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Review

Use of haemoglobin adducts in exposure monitoring and risk assessment

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Abstract

Many industrial bulk chemicals are oxiranes or alkenes that are easily metabolised to oxiranes in mammalian systems. Many oxiranes may react with DNA and are therefore mutagenic in vitro. Some oxiranes have been shown to be carcinogenic in rodents in vivo as well. Despite the very limited evidence of the carcinogenicity of oxiranes in humans, they should be considered potential human carcinogens. As a consequence, exposure to these compounds should be minimised and controlled. Twenty-five years ago, Ehrenberg and co-workers suggested that exposure to oxiranes might be determined through the measurement of the adducts they form with haemoglobin (Hb). Ten years later, a modification of the Edman degradation was developed at Stockholm University that allowed determination of adducts with the N-terminal valine of Hb by GC–MS. In our laboratory, this methodology was modified and adapted for analysis on an industrial scale. Since 1987, exposure of operators in our facilities to ethylene oxide (EO) has been routinely monitored by determination of *N*-(2-hydroxyethyl)valine in Hb. Biological monitoring programmes for propylene oxide (PO) and 1,3-butadiene (BD) were developed later. In this review, the methodology and its results are discussed as a tool in human risk assessment of industrial chemicals. Two major advantages of Hb adduct determinations in risk assessment are (1) the qualitative information on the structure of reactive intermediates that may be obtained through the mass spectrometry, which may provide insight in the molecular toxicology of compounds such as BD, and (2) the possibility of reliable determination of exposure over periods of several months with limited number of samples for compounds such as ethylene oxide (EO), propylene oxide (PO) and BD which form stable adducts with Hb. Since good correlations between the airborne concentrations of these chemicals with their respective adducts have been established, Hb adducts can also be used to quantitate airborne exposure which is of paramount importance as exposure assessment is usually one of the weaker parameters in risk assessment.

Keywords: Exposure monitoring; Risk assessment; Haemoglobin adducts

Contents

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1. Introduction

Lower olefins, such as ethylene (ethene, CAS no. 74-85-1), propylene (propene, CAS no. 115-07-1) and 1,3-butadiene (BD) (CAS no. 106-99-0), are important industrial bulk chemicals. Ethylene is mainly used for the production of polyethylenes, ethylene oxide, vinyl chloride and dichloroethane. Propylene is mainly used to synthesise polypropylene, propylene oxide, cumene, isopropanol and acrylonitrile. BD is mainly used for the production of resins and elastomers, such as butadiene rubbers and styrene–butadiene rubbers. The versatility of these chemicals is, of course, based on the olefinic double bond that allows polymerisation and a wide range of other chemical reactions. In mammalian systems in vivo, however, this double bond may be oxidised yielding direct-alkylating oxirane compounds. In humans as well as rodents, ethylene is oxidised to ethylene oxide (EO) (CAS no. 75-21-8), propylene to propylene oxide (PO) (CAS no. 75-56-9), and BD to butadiene monoxide (BMO, 1,2-epoxy-3-butene) (CAS no. 930-22-3) [1–7]. EO and PO are also major industrial chemicals that are produced mainly for the manufacturing of glycols and glycol ethers. BMO, in contrast, is manufactured as an industrial chemical only on a very limited scale.

EO is a rodent carcinogen [8] and was classified as a human carcinogen by the International Agency for the Research on Cancer (IARC) despite the fact that there is only limited evidence in humans for its carcinogenicity [9]. The American Conference of Governmental Industrial Hygienists (ACGIH) classified EO as a suspect human carcinogen [10]. Recent studies on DNA adducts and mutagenic potential indicated that, although EO has a clear carcinogenic and genotoxic potential, its potency is so low that exposure to 1 ppm (1.8 mg m^{-3}) EO would not significantly contribute to human cancer risk [11,12]. PO is also a rodent carcinogen [13]. Several authoritative bodies have evaluated the genotoxicity of PO and concluded that there is no [14] or insufficient [15] evidence for the carcinogenic effects of PO in humans. As a consequence, IARC downgraded the carcinogenic classification of PO from a probable (2A) to a possible (2B) human carcinogen [15]. The ACGIH has classified PO as an animal carcinogen (3A) [10]. Despite the similarity of the reaction kinetics of EO and PO [16], the mutagenic potency and genotoxic effects of PO are much lower than for EO [17–19]. Nevertheless, pressures remain to lower the occupational exposure limit (OEL) for PO. Both IARC and ACGIH have evaluated ethylene and propylene as ‘not classifiable as to its carcinogenicity to humans’ based on the absence of epidemiological

evidence of their carcinogenic potential and the inadequate evidence in experimental animals [10,21,22]. BD, finally, is different. Unlike ethylene and propylene, BD was clearly carcinogenic in both mice and rats [23], but whether or not BD is also a human carcinogen is still under debate since epidemiological data are equivocal and seemingly contradictory (for review see Ref. [24]).

