Haemoglobin adducts as biomarkers of occupational exposure to 1,3-butadiene

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Adducts of 1,2-epoxy-3-butene (EB) with haemoglobin were monitored in 17 workers from the 1,3-butadiene (BD) production unit of a petrochemical plant and in nine referents employed at the same factory but not exposed to BD. The air concentrations of BD were determined using stationary and personal monitoring. The ambient level of exposure of the plant workers handling butadiene containers (sampling and voiding) was on average 11.2 ± 18.6 (mean ± SD) mg/m³. Maintenance and laboratory workers were exposed to levels ≤1.2 mg/m³. The particular haemoglobin adduct measured was 2-hydroxy-3-butenylvaline, formed by reaction of N-terminal valine with carbon 1 in EB. The adduct levels were increased (0.16 ± 0.099 pmol/g; n = 10) in plant workers compared with the levels in maintenance and laboratory workers and controls (–0.05 pmol/g; seven laboratory workers and nine controls evaluated). Thus, the method used for adduct determination—derivatization of 200–300 mg globin samples with pentafluorophenyl isothiocyanate according to the N-alkyl Edman method and detection of the thiohydantoin derivatives by tandem mass spectrometry—is sufficiently sensitive to allow monitoring of exposure to BD down to the p.p.m. level.

Introduction

The increasing interest in studying the carcinogenic and genotoxic properties of 1,3-butadiene (BD) is justified on the basis of its large-scale use in the petrochemical and rubber industries. The worldwide production of BD exceeds 5.5 million tonnes. Styrene-butadiene rubber and latex, and polybutadiene rubber production are the two largest uses of BD in the petrochemical and rubber industries. Reaction products (adducts) with haemoglobin or serum albumin in blood can be used to detect and quantify exposure to chemicals that are reactive per se or that are transformed in the body to reactive metabolites (see Skipper and Tannenbaum, 1990). The analysis of protein adducts is highly sensitive and specific to the chemical, and allows studies at low exposure levels. Studies of blood protein adducts in humans give considerably improved exposure assessments and provide a useful endpoint in epidemiological studies. The dose response for formation of adducts of EB with N-terminal valine in haemoglobin and the stability of the adducts have been investigated in butadiene-exposed mice and rats (Osterman-Golkar et al., 1991, 1993; Albrecht et al., 1993). The haemoglobin adducts are chemically stable. In agreement with the relative blood concentrations found in mice and rats (Himmelstein et al., 1994), the adduct levels are ~3–8 times higher in the former species (Albrecht et al., 1993; Osterman-Golkar et al., 1993). Because of their chemical stability, EB adducts accumulate during prolonged exposure and reach a steady state level. The steady state level established following prolonged occupational exposure to BD may be used as a proportional measure of the dose received during the 4 months (the life span of erythrocytes in humans) prior to blood sampling (Osterman-Golkar et al., 1976).

The present study of haemoglobin adducts in BD-exposed workers is part of an international collaborative project aimed at an assessment of the cancer risk associated with
environmental and occupational exposures to BD. Chromosome aberrations, micronuclei and sister chromatid exchanges in peripheral lymphocytes were analysed in the same workers (Sorsa et al., 1994). No exposure related effects were seen in any of these cytogenetic endpoints.

Materials and methods

Study design

The study was performed in a Portuguese petrochemical plant where BD is produced. No previous data exist about the exposure levels on the plant, but since the work is mainly outdoors, low exposure levels were expected. A plant worker group (n = 10), a laboratory and maintenance worker group (n = 7) and a control group (n = 10) were chosen. The participants in the study were all volunteers. They were asked to complete a questionnaire which solicited information on the following subjects: sex, age, job title, tobacco usage, medications and exposure to chemicals at their workplace. Blood (and urine) samples were collected from all participants after a work shift.

