BIOMARKERS, 2000, VOL. 5, NO. 4, 252–262

Analysis of *S*-phenyl-L-cysteine in globin as a marker of benzene exposure

R. HANWAY[1], A. CAVICCHIOLI[1], B. KAUR[1], J. PARSONS[1], J. H. LAMB[1], L. D. BUCKBERRY[2] and P. B. FARMER[1]*

[1] MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester LE1 9HN, UK. e-mail: PBF1@le.ac.uk

[2] Department of Chemistry, De Montfort University, The Gateway, Leicester LE1 9BH, UK

Received 10 January 2000, revised form accepted 31 March 2000

An assay has been developed to determine *S*-phenylcysteine (SPC) in globin as a potential biomarker for exposure to benzene. The sensitivity of the assay is less than 20 pmol SPC g^{-1} globin. Following acidic hydrolysis of the protein, the modified amino acid is purified by reverse phase cartridge chromatography and HPLC, prior to conversion to the *tert*-butyldimethylsilyl derivative and GC–MS selected ion recording. Quantitation is achieved using the internal standard [$^2H_5$]-SPC, and calibration lines were established using a synthetic peptide Leu-His-SPC-Asp-Lys. Control human globin was found to contain *ca* 30 pmol SPC g^{-1} globin in two populations. The source of the apparent background level of SPC is unknown.

Keywords: *S*-phenyl-L-cysteine, benzene, biomonitoring, globin.

Introduction

Human exposure to benzene is a topic of especial current interest because of the widespread occurrence of this compound and its known myelotoxicity, clastogenicity and carcinogenicity (Snyder *et al*. 1993). Both occupational and environmental exposures occur, the latter being mainly associated with the presence of benzene in petroleum and cigarette smoke (Wallace 1989). The approaches that have been used to monitor human exposure to benzene have fallen into three categories: (a) 'external' monitoring of benzene concentrations in air, food, water, etc.; (b) 'internal' monitoring of benzene or one of its metabolites in exposed subjects, and (c) biologically effective dose monitoring by measuring one of the interaction products formed between active benzene metabolites and a target molecule in an exposed individual. Although external monitoring (a) is extremely valuable in assessing the sources and magnitude of the exposures, it does not inform on an individual's response to the exposure, which may vary considerably from person to person. Internal monitoring (b) does give some information on this individual variation, but because of the nature of the pharmacokinetics of benzene metabolism, can only reflect recent exposures (normally up to 1–2 days earlier than the measurements). Biologically effective dose monitoring (c) allows a more historical view to be taken of the exposure monitoring. Depending on the lifetime of the molecule with which the covalent interaction of the metabolite has taken place, it may be possible to detect benzene exposures that occurred several months before the sampling. One possible disadvantage in the case of benzene is that,

* Corresponding author: P. B. Farmer, MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester LE1 9HN, UK.

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2000 Taylor
http://www.tandf.co.uk/journals

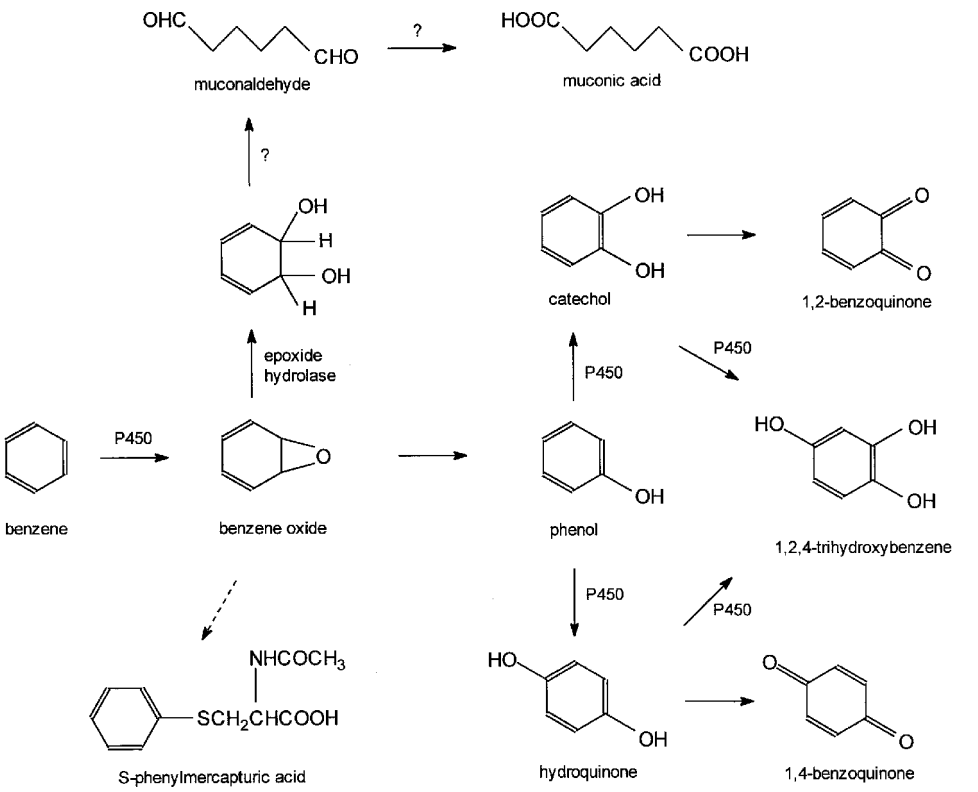

Figure 1. Metabolism of benzene.

despite the large amount of investigations that have been carried out, it is not yet clear which metabolite is of most carcinogenic significance.

The metabolism of benzene is complex (reviewed in Snyder and Hedli 1996) (figure 1). It involves as the first stage a P450 (CYP2E1) mediated oxidation to the reactive intermediate benzene oxide (Lindstrom *et al.* 1997, Lovern *et al.* 1997). This further yields a range of other products including phenol, hydroquinone, catechol, 1,2-benzoquinone, 1,4-benzoquinone, Z_1Z-muconaldehyde, and *S*-phenylmercapturic acid. Of these, because of their chemical reactivity, benzene oxide, benzoquinone/hydroquinone and Z_1Z-muconaldehyde may be considered as the most likely components to interact with DNA and thus to be involved in the initiation stage of carcinogenesis.

