chromatographed after neutralization and without the addition of internal standard.

The concentration of *S*-PMA in the urine samples (30–1152 μg $l^{-1}$) had been determined previously by GC/MS.

# Results

## *Immunogen preparation*

Hydrolysis of the *S*-PMA was complete after 3 h. A single spot, which gave a positive reaction with ninhydrin, was observed after TLC of the reaction mixture. The loss of the acetyl group was confirmed by NMR. Phenylcysteine was coupled to BSA with an average hapten–protein incorporation ratio of 25:1. Comparable hapten–protein loading densities were achieved with the phenylcysteine–KLH conjugate.

## *S-PMA immunoassay*

A standard curve for an ELISA based on 3G9, with a limit of detection of 8 μg $l^{-1}$ is illustrated in Figure 1. Values of 28 ± 4, 98 ± 9 and 598 ± 45 μg $l^{-1}$ were obtained for the repeat analysis of a low, medium and high QC ($n$ = 12, inter-assay variation). Intra-assay CVs for the low, medium and high QCs were 24.2%, 5.2% and 13.2% respectively ($n$ = 6). The antibody did not cross-react with acetylcysteine, hydroxyethyl and hydroxypropylmercapturic acid. Some cross-reactivity was observed with relatively high concentrations of *S*-PMA methyl ester and benzylmercapturic acid ($IC_{50}$ values were 35 and 18 mg $l^{-1}$ respectively). Cross-reactivity with other possible benzene metabolites was not investigated.

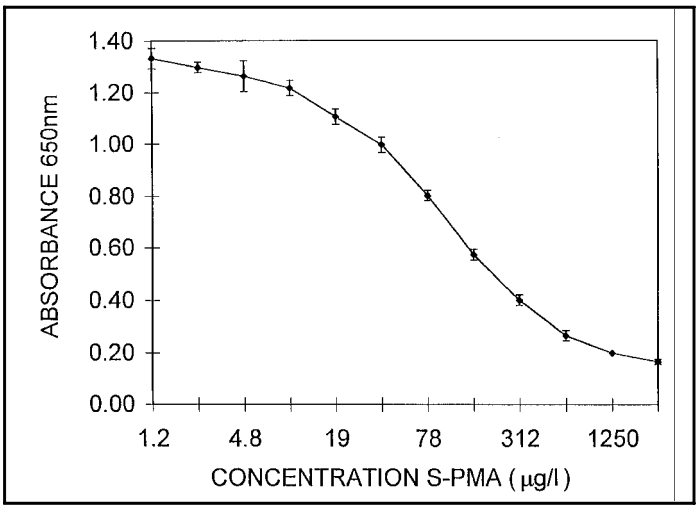


**Figure 1.** *S*-PMA standard curve developed by ELISA as described in 'Methods'. Points represent the mean of three replicate values, error bars are standard deviations.

## *Immunoaffinity chromatography*

Protein-G purification yielded 30 μg of 3G9 per ml of cell supernatant. A further 20-fold concentration of antibody was achieved during desalting.

A weight of 4.06 mg (89%) of 3G9 was coupled to 0.21 g (0.75 ml of swollen gel) of CNBr-activated sepharose. No protein was detected in the final coupling/acetate buffer washes (OD readings at 280 nm), indicating that all the antibody was covalently attached to the solid support.

The immunoaffinity column had a maximum binding capacity of 1.5 μg of *S*-PMA. No *S*-PMA was observed in the