As stated, the lower olefins and some of their epoxides are major industrial chemicals. Ethylene is even the most widely used petrochemical in the world, and although produced and handled in smaller quantities, still thousands of operators in the petrochemical industry are potentially exposed to EO, propylene, PO and BD. Since EO, PO and BD are potential human carcinogens, exposure to these compounds should be minimised and controlled by proper exposure measurements. For proper risk assessment estimates of exposure are also imperative. Estimation of exposure by (personal) air monitoring is complicated since the exposure usually occurs intermittently and at low and variable levels. Moreover, air monitoring data are indicators of *potential* exposure and not of the *actual* dose received by the exposed persons. The determination of biomarkers, such as adducts in proteins, may overcome these problems. Since blood is the only human tissue that is readily available for epidemiological and biomarker studies, the proteins albumin and haemoglobin (Hb), which are both abundantly present in blood, have been used to determine adduct levels [25,26]. The benefits of Hb adduct determination as internal dose monitor for occupational exposure were first suggested in 1976 by Ehrenberg and co-workers [27]. Originally, adducts to histidine residues in Hb were determined in blood samples from persons occupationally exposed to EO or PO and to obtain the necessary sensitivity, GC–MS methods were developed [28,29]. However, a more general application of this methodology was hampered by the complicated and tedious methods for sample preparation in combination with the high risk of introducing artefacts. Radio-immunoassays that were developed overcame a number of these problems, but suffered from the inherent use of radioactivity [30]. A major break-through was realised in 1986 by Törnqvist and co-workers with a modification of the Edman-degradation method that made reliable determination of Hb adducts to the N-terminal valine by GC–MS feasible [31,32]. This work was sponsored by Shell, and, as a consequence, our laboratory got access to this methodology at an early stage. We adapted the method to make it suitable for routine monitoring on an industrial scale, and, from 1987 onward, biomonitoring of exposure to EO has been a routine operation in our work force.

One of the advantages of the determination of adducts with the N-terminal valine of Hb of small molecules such as EO, PO and BMO, is that these adducts are chemically stable. EO forms a single adduct at the N-terminal valine: *N*-(2-hydroxyethyl)valine (HOEtVal). Racemic PO forms *N*-(3-hydroxypropyl)valine (HOPrVal), which exists as two diastereomers. Racemic BMO forms two pairs of diastereomeric N-terminal valine adducts (HOButVal): *N*-(2-hydroxy-3-butenyl)valine and *N*-(1-(hydroxymethyl)-2-propenyl)valine, resulting from attack on the C_1 and C_2 of BMO, respectively. In contrast to most other proteins, such as serum albumin, which are subject to turnover with molecules being removed with apparent first-order kinetics, Hb disappears from the circulation with apparent zero-order kinetics [33–36]. These apparent zero-order kinetics are explained by the stability of Hb in the erythrocyte, which is not affected by the adducts of EO, PO and BMO with its N-terminal valine. Hb in stable in erythrocytes and, as a consequence, its removal is entirely determined by the life-span of the erythrocytes [37,38]. This means that exposure dose as measured by determination of Hb-adduct concentrations is integrated over the average life-span of human erythrocytes, which is approximately 4 months. As Hb adducts provide a measure of the internal dose (defined as the amount of chemical absorbed into an organism [39]) of EO, PO and BMO in experimental animals and humans alike, some of the difficulties arising from the extrapolation from the high doses used in experimental animals to the low doses that are common in human exposure situations as well as from the extrapolation across species may be eliminated by determination of Hb adducts. In addition, Hb adducts may serve as a surrogate monitor for DNA adducts [11,39,40].