The most widely used internal biomarkers of benzene exposure have been benzene itself (in blood or breath) (Perbellini *et al.* 1988) and urinary phenol (Van Haaften and Sie 1965, Bechtold *et al.* 1991), *trans,trans*-muconic acid (Inoue *et al.* 1989, Bechtold *et al.* 1991, Boogard and Van Sittert 1995, Melikian *et al.* 1999) and *S*-phenylmercapturic acid (S-PMA) (Stommel *et al.* 1989, Boogard and Van Sittert 1995, Melikian *et al.* 1999). The latter holds a particular advantage in that low backgrounds of this compound are found in urine, allowing a more selective and sensitive assay of benzene exposure. A recently published immunoaffinity chromatography-HPLC procedure for S-PMA has allowed quantification of benzene exposures at an 8 h TWA of around 1 ppm (Ball *et al.* 1997).

The use of sulphur conjugates in protein has also been favoured for biologically

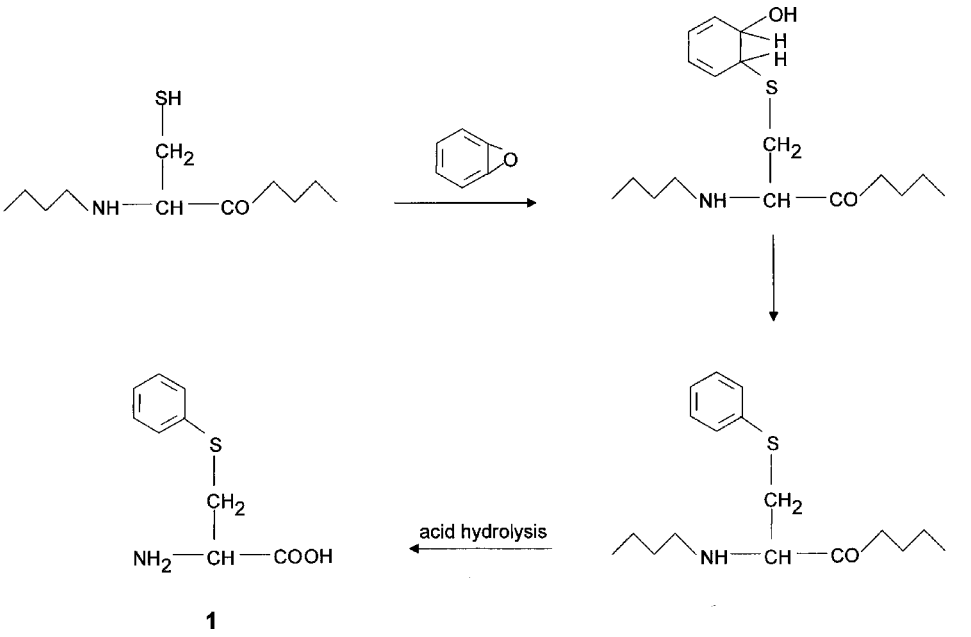

Figure 2. *S*-Phenylcysteine production in proteins following exposure to benzene oxide.

effective dose monitoring. The product that has so far been monitored is *S*-phenyl-L-cysteine (**1**,SPC) (figure 2), which is presumed to reflect the dose of the primary electrophilic metabolite benzene oxide in the proteins globin and albumin. [The initial adduct from benzene oxide is assumed to be *S*-(1,2-dihydro-2-hydroxyphenyl)cysteine. It is unclear if this spontaneously dehydrates in the protein, or whether SPC is formed during the analytical work-up procedures]. Three approaches for the analysis of SPC have been reported. In the first, the protein is hydrolysed to its constituent amino acids, and SPC is purified, derivatized and analysed by GC–MS (Bechtold *et al*. 1992a, b, Melikian *et al*. 1992, Bechtold and Henderson 1993, Bechtold and Strunk 1996). The second method involves the reaction of the adducted protein with a mixture of trifluoroacetic anhydride and methane sulphonic acid which yields *S*-phenyl trifluorothioacetate from SPC (Yeowell-O'Connell *et al*. 1996a, b, 1998, Lindstrom *et al*. 1998). This product may then be analysed by negative ion chemical ionization GC–MS. The third approach which has so far only been applied to animal albumin samples involves a Raney nickel treatment in deuterium oxide, which yields monodeuterobenzene (Bechtold and Strunk 1996). Satisfactory dose–response relationships have been obtained for animals treated with benzene, and Yeowell-O'Connell *et al*. (1998) have shown using the trifluoroacetic anhydride method that benzene oxide–cysteine adducts in globin are significantly correlated with benzene exposure. However the background level of adducts in apparently unexposed human subjects was unexpectedly high, indicating artifactual formation of the adduct, or unknown exposures to benzene, or endogenous production of adducts. In order to gain more information on the sources of SPC in proteins we have developed a method which increases the sensitivity of the published procedure for GC–MS determination of SPC after acidic hydrolysis of globin, and have applied this to control and occupationally-exposed human populations.