BD exposure concentrations were determined for each plant worker over at least one full shift. For some of the workers, three shifts were monitored over the course of 3 workdays (in-depth surveys). Area samples were collected to determine the BD concentrations in workplace air in the general process area in the vicinity of the distillation columns.

Air sampling and analytical methods

During the in-depth surveys, both personal and area samples were taken. The samples were collected with SKC 222-3 portable low-flow air sampling pumps on coconut-shell charcoal tubes at a flow rate of 50 ml/min. Front section contained 100 mg and the rear section 50 mg of charcoal respectively. All sections were analysed separately and the rear section was used to quantify the breakthrough. The charcoal tubes were connected to the pumps with plastic tubing. Air sample volumes were limited to a maximum of 25 l. The passive sampling was done using 3M 3500 dosimeters. The diffusion rate for BD is 43 ml/min (as stated by the manufacturer). Altogether 27 charcoal tubes and 46 dosimeters were sampled.

The samples were desorbed in acetonitrile and analysed by means of high resolution gas chromatography using an aluminium oxide-fused silica capillary column and flame-ionization detection. Some samples were used for positive identification of BD using mass spectrometry. A detailed description of the sampling and analysis of BD is given elsewhere (Peltonen, 1995).

In order to ensure the quality of the results, blank samples and quality control spikes were generated, analysed and reported, in accordance with NIOSH quality assurance and quality control procedures (US National Institute for Occupational Safety and Health, 1984). Field samples were refrigerated during shipment and storage and were found to remain stable for at least 2 weeks when kept at ~20°C.

Haemoglobin adduct quantification

Blood sampling and isolation of globin. All blood samples collected were post shift samples. Plasma, ‘buffy coat’ and erythrocytes were separated by centrifugation (1100 g). The erythrocytes were washed twice with PBS buffer and stored at ~20°C. Globin was isolated from the erythrocytes as described by Mowrer et al. (1986).

Preparation of standards. A mixture of 2-hydroxy-3-butenylvaline and 1-hydroxyethylallylvaline was prepared by incubation of 1 mmol 14C-labelled valine (prepared by mixing 1 μCi l-[14C]valine obtained from Amersham, Buckinghamshire, UK, with 1 mmol of l-valine) in 1 ml of 1 M sodium hydroxide with 1.5 mmol EB (purity 98%; Aldrich, Steinheim, Germany). The mixture was left at room temperature for 2 weeks with occasional shaking. A part of the mixture was used to prepare Dowex 50x4 column (35×2 cm) which was eluted with 1 M hydrochloric acid. Fractions of 5 ml were collected. Valine, 2-hydroxy-3-butenylvaline and l-hydroxymethylallylvaline were eluted after 410, 430 and 500 ml respectively. The 2-hydroxy-3-butenylvaline was re-chromatographed on the Dowex column to remove traces of valine and 1-hydroxyethylallylvaline. The pentafluorophenylthiohydantoin (PFPTH) derivative of 2-hydroxy-3-butenylvaline was prepared according to Tömquist et al. (1986) and dissolved in toluene. The concentration was determined by means of radioactivity measurements. The purity of the derivative was checked by HPLC. The structural assignment was based on the mass fragmentation pattern of the PFPTH derivative (see Osterman-Golkar et al., 1991). A tripeptide (ValGlyGly ethyl ester) containing radiolabelled valine was prepared from valine (7 μCi/mmol) and glycylglycine ethyl ester. A 1 mmol aliquot of l-valine (Sigma, St Louis, MO) was dissolved in 5 ml of water. L-[3,4-14C]Valine (Du Pont NEN, Boston, MA) was added to give a sp. act. of 7 μCi/mmol and the mixture was evaporated to dryness. FMOC (9-fluorenylmethyloxycarbonylformate; Fluka Chemie AG, Buchs, Switzerland) was added to 0.2 mmol of the radiolabelled valine according to Meinenhofer et al. (1979). The crude product was dissolved in dichloromethane and recovered by addition of hexane. The valine–FMOC was then coupled to glycylglycine ethyl ester hydrochloride (Sigma) by using EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; Bio-Rad, Richmond, CA) according to Sheehan et al. (1965). The valine–FMOC was dissolved in 1 ml dichloromethane, and 0.2 mmol glycylglycine ethyl ester hydrochloride, 0.2 mmol triethylamine (Merck, Darmstadt, Germany) and finally 0.22 mmol EDAC was added. The suspension was left to react for 24 h and was then washed successively with water, 0.1 M hydrochloric acid, water, 0.1 M sodium bicarbonate and water. The suspension was evaporated and then kept at ~20°C. Uncoupling of FMOC was carried out by suspending the reaction product in dichloromethane containing 20% piperidine (Merck). After 5 min the product was evaporated and dissolved in water and extracted three times with diethyl ether.