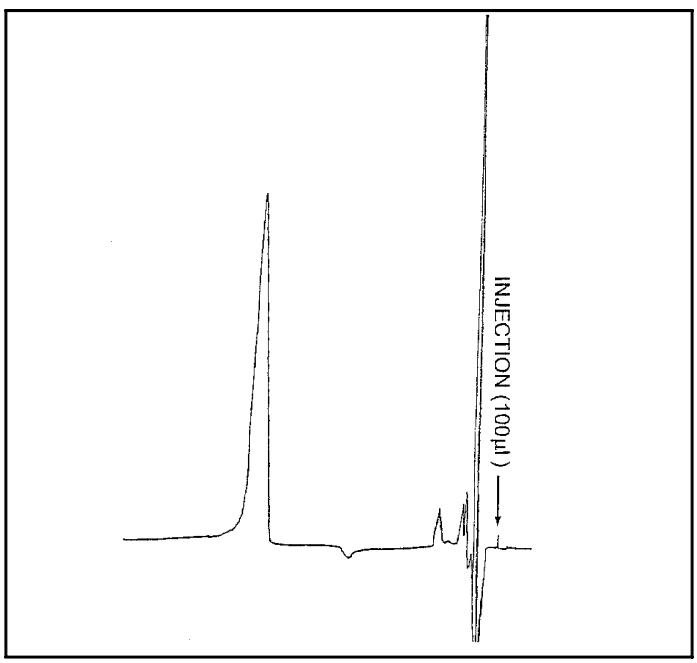


**Figure 2.** HPLC chromatogram of *S*-PMA after immunoextraction from the urine of a benzene-exposed worker. The urine contained 1152 μg $l^{-1}$ of *S*-PMA determined by GC/MS. HPLC was performed on a C18 column using 17% acetonitrile, 0.03 mol $l^{-1}$ phosphate buffer, pH 2.1, flow rate 1 ml $min^{-1}$. The absorbance was set at 0.05 AUFS and the retention time of the *S*-PMA was 14 min.

| GC/MS (μg $l^{-1}$) | IAC/HPLC (μg $l^{-1}$) |
|---|---|
| 1152 | 1157 |
| 580 | 297 |
| 492 | 476 |
| 154 | 143 |
| 60 | 63 |
| 30 | 32 |

**Table 1.** Comparison of *S*-PMA concentrations determined by GC/MS and immunoaffinity chromatography (IAC)/HPLC in the urine of six different benzene-exposed workers.

final washes when the fractions were analysed by ELISA. After immunoenrichment of a PBS solution (10 ml) containing 0.05 μg of analyte, 0.54 μg (108%) of *S*-PMA was recovered. Recoveries of 80% (SD = 6.7, $n = 6$) were obtained after immunoaffinity chromatography of 5 ml mid-stream urine containing 50 μg $l^{-1}$ of *S*-PMA. Recoveries of 94% and 80% were obtained after immunoaffinity chromatography of 10 and 20 ml urine containing 25 and 5 μg $l^{-1}$ respectively.

Twenty-three urine samples containing 12–168 μg $l^{-1}$ *S*-PMA analysed by the method of Van Sittert *et al.* (1993) were analysed by immunoaffinity chromatography – GC/MS. The correlation coefficient between the two methods was 0.98. Recovery of *S*-PMA after acidification and neutralization of the urine was 28.3% (SD = 6.2, $n = 23$). GC/MS chromatograms of immunoextracted samples contained fewer peaks than those samples analysed by the method of Van Sittert *et al.*

Results of HPLC analyses of immunoaffinity column eluates from the urine of workers exposed to benzene are shown in Table 1. The corresponding data obtained by GC/MS are also presented. The concentration of urinary PMA ranged from 30 to 1152 μg $l^{-1}$. The correlation coefficient was 0.964 ($n = 6$). A typical HPLC chromatograph is illustrated in Figure 2. *S*-PMA has a retention time of 14 min under the HPLC conditions employed.

## Discussion

A monoclonal antibody, 3G9, which specifically binds *S*-PMA, has been generated. 3G9 retains its immunoreactivity after covalent coupling to a solid support, enabling the production of an immunoaffinity chromatography column. The chromatography column can be used to extract low levels of *S*-PMA (5 μg $l^{-1}$) selectively from urine. The performance of the immunoaffinity column at low concentrations of *S*-PMA (12–168 μg $l^{-1}$) has been validated in a comparative study with the method of Van Sittert *et al.* (1993). The two methods show excellent agreement, the correlation coefficient was 0.98 ($n = 23$) over this range. Furthermore, bioconcentration of *S*-PMA from the urine of benzene-exposed workers has permitted the quantification of *S*-PMA by HPLC at 8h TWA airborne exposures of around 1 ppm.

Compounds of low molecular weight are not normally immunogenic but can be rendered antigenic by coupling to a carrier protein such as KLH. It is generally accepted that antibody specificity is highest for that part of the molecule furthest from the carrier protein (Harrison *et al.* 1991, Aston *et al.* 1992) and that the inclusion of a spacer arm between the hapten and the protein decreases steric shielding by the bulky carrier thereby improving hapten presentation (Harrison *et al.* 1991, Aston *et al.* 1992). Marco *et al.* (1993) have reported the production of a polyclonal antiserum specific for the mercapturic acid metabolites of naphthalene using a mercapturic acid metabolite conjugated through the free carboxylic acid of the acetyl cysteine moiety. However, in this laboratory (R. L. Ball unpublished data) when immunizations were performed with *S*-PMA, coupled through the carboxylic acid group to KLH, a large immune response was induced which recognized conjugated hapten but did not bind *S*-PMA in free solution. It was postulated, therefore, that the carboxylic acid plays a critical role in antigen–antibody binding. Conjugation of mercapturic acids through the sulphydryl moiety led to the generation of antibodies which recognized the acetylcysteine moiety and generally bound mercapturic acids *per se* (R. L. Ball, unpublished data). To preserve antibody specificity therefore, a conjugate in which *S*-PMA was linked through the methyl group of the acetyl cysteine moiety to KLH was designed.