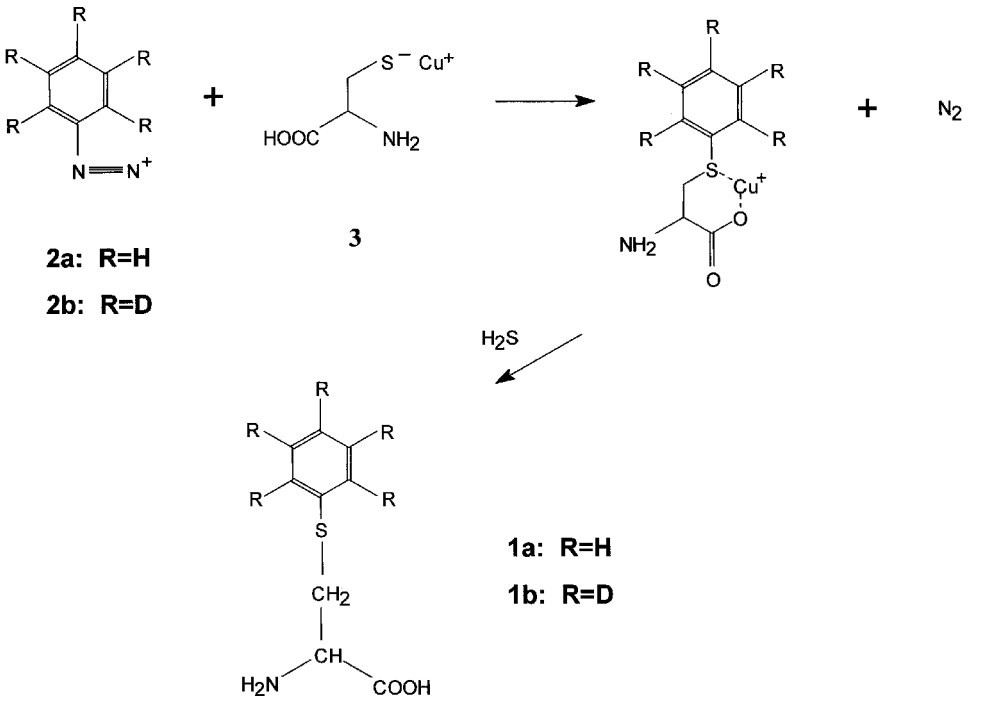

Figure 3. Synthesis of $[^2H_0]$-*S*-phenylcysteine and $[^2H_5]$-*S*-phenylcysteine.

Materials and methods

Materials

Except where otherwise mentioned all chemicals were from Fisher Scientific UK (Loughborough, UK). Hydrochloric acid (s.g. 1.18) was SLR grade; ammonium acetate, acetone and diethyl ether were of Analar grade. For HPLC the water used was ultrapure (18 Ω grade) and the methanol was HPLC grade. *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing 1% *tert*-butyldimethylchlorosilane (TBDMCS) was obtained from Pierce and Warriner (UK) Ltd (Chester, UK) and the acetonitrile used for derivatization was of HPLC grade. Aniline, $[^2H_5]$-aniline, L-cystine and hydrogen sulphide were obtained from Aldrich Chemical Company (Gillingham, UK), and cupric oxide, zinc metal powder, sodium nitrate, citric acid and sodium carbonate from BDH Chemicals Ltd (Poole, UK). Fmoc-chloride was purchased from Sigma (Poole, UK). SepPak C_{18} cartridges (Millipore, Croxley Green, UK) were washed successively with methanol (4 ml) and water (4 ml) before use.

The pentapeptide Leu-His-SPC-Asp-Lys was synthesized by the Protein and Nucleic Acids Chemical Laboratory, University of Leicester (Leicester, UK).

Instrumental analysis

NMR spectra were recorded on a Bruker ARX 250Mhz instrument with D_2O and trifluoroacetic acid as solvent. Fast atom bombardment (FAB) mass spectra were obtained using a VG70-SEQ mass spectrometer with argon atoms at 8 KeV acceleration. Samples were introduced in a 0.2 mM *p*-toluenesulphonic acid/glycerol matrix. Gas chromatography–mass spectrometry (GC–MS) and tandem mass spectrometry (GC–MS/MS) were carried out using a VG70-SEQ instrument linked to a Hewlett Packard 5890 Series II gas chromatograph, or a VG Auto Spec Ultima Q instrument linked to a Carlo Erba CE8060 gas chromatograph. HPLC was performed using Gilson 305 pumps and a Techsphere ODS-5μ column (25 cm×4.6 mm), monitoring the column elute by ultra-violet (UV) detection (Applied Biosystems) at 248 nm and 280 nm. Semi-preparative HPLC was carried out with a Hypersil BDS C_{18} column (25 cm×10 mm).

Synthesis of S-phenylcysteine (figure 3)

Aniline (4.24 g, 45 mmol), concentrated sulphuric acid (2.0 ml) and water were cooled in an ice–salt bath (–5 to –7 °C) and sodium nitrite (2.81 g, 40.7 mmol) in water (10 ml) was added dropwise, always

maintaining the temperature below 5 °C. In a separate reaction vessel, L-cystine (4.94 g, 20.6 mmol), sulphuric acid (2 M 100 ml) and zinc dust (4.04 g, 61.8 mmol) were heated under reflux at 100 °C for 2 h. The mixture was filtered whilst hot and a saturated solution of aqueous copper(I) oxide suspension (containing 7.13 g, 49.8 mmol) was then added to the filtrate until no further dissolution occurred. The diazotized aniline solution **(2a)** was then added dropwise with stirring to the cold (<−5 °C) cysteinyl cuprous mercaptide **(3)**. A vigorous evolution of nitrogen occurred with the concomitant formation of a yellow buff precipitate. This precipitate redissolves on stirring for 1 h. Subsequent heating at 70 °C for 1 h caused a colour change from yellow to green and undissolved material was removed by decanting. The reaction mixture was extracted with ether (4 × 40 ml) and then saturated with hydrogen sulphide. The reaction mixture was then heated under reflux to remove any excess hydrogen sulphide. Dropwise addition of 0.880 ammonia solution caused the precipitation of the crude product which was purified by silica chromatography, using the eluting solvent system dichloromethane:methanol:0.880 ammonia (4:2:1 v:v:v). The product **(1a)** was obtained with yield 0.8 g, 9.2 %, m.p. 179–180 °C (Lit. 180–181 °C; Parke and Williams 1951), R_f (dichloromethane:methanol:0.880 ammonia, 4:2:1, v:v:v) on DC-Alufolien Kieselgel 60^{F254}, 0.65. NMR (360 MHz, D_2O): δ 3.50, 3.65 (2H, dd, cystβ and cystβ′), 4.25 (1H, q, cystα, Jα,β and Jα,β′ = 4.2 and 7 Hz), 7.4–7.55 (5H, m, aromatic); FAB (positive): *m/z* 198 $(MH)^+$.