The tripeptide, 0.1 mmol dissolved in water, was reacted with EB (0.2 mmol). After 16 h at room temperature the reaction mixture was extracted several times with toluene to remove excess EB. The adduct content was determined by derivatization as described for globin samples (below) and determination of the radioactivity of the PFPTH derivative.

Derivatization for MS/MS. Globin samples (200–300 mg) from the workers were dissolved in 6 ml of formamide and internal standard globin (3 mg) was added. The internal standard contained 8.75 pmol [14C]-hydroxypropylvaline/mg globin according to radioactivity determinations (Segerback et al., 1992). The normal background level of hydroxypropylvaline, ~2 pmol/g globin, and the contribution from smokers (0.03 pmol/g of 14C-label per 10 cigarettes/day) are low compared with the added amount and were therefore ignored. The samples were derivatized with pentafluorophenyl isothiocyanate (PFPTH; 10 μl/50 mg globin) according to Tömquist et al. (1988) and the PFPTH derivatives of the valine adducts were extracted into diethyl ether. The ether was then evaporated and the samples were dissolved in toluene, purified, evaporated to dryness and redissolved in 50 μl of toluene for analysis. Seven samples were reanalysed using as the internal standard a globin containing (14H2)hydroxyethylvaline (5 pmol/sample; cf. Farmer et al. (1986)).

MS/MS analyses. The MS/MS analyses were carried out using a Finnigan TSQ-700 instrument in the negative ion chemical ionization mode. The technical details were as follows: column, RESTEK XT-5 (30 m, 0.32 i.d., 1.0 μm df); retention gap, methyl deactivated silica (CHROMPACK; 2.5 m, 0.53 mm i.d.;) injector, VARIAN 1093 SI (septum-equipped programmed); carrier gas, helium, 8 p.s.i.; reagent gas, argon; collision cell pressure, 1 mtorr; collision energy, 4 eV; injector temperature programme, 180°C/min from 60 to 320°C, 19 min at 320°C; GC temperature programme, 1 min at 100°C, 20°C/min to 240°C, 10°C/min to 320°C, 7 min at 320°C. Two-microliter samples, in toluene, were injected on column. The retention time of the internal standard was ~12 min; the retention times of the two peaks of the PFPTH derivatives of the analyte were 10 and 17 s, respectively, longer. The quantifications were based on measurement of peak areas for the fragment m/z 318, a daughter fragment both of m/z 362 of the analyte and of m/z 318, a daughter fragment both of m/z 374 of the analyte and of m/z 374 of the internal standard. The linearity of response was studied by adding various amounts (range 0.01–5 pmol) of the alkylated tripeptide and the internal standard to 200 mg samples of control globin. The peak area of the analyte increased linearly with tripeptide concentration. Assuming that the proportions of adducts with carbons 1 and 2 in EB are equal in the tripeptide and in the human samples, total adduct levels in the human samples may be calculated. In this study we used the response factors of 2-hydroxy-3-butenylvaline–PFPTH for quantification of adducts at carbon 1 of EB. The PFPTH derivative was added to the standard globins containing hydroxypropylvaline- or [14H2]hydroxyethylvaline, and the adducts were determined under the above-mentioned conditions. Thus, the quantifications of adduct levels in samples of the workers are based on the assumption that the derivatization yield of 2-hydroxy-3-butenylvaline–PFPTH is 100%. However, derivatization of similar haemoglobin adducts (hydroxypropylvaline, hydroxyphenethylvaline) under similar reaction conditions show yields of ~80–90%, suggesting that this assumption is reasonable.