An ELISA (limit of detection 8 μg $l^{-1}$) based on 3G9 has a relatively high affinity for *S*-PMA compared with structurally similar mercapturic acids. 3G9 was therefore selected for the preparation of an immunoaffinity chromatography column. Antibody was covalently attached to CNBr-activated sepharose. The amount of antibody coupled to the solid support (5.4 g $l^{-1}$ gel) was less than the quoted binding capacity (8–10 g of protein per l) of the gel. In this study, no attempt was made to optimize the antibody coupling chemistry or the chromatography conditions.

Immunoreactivity was assessed by monitoring binding of *S*-PMA applied to the column in aqueous buffer. The column adsorbed 1.5 μg of *S*-PMA and the analyte did not appear to leech from the column during washing. High recoveries (up to 100% of the load) were obtained where the retentate was eluted from the column with methanol. Moreover, the column retained functionality even after a very high percentage solvent (95%) elution step. With repeated use (10 times) there was no noticeable loss in column performance. Observations show no evidence of changes in the antibody binding characteristics with time.

The column also proved to be effective in selectively adsorbing and concentrating *S*-PMA from mid-stream urine. Loading analyte in this matrix appeared to have no detrimental effect on column performance. Furthermore, recovery of analyte appears to be independent of sample volume. At present urine samples are collected in polythene bottles and acidified to pH 2. Acidification and neutralization of urine exercised a strong effect on column performance. Recovery of *S*-PMA (28.3%) was lower than expected and is probably due to high levels of salt disrupting antigen–antibody binding. Improving sample recovery will further increase assay sensitivity. Recovery may be improved if the urines are desalted prior to extraction. Alternatively, samples may be stored frozen (where it has been shown that *S*-PMA in urine is stable for at least 2 years) rather than at acid pH after collection.

We have shown that 3G9 is predominantly reactive with *S*-PMA. GC/MS chromatograms of immunoextracted samples contained fewer peaks than those samples analysed by the method of Van Sittert *et al.* (1993). Due to the loss of sample matrix it was necessary to modify the reported GC conditions. Changes were made in the injection protocol to prevent loss of sample during the solvent purge. The excellent agreement between the data derived by post-immunoenrichment HPLC and those obtained by GC/MS analyses also suggest that no endogenous components of urine are recognized by 3G9 or interfere with antibody–analyte binding. The HPLC chromatogram (Figure 2) is free from interfering peaks.

The sensitivity of the GC/MS assay is limited by the purity of the sample. The limit of detection of the present assay for *S*-PMA is 0.3 ppm, 8 TWA, of benzene (Boogaard and Van Sittert 1995). Immunopurification reduces the background noise in the MS fragmentographs and, therefore, the data demonstrate the potential of immunoaffinity chromatography to increase GC/MS assay sensitivity.

Jongeneelen *et al.* (1987) has reported that HPLC generally lacks the sensitivity for application in biomonitoring. However, the data presented in this report indicate that immunoextraction may facilitate the measurement of *S*-PMA by HPLC from the urine of benzene-exposed workers. Results obtained by immunoaffinity chromatography/HPLC correlate (corr. coeff. 0.96, $n = 6$) with those obtained by GC/MS (Table 1) over a range of values (30–1152 $\mu g\ l^{-1}$). The immunoaffinity chromatography/HPLC analysis of urine from benzene-exposed workers could be further improved by the use of an internal standard. Preliminary studies show 3G9 binds *S*-phenyl-*N*-trifluoroacetylcysteine and *S*-phenyl-*N*-trifluoracetylcysteine and *S*-PMA can be separated by HPLC using the conditions employed.

Immunoaffinity chromatography/HPLC analysis is simple and easy to perform compared with GC/MS. It is estimated that 75 samples per day can be analysed by IAC/HPLC. In addition, HPLC determination can be readily performed in many laboratories. Our findings confirm the potential utility of immunoaffinity chromatography/HPLC in monitoring exposure to benzene.



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