Synthesis of {$^2H_5$}-S-phenyl-L-cysteine

The synthesis was repeated as for the [$^2H_0$]-SPC substituting [$^2H_0$]-aniline with [$^2H_5$]-aniline. The product **(1b)** was obtained with yield 30 mg, 8%, m.p. 193–196°C, R_f (dichloromethane: methanol:ammonia, 4:2:1, v:v:v) on DC-Alufolien Kieselgel 60^{F254}, 0.67. NMR (360 MHz, D_2O): δ 3.15, 3.25 (2H, dd, cystβ and cystβ′), 3.95 (1H, q, cystα, Jα,β and Jα,β′ = 4.2 and 7 Hz); FAB (positive): *m/z* 203 $(MH)^+$.

Synthesis of Fmoc-SPC

SPC (500 mg, 2.54 mmol) was dissolved in a mixture of 10% aqueous sodium carbonate (10 ml) and dioxane (5 ml). Any undissolved material was removed and the remaining solution cooled in an ice-bath and stirred. A solution of Fmoc chloride (748.6 mg, 2.89 mmol) in dioxane (2.5 ml) was added dropwise over 5 min. The ice-bath was removed, and the solution stirred for 50 min after which time TLC indicated minimal starting material remaining. The mixture was then poured into water (68 ml), and the solution acidified with citric acid. The resulting solution was extracted into chloroform (3 × 17 ml) and the combined chloroform extracts washed thoroughly with water, dried (Na_2SO_4) and concentrated. The crude product was purified by silica chromatography, using the elution solvent system dichloromethane:methanol (9:1, v:v). The product was characterized by positive FAB mass spectrometry which showed *m/z* 420 $(M+H^+)$, *m/z* 442 $(M+Na^+)$, *m/z* 458 $(M+K^+)$.

The product was further purified by chromatography on ion exchange resin (Dowex 50WX2-100). The purified material, yield 425 mg, 43 %, was characterized by NMR. It was found to be 95 % pure, Rf (dichloromethane: methanol, 9:1, v:v) on DC-Alufolien Kieselgel 60^{F254}, 0.25. FAB (positive) *m/z* 420 (MH^+).

The product was used for the preparation of the pentapeptide Leu-His-SPC-Asp-Lys.

Globin isolation and hydrolysis

Globin was isolated from erythrocytes by a procedure involving freezing and/or dilution with water, centrifugation at 20 000 *g*, dialysis of the supernatant, centrifugation and precipitation of globin from the supernatant by the addition of 1% HCl in acetone (Tavares *et al*. 1996). The precipitate was washed successively with 1% HCl in acetone, acetone and diethyl ether and then dried. Aliquots were hydrolysed by adding 3 ml of 6 M HCl to 10 or 50 mg of protein, or by adding 1 ml of 6 M HCl to 2.5 mg of protein and transferring the dissolved solid to hydrolysis tubes. After purging with dry nitrogen and evacuation, the tubes were sealed and heated at 110 °C for 24 h. The hydrolysates were brought to dryness on an evacuated centrifuge, reconstituted in water (2 ml) and re-evaporated to dryness.

Extraction, derivatization and mass spectral analysis

The hydrolysate was applied to a C_{18} SepPak cartridge column in water (2 ml) and the SPC then eluted with 30% methanol/water (4.5 ml). The eluate was dried and the SPC further purified by HPLC. For hydrolysates from 10 and 50 mg samples of globin a semi-preparative separation was first carried out. The SPC fraction was collected, dried and then subjected to analytical HPLC (see Instrumental Analysis for instrumental details). The solvent systems for both analyses were the same:

Solvent A: 10 mM ammonium acetate buffer, pH 4.10
Solvent B: Methanol: 100 mM ammonium acetate buffer pH 4.10 (9:1)

The solvent gradient systems were: 86% solvent A, decreasing to 20% at 5% per minute, over 13.2 min. Run duration 30 min. For the semi-preparative separation SPC was eluted at 12–13 min, and for the analytical separation at 9.5–11.3 min.

For hydrolysates of 2.5 mg samples of globin only the analytical HPLC was used.

The dried HPLC fraction from the analytical column was treated with MTBSTFA (20 µl) and acetonitrile (60 µl), sonicated for 30 min and heated to 65 °C for 45 min. The resulting $[^2H_0]$- and $[^2H_5]$-derivatized SPC was subjected to GC–MS (EI^+) selected ion recording (SIR) (*m/z* 368 and 373 $(M–C_4H_9)^+$ for $[^2H_0]$- and $[^2H_5]$-SPC respectively) or to GC–MS/MS (EI^+) multiple reaction monitoring (MRM). For the latter the ions *m/z* 368 and 373 dissociated by loss of CO, without the addition of any collision gas, and the fragmentations monitored were therefore (368 → 340) and (373 → 345) respectively. The injection volume was 1 µl from the 80 µl derivatization mixture. Chromatography was performed on a capillary column (30 m × 0.25 mm i.d., film thickness 25 µm, coated with PTE-5). GC–MS/MS was carried out with no added gas in the collision cell at a collision energy of *ca* 10 eV. The injector port temperature and source temperature were 270 °C and 250 °C respectively. The initial column temperature was 80 °C for 1 min followed by a 10 °C min^{-1} temperature gradient to 280 °C.

Quantitation

Quantitation of SPC in the hydrolysed globin was carried out by reference to calibration curves determined from the analysis of 2.5, 10 or 50 mg samples of control human globin (the amount being the same as the aliquot size of globin used for the analytical samples). The internal standard $[^2H_5]$-SPC (2–5 ng) was added to the human globin together with varying amounts of $[^2H_0]$-SPC or the pentapeptide Leu-His-SPC-Asp-Lys, containing between 0 and 1.2 ng SPC.