Results and discussion

Two different air sampling methods were used in this study. One is based on an active sampling of BD from air utilizing a personal pump and a commercial SKC-221 charcoal tube. The sections of the tube were analysed separately and no breakthrough was detected. The other sample collection tech-
nique takes advantage of passive diffusion of BD from air into a charcoal bed (commercial 3M monitors). Both sample types were analysed in the same manner. The charcoal tubes were used to check the passive monitors. The results of the two different sample collection methods showed a good correlation (Figure 1).

A total of 46 full shift samples (27 personal and 19 area samples) were collected during one survey of the monomer industry (comprising four different sampling days). The BD concentrations detected are presented in Table I. A review of personal exposures by job title suggested that those jobs requiring workers to handle containers of BD present the greatest potential exposure. All the area samples were taken at the location of the plant perimeter. The area samples showed clearly elevated BD concentrations, which were higher than the exposure levels recorded for laboratory workers. This seems to be logical since the laboratory was located ~100-1000 m from emission sources of BD. Laboratory-related emission sources of BD were not detected.

Blood samples from 26 of the 27 subjects were analysed for 2-hydroxy-3-butenylvaline, the product of reaction between carbon 1 of EB and the N-terminal valine in haemoglobin. Analyses of 1-hydroxymethylallylvaline, the product of reaction of carbon 2 of EB, were not attempted. Figure 2a and b shows typical MS/MS ion chromatograms of samples from a control person and a plant worker respectively. There were peaks at the retention times for the EB adducts also in samples at the location of the plant perimeter. The area samples showed different sample collection methods showed a good correlation (R = 0.985). The limit of detection is ~0.03–0.05 pmol/g globin. The adduct levels recorded in laboratory and maintenance workers (mean 0.05 pmol/g globin) were not increased above the background found in controls (mean 0.06 pmol/g globin). Increased adduct levels, on average 0.16 ± 0.099 (mean ± SD) pmol/g globin, were recorded in subjects exposed to BD in the BD production areas (Figures 2b and 3). The highest values were found among the non-smokers.

Mainstream smoke contains ~0.06 mg BD/cigarette (range 0.016–0.075 mg BD/cigarette; Brunnemann et al., 1990). The amount of BD in the mainstream smoke of 30 cigarettes, ~1.8 mg, corresponds to the amount (~1.6 mg) which would be inhaled during 8 h of work at an air concentration of ~0.1 p.p.m. BD, assuming an alveolar ventilation rate of 0.21 kg⁻¹ min⁻¹ and a body wt of 70 kg. The smokers reported a cigarette consumption of between 8 and 20 cigarettes/day (smoking is not allowed in the production area or in the laboratory of the factory). Accordingly, the contribution from smoking to the adduct level in haemoglobin of production plant workers is expected to be smaller than the contribution from exposure to BD (median exposure level ~1 p.p.m.; see Table 1). The adduct levels in smoking and non-smoking laboratory and maintenance workers were similar (~0.05 pmol/g). Among the control subjects, smokers showed somewhat higher values than non-smokers.