Human sample collection

Human blood samples were collected during two European Union collaborative studies. Occupationally-exposed and control samples were collected in Estonia (acknowledgements to K. Peltonen and T. Veidebaum), and two populations with different exposure to environmental pollution were sampled in Greece (acknowledgements to S. Kyrtopoulos). Full details of these populations will be described in a further publication, but in brief, the Estonian subjects consisted of workers from an industrial shale factory in Kohtla-Jarve (14 males and 4 females, 14 smokers and 4 non-smokers) and from a control population (6 males and 4 females, 3 smokers and 7 non-smokers). Benzene blood levels were in the range 18–1688 nmol l^{-1} (median 73 nmol l^{-1}) in the exposed group and 3-7 nmol l^{-1} (median 4 nmol l^{-1}) in the control group. The Greek samples were taken from non-smoking volunteers living in different areas of Athens and near the town of Halkida. No benzene exposure measurements were taken for the Greek population. Erythrocytes were collected by centrifugation, washed and stored at –80 °C prior to globin analysis.

Results

The synthetic procedure used for $[^2H_0]$-SPC and $[^2H_5]$-SPC (**1a, 1b**) was derived from that of Buckberry (1990). Initial experiments for the GC–MS quantitation used calibration lines derived from mixtures of $[^2H_0]$- and $[^2H_5]$-SPC (0–0.2 ng and 2 ng respectively) together with control globin (2.5 mg). Subsequently, the pentapeptide Leu-His-SPC-Asp-Lys, which mimics the cysteine β-93 sequence in human globin, was used as the unlabelled component in the calibration line mixtures, together with 5 ng $[^2H_5]$-SPC and 50 mg of control globin.

The detection procedure for $[^2H_0]$- and $[^2H_5]$- SPC was GC–MS selected ion recording (SIR) of the bis-*tert*-butyldimethylsilyl (bis-*t*-BDMS) derivatives. The full spectra of a mixture of these two derivatives are shown in figure 4. These derivatives exhibited excellent GC properties and yielded strong $[M–C_4H_9]^+$ ions at *m/z* 368 and 373 for $[^2H_0]$-SPC and $[^2H_5]$-SPC respectively.

SPC was first isolated from globin by acidic hydrolysis followed by reverse phase cartridge column chromatography. GC–MS SIR of the product isolated from globin using this single purification step was not successful owing to unknown contaminants of the same nominal mass as SPC. In order to try to overcome this problem, initial experiments involved the incorporation of the technique GC–MS/MS. However, this technique, which is normally very selective, did not

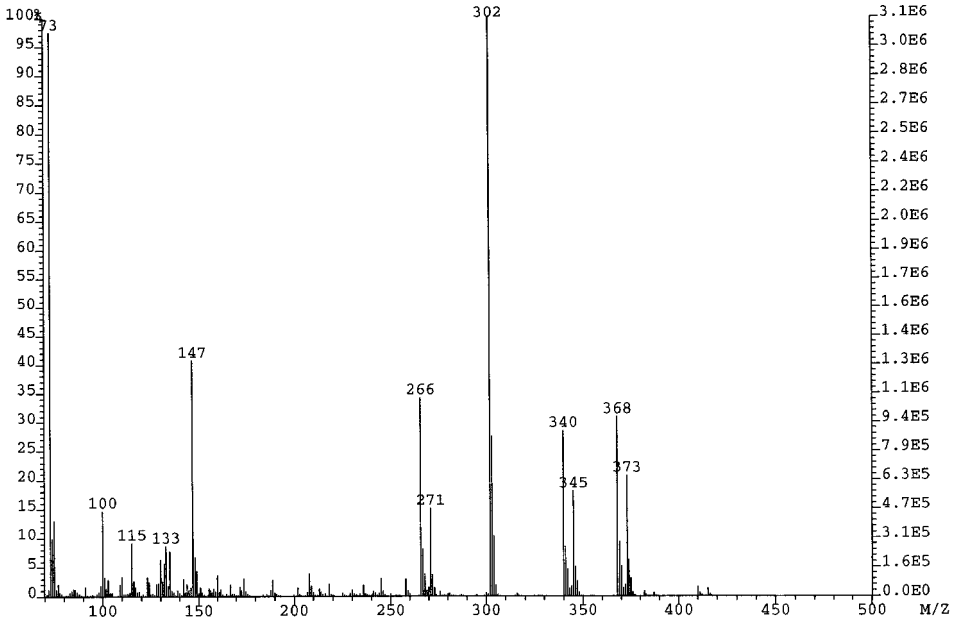

Figure 4. EI mass spectra of the bis-t-butyldimethylsilyl dervative of a mixture of $[^2H_0]$-*S*-phenylcysteine and $[^2H_5]$-*S*-phenylcysteine.

always yield contamination-free MRM traces, and on occasions gave irreproducible results. Subsequent experiments therefore involved the addition of an analytical HPLC stage, followed by GC–MS SIR detection. By using this HPLC–GC–MS SIR method, satisfactory analyses resulted. The appropriate retention time for SPC on HPLC was elucidated using UV-detection following injection of the standard. Considerable attempts were made to maximize the yields of SPC through these procedures, using 2.5 mg aliquots of globin. The recovery of the standard compound SPC from cartridge chromatography was 71.1% ± SD 6.3 %. However in the presence of hydrolysed globin the recovery was higher, being 83.8 % ± SD 1.5 % for the combination of the cartridge chromatography and the analytical HPLC purification stages. The recovery of SPC from a mixture with globin for acidic hydrolysis, cartridge chromatography and analytical HPLC was found to be 54.2 % ± SD 11.8 %.

Initial analysis of human samples was carried out using 2.5 mg aliquots of globin and only analytical HPLC separation of the SepPak eluent. Calibration lines were established for quantitating SPC in globin. The sensitivity and accuracy of this analytical method is shown in figure 5, which depicts an example of such a calibration line for which 2 ng $[^2H_5]$-SPC was used as internal standard to quantitate $[^2H_0]$-SPC in the range 0–0.15 ng. Subsequently the pentapeptide calibration standard Leu-His-SPC-Asp-Lys was used in place of $[^2H_0]$-SPC in order to reflect more faithfully the analysis of SPC in globin, and similar linear calibration lines were obtained. This showed that the hydrolysis stage was occurring effectively. The use of the pentapeptide as unlabelled standard represents an improvement over the use of $[^2H_0]$-SPC as it provided a safeguard against poor hydrolysis yields. The reproducibility of the analysis was evaluated using 6 replicate analyses of SPC in a human globin sample (2.5 mg). The value

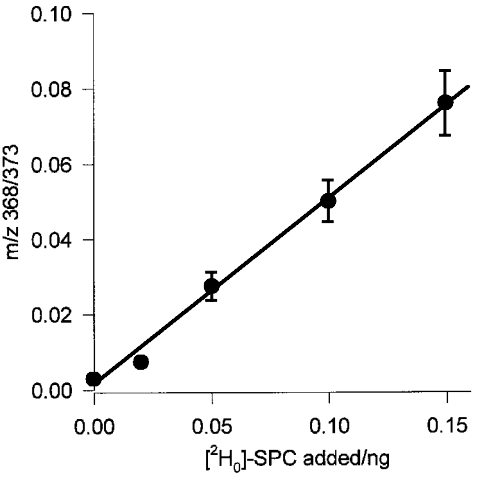

Figure 5. Calibration line for the analysis of *S*-phenylcysteine. Samples contained 2.5 mg control globin, 2 ng [$^2H_5$]-*S*-phenylcysteine and a range of amounts of *S*-phenylcysteine (0–0.15 ng).

obtained was 44.41 ± SD 5.79 pmol SPC g^{-1} globin ($n = 6$).

Preliminary analyses of 18 occupationally-exposed Estonian workers and 10 controls were carried out using 2.5 mg globin samples, and revealed a large interindividual variation in the results of the exposed workers. In a group of benzene plant workers ($n = 10$) the adduct levels were in the range 38.37–124.35 pmol SPC g^{-1} globin (mean 55.62 ± SD 25.81 pmol SPC g^{-1} globin, median 45.57 pmol g^{-1} globin) In a group of cokery workers ($n = 8$) the range was 22.47–101.51 pmol SPC g^{-1} globin (mean 40.15 ± SD 28.22 pmol SPC g^{-1} globin, median 26.85 pmol g^{-1} globin). SPC was also detected in the control population ($n = 10$) with an overlapping range of values 14.75–43.83 pmol SPC g^{-1} globin (mean 29.00 ± SD 10.33 pmol SPC g^{-1} globin, median 27.85 pmol g^{-1} globin). Only the workers from the benzene plant had a statistically higher level of adducts than the control group ($p < 0.05$). Although on most occasions contamination-free GC–MS traces were obtained (e.g. figure 6(a) for an occupationally-exposed Estonian subject), some extraneous compounds of similar GC retention time to SPC sometimes distorted the SIR peak shape. For this reason, and also to improve the signal:noise ratio, subsequent analyses were carried out using 50 mg samples of globin using both a semi-preparative and also an analytical HPLC separation for the purification of SPC. Contamination-free traces then resulted, an example of which is shown in figure 6(b) for the globin from one of the Greek subjects. Satisfactory analytical traces were also achieved with the use of 10 mg aliquots of globin and GC–MS carried out on the VG Auto Spec Ultima Q mass spectrometer. The mean values for SPC levels from 22 members of the Greek population, analysed using 10 or 50 mg samples of globin, was 35.8 ± SD 26.5 pmol SPC g^{-1} globin (median 23.8 pmol g^{-1} globin, range 7.7–114.2 pmol SPC g^{-1} globin) .

Discussion

We have developed an EI GC–MS assay for SPC in haemoglobin which has a sensitivity of less than 20 pmol SPC g^{-1} human globin. This is sufficient to detect 'background' levels of SPC in human samples, and is of considerably greater