Two studies on EB–haemoglobin adducts in occupationally exposed humans have been published previously (Osterman-Golkar et al., 1993; van Sittert and van Vliet, 1994). Osterman-Golkar et al. (1993) reported preliminary data on haemoglobin adduct levels in nine workers at a US chemical production plant and a few outside controls. The workers, all non-smokers, were divided into two groups on the basis of work location. Increased adduct levels [1.1–2.6 pmol/g globin; N-(2-hydroxy-3-butenylvaline + N-(1-hydroxymethylallyl)valine] were found in four workers from BD production areas. Exposures to BD were estimated from an environmental sampling survey which was conducted between 3 and 9 months prior to the collection of blood samples. Time-weighted average (TWA) 8 h area samples showed a mean value of ~3.5 p.p.m., although most samples contained <1 p.p.m. Most of the samples with higher levels were collected in areas not frequented by workers, suggesting that the average exposure of workers to BD was <3.5 p.p.m. As control groups, five workers from two non-production areas—the power plant and the central control area—and two external controls were evaluated. An environmental sampling survey indicated that exposure to BD in the central control area was ~0.03 p.p.m. No data were available on the power plant. Adduct levels in control samples were below the detection limit of 0.5 pmol/g globin. The same paper reports on adduct levels in two heavy cigarette smokers.

Figure 1. The correlation of the two different sampling methods of BD. The number of the samples was 20, correlation coefficient 0.985, SD 0.975.

<table>
<thead>
<tr>
<th>Job</th>
<th>BD concentrations [mg/m³]a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples</td>
</tr>
<tr>
<td>Bomb voiding and sampling</td>
<td>19</td>
</tr>
<tr>
<td>Maintenance and laboratory work</td>
<td>8</td>
</tr>
<tr>
<td>Area samples</td>
<td>19</td>
</tr>
</tbody>
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*1 mg/m³ = 0.445 p.p.m.
Fig. 2. Ion chromatograms for adducts of EB [N-(2-hydroxy-3-butenyl)valine] in globin from (a) a control subject and (b) a BD-exposed worker. The peak areas correspond to levels <0.1 pmol/g globin in the control and 0.15 pmol/g globin in the exposed subject respectively.

Fig. 3. Haemoglobin adduct levels [N-(2-hydroxy-3-butenyl)valine] in 17 workers at a BD production unit of a petrochemical plant and in nine referents employed at the same factory but supposedly not exposed to BD.
state level corresponds to exposure for 9 weeks, i.e., \( -9 \times 5 \times 8 \) (= 360) h in the case of occupational exposure and \( 9 \times 7 \times 24 \) (= 1512) h if the compound is a ubiquitous contaminant in the environment. Studies in Sweden on urban air pollution show an average level of 0.3 p.p.b. of BD (Boström et al., 1992). Exposure to this air level would give 0.45 p.p.m. of BD during 9 weeks. Exposure to 1 p.p.m. (median air concentration for the plant workers) would give 3 p.p.m. Thus, the adduct levels recorded for controls are higher than would be expected based on the adduct levels observed in these two worker categories. The origin of this background remains to be clarified. It is possible that the low levels of adducts detected in controls can be associated with occasional visits to plant areas with elevated BD concentrations.

If compared at equal BD doses (p.p.m.), the EB adduct levels observed in BD-exposed workers are considerably lower than those in rats and mice (Osterman-Golkar et al., 1993).

The spread of values within the groups of workers may reflect different exposures, as well as interindividual differences in activation of BD or inactivation of EB.

In conclusion, the method used for adduct determination—derivatization of 200–300 mg globin samples with PFPITC according to the N-alkyl Edman method and detection of the thiohydantoin derivatives by tandem mass spectrometry—is sufficiently sensitive to allow monitoring of exposure to BD at the p.p.m. level or higher. Further studies are required to determine the true background levels and to evaluate the impact of smoking, both as a source of exposure to BD and its possible influence on BD metabolism. Although indicative of an overall low efficiency of BD in humans, a risk estimation of the true background levels and to evaluate the background remains to be clarified. It is possible that the low levels of adducts detected in controls can be associated with occasional visits to plant areas with elevated BD concentrations.

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