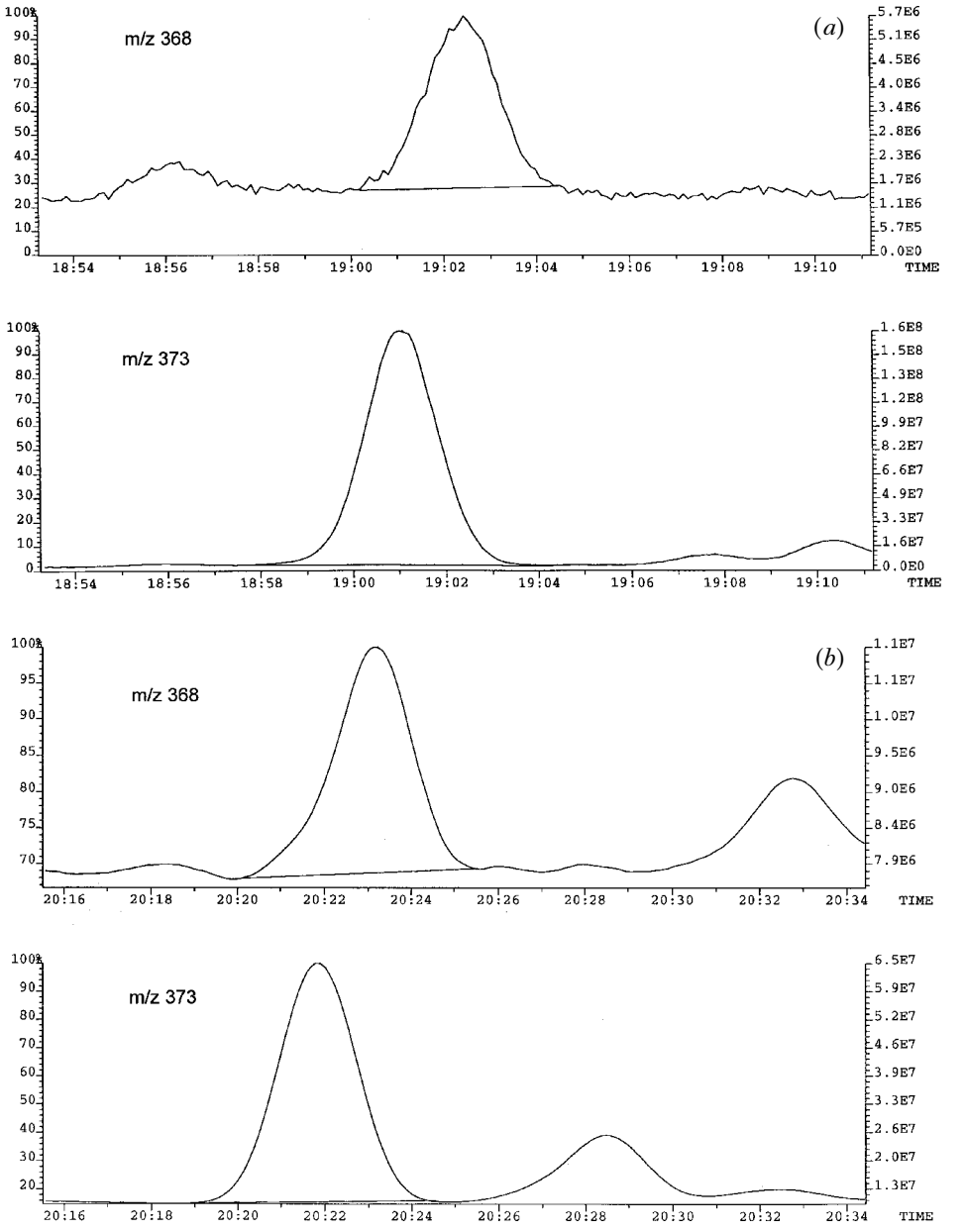

Figure 6. GC–MS selected ion recording traces for the analysis of *S*-phenylcysteine in (a) globin (2.5 mg) from an Estonian subject occupationally-exposed to benzene (b) globin (50 mg) from a non-occupationally exposed Greek subject.

sensitivity than the method of Bechtold *et al.* (1992), who reported a detection limit of *ca* 500 pmol SPC g^{-1} protein using analytical standards, and who were unable to detect SPC in the globin of workers exposed to benzene concentrations as high as *ca* 28 ppm for 8 h per day, 5 days per week. The method appears comparable in sensitivity to that of Yeowell-O'Connell *et al.* (1996a), but in contrast in our

method (a) the adduct itself is monitored, rather than a chemical breakdown product of it; (b) the calibration standard is an adducted peptide (a very close analogue of the adducted protein being analysed); (c) EI is used rather than CI for mass spectral determinations. Despite these changes and possible advantages, our method is more time-consuming than that of Yeowell-O'Connell *et al*. (1996a). One way by which our sample preparation time could in future be shortened would be to employ an immunoaffinity separation using an anti-SPC antibody.

Of particular interest from our use of this method for the determination of SPC in human globin samples was the detection of background levels of this adduct (*ca* 30 pmol SPC g^{-1} globin) in 'control' populations. This has also been observed by Yeowell-O'Connell *et al*. (1998), who detected a median adduct level of 32.0 pmol SPC g^{-1} globin in a control Chinese population ($n = 42$). The origin of this background is unknown and clearly its presence limits the ability of the method to detect occupational exposure to benzene. Relatively small increases in SPC levels were consequently also seen by Yeowell-O'Connell *et al*. (1998) for benzene-exposed workers, whose adduct levels had median values of 46.7 and 129 pmol g^{-1} globin for exposure to < 31 ppm and > 31 ppm respectively. Our haemoglobin samples were dialysed prior to precipitation of globin, which should remove low molecular weight contaminants. Also, our SPC samples were chromatographed extensively during our work up procedure and detected by capillary GC–MS–SIR (or GC–MS/MS MRM), thereby removing the likelihood of contamination. However, it is still conceivable, though very unlikely, that a contaminant contributes to our background SPC levels.

We strongly suspect that the 'background' peak in control population truly represents SPC. Possibilities for the source of this are: unexpectedly high exposures to benzene in these populations (unlikely because of the magnitude of such an exposure would need to be much above normal recognized levels), endogenous production of benzene, biosynthetic incorporation of naturally occurring SPC into the haemoglobin, or an analytical artifact which chemically produces SPC during the protein work-up. A further possible explanation is that the SPC is produced via interaction of cysteine with compounds other than benzene, for example from a substituted (e.g. halogenated) benzene by an addition–elimination mechanism with the cysteinyl sulphur as the nucleophile. These possibilities are currently under active investigation in our laboratory.

Acknowledgements

The authors acknowledge financial support from the EC PECO programme CIPA-CT92-3016 the EC Environment Programme ENV4-CT96-0203 and the EC Biomed 2 programme BMH4-CT98-3785. They also acknowledge Prof. T. Veidebaum, Dr K. Peltonen and Prof. S. Kyrtopoulos for provision of human samples.

References

Ball, L., Wright, A. S., van Sittert, N. J. and Aston, P. 1997, Immunoenrichment of urinary S-phenylmercapturic acid. *Biomarkers*, **2**, 29–33.

Bechtold, W. E. and Henderson, R. F. 1993, Biomarkers of human exposure to benzene. *Journal of Toxicology and Environmental Health*, **40**, 377–386.

Bechtold W. E. and Strunk, M. R. 1996, *S*-Phenylcysteine in albumin as a benzene biomarker. *Environmental Health Perspectives*, **104**, suppl. 6, 1147–1149.

Bechtold, W. E., Lucier, G., Birnbaum, L. S., Yin, S. N., Li, G. L. and Henderson, R. F. 1991, Muconic acid determinations in urine as a biological exposure index for workers occupationally exposed to benzene. *American Industrial Hygiene Association Journal*, **52**, 473–478.

Bechtold, W. E., Sun, J. D., Birnbaum, L. S., Yin, S. N., Li, G. L., Kasicki, S., Lucier, G. and Henderson, R.F. 1992a, *S*-Phenylcysteine formation in hemoglobin as a biological exposure index to benzene. *Archives of Toxicology*, **66**, 303–309.

Bechtold, W. E., Willis, J. K., Sun, J.-D., Griffith, W. C. and Reddy, T. V. 1992b, Biological markers of exposure to benzene: *S*-phenylcysteine in albumin. *Carcinogenesis*, **13**, 1217–1220.

Boogaard, P. J. and Van Sittert, N. J. 1995, Biological monitoring of exposure to benzene: a comparison between *S*-phenylmercapturic acid, *trans,trans*-muconic acid and phenol. *Occupational and Environmental Medicine*, **52**, 611–620.

Buckberry, L. D. 1990, The biochemistry of mammalian C-S lyases. PhD Thesis, University of Nottingham.

Inoue, O., Seiji, K., Nakatsuha, H., Watanabe, T., Yin, S. N., Li, G-L., Cai, S. X., Jin, C. and Ikeda, M. 1989, Urinary *t,t*-muconic acid as an indicator of exposure to benzene. *British Journal of Industrial Medicine*, **46**, 122–127.

Lindstrom, A. B., Yeowell-O'Connell, K., Waidyanatha, S., Golding, B. T., Tonero-Velez, R. and Rappaport, S. M. 1997, Measurement of benzene oxide in the blood of rats following administration of benzene. *Carcinogenesis*, **18**, 1637–1641.

Lindstrom, A. B., Yeowell-O'Connell, K., Waidyanatha, S., McDonald, T. A., Golding, B. T. and Rappaport, S. M. 1998, Formation of hemoglobin and albumin adducts of benzene oxide in mouse, rat and human blood. *Chemical Research in Toxicolology*, **11**, 302–310.

Lovern, M. R., Turner, M. J., Meyer, M., Kedderis, G. L., Bechtold, W. E. and Schlosser, P. M. 1997, Identification of benzene oxide as a product of benzene metabolism by mouse, rat and human liver microsomes. *Carcinogenesis*, **18**, 1695–1700.

Melikian, A. A., Prahald, A. K. and Coleman, S. 1992, Isolation and characterization of two benzene-derived hemoglobin adducts *in vivo* in rats. *Cancer Epidemiology, Biomarkers and Prevention*, **1**, 307–313.

Melikian, A. A., O'Connor, R., Prahalad, A. K., Hu, P., Li, H., Kagan, M. and Thompson, S. 1999, Determination of the urinary benzene metabolites *S*-phenylmercapturic acid and *trans,trans*-muconic acid by liquid chromatography-tandem mass spectrometry. *Carcinogenesis*, **20**, 719–726.

Parke, D. V. and Williams, R. T. 1951, Studies in detoxication. 38. The metabolism of benzene. (a) The determination of phenylmercapturic acid in urine. (b) Mercapturic acid excretion by rabbits receiving benzene. *Biochemical Journal*, **48**, 624–629.

Perbellini, L., Faccini, G. B., Pasini, F., Cazzoli, F., Pistoia, S., Rosellini, R., Valsecchi, M. and Brugnone, F. 1998, Environmental and occupational exposure to benzene by analysis of breath and blood. *British Journal of Industrial Medicine*, **45**, 345–352.

Snyder, R. and Hedli, L. L. 1996, An overview of benzene metabolism. *Environmental Health Perspectives*, **104**, suppl. 6, 1165–1171.

Snyder, R., Witz, G. and Goldstein, B. D. 1993, The toxicology of benzene. *Environmental Health Perspectives*, **100**, 293–306.

Stommel, P., Muller, G., Stucker, W., Verkoyen, C., Schobel, S. and Norpoth, K. 1989, Determination of *S*-phenylmercapturic acid in the urine, an improvement of the biological monitoring of benzene exposure. *Carcinogenesis*, **10**, 279–282.

Tavares, R., Borba, H., Monteiro, M., Proenca, M. J., Lynce, N., Rueff, J., Bailey, E., Sweetman, G. M. A., Lawrence, R. M. and Farmer, P. B. 1996, Monitoring of exposure to acrylonitrile by determination of *N*-(2-cyanoethyl)valine at the N-terminal position of haemoglobin. *Carcinogenesis*, **17**, 2655–2660.

Van Haaften, A. B. and Sie, S. T. 1965, The measurement of phenol in urine by gas chromatography as a check on benzene exposure. *American Industrial Hygiene Association Journal*, **26**, 52–58.

Wallace, L.A. 1989, Major sources of benzene exposure. *Environmental Health Perspectives*, **82**, 165–169.

Yeowell-O'Connell, K., McDonald, T. A. and Rappaport, S. 1996a, Analysis of hemoglobin adducts of benzene oxide by gas chromatography–mass spectrometry. *Analytical Biochemistry*, **237**, 49–55.

Yeowell-O'Connell, K., Rothman, N., Smith, M. T., Hayes, R. B., Li, G., Bechtold, W., Dosemeci, M., Zhang, L., Yin, S. Y. and Rappaport, S. M. 1996b, Hemoglobin adducts of benzene oxide in workers exposed to high levels of benzene in China. *Proceedings of the American Association for Cancer Research*, **37**, 251.

Yeowell-O'Connell, K., Rothman, N., Smith, M. T., Hayes, R. B., Li, G., Waidyanatha, S., Dosemeci, M., Zhang, L., Yin, S., Titenko-Holland, N. and Rappaport, S. M. 1998, Hemoglobin and albumin adducts of benzene oxide among workers exposed to high levels of benzene. *Carcinogenesis*, **19**, 1565–1571.