In our laboratory, we have established relationships between ambient exposures to EO and PO and

the formation of their respective adducts with Hb by correlation of the Hb adduct data with the corresponding personal air monitoring data in various groups of potentially exposed operators [41,42]. A relationship between airborne BD concentrations and the Hb adducts of its primary metabolite, BMO, has also been established [6]. In this paper the methodology of the analytical procedures for the Hb adduct measurement and its application to human risk assessment in petrochemical industry is reviewed and discussed.

2. Experimental

2.1. Study populations and personal air monitoring

Studies were carried out in a variety of chemical plants and on workers with potential exposure to EO, PO or BD. EO exposure was determined in workers of a plant manufacturing ethylene glycol and ethylene glycol ethers during a maintenance and inspection shut-down. Similarly, PO exposure was determined during a maintenance and inspection shutdown in workers of styrene-PO production plant. BD exposure was measured in workers engaged in loading operations and the manufacturing of BD itself and various of its polymers. Details of these studies have been published elsewhere [6,41–43]. In all studies potential exposure to EO, PO or BD was determined by personal air monitoring and internal exposure was determined by Hb adduct measurements. The sampling strategy, however, was basically different in the studies on EO and PO compared to that on BD. In the studies on EO and PO exposure, a blood sample was first collected from the operators at the very beginning of the study. From that point on personal air monitoring was applied to each operator during the full shift in each shift until the end of the study, which was different for the individual operators and varied from 3 to 18 days later (average 5 days). On the last day of the study, another blood sample was collected. The total airborne exposure to EO or PO for each operator was correlated with the increase in the blood concentration of HOEtVal or HOPrVal, respectively, over the period of the study. In the study on BD exposure, personal air monitoring was applied randomly during 10 full shifts over a period of 2 months. On the last day of the study, a single blood sample was collected for determination of HOButVal. In all studies, air samples were collected using passive gas diffusion monitors (3M, Minneapolis, USA), which were attached to one of the lapels of the workers' overalls, and/or by actively pumping air from the breathing zone over activated charcoal tubes. Air measurements were carried out according to the recommendations made by the manufacturers [44] of the monitors and compliant to generally accepted guidelines [45,46]. Details about exposure times (full shifts) and relevant circumstances were recorded by the plants' occupational hygienists. When completed, the air samples were closed air-tight and transported to the laboratory in a cool-box with ice-packs. Upon arrival the samples were immediately stored frozen at −20°C until analysis. Air samples were analysed by generally accepted methods as described previously [6,41–43,47].

2.2. Biomonitoring

Warning: Collection and analysis of blood are potentially hazardous operations. All sampling and handling of blood and blood-contaminated equipment should be performed very carefully because of the risk of viral contamination (in particular with HIV and hepatitis B). Therefore, all operators should wear disposable gloves and aprons and wear safety glasses and use only disposable materials that should be discarded after use.

2.2.1. Chemicals

2.2.1.1. Standards. Calibration standards and internal standards were prepared by reacting whole blood or erythrocytes with radiolabelled and deuterated epoxides, respectively [6,31,48]. Aliquots of the radiolabelled Hb thus obtained were hydrolysed and analysed. Using the specific activity of the radiolabelled epoxides the amount of adducts to the N-terminal valine was quantified [6,31]. Control globin was obtained from blood of workers with no known exposure to ethylene, EO, propylene, PO or BD.

2.2.1.2. Other chemicals. Dimethylsulfoxide (DMSO) was obtained from BDH (Poole, UK). Pentafluorophenylisothiocyanate (Edman reagent), ammonium hydroxide, concentrated hydrochloric

acid (37%), diethyl ether, ethyl acetate, formamide, methylformamide, *n*-pentane, isopropanol, sodium chloride, sodium carbonate, sodium hydroxide, methanol and toluene were of p.a. grade and purchased from Fluka (Buchs, Switzerland). Octafluoronaphthalene was also of p.a. grade and obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). *N*,*N*-diisopropylethylamine, *N*,*N*,*N*-triethylamine and pentafluorobenzyl bromide were purchased from Pierce (Rockford, USA). Methane, helium and argon were bought from HoekLoos (Schiedam, The Netherlands) in the highest purity available.

2.2.2. Pre-analytical phase

2.2.2.1. Sampling. Blood samples of approximately 5 ml were collected from the antecubital vein into vacutainer tubes containing sodium edetate as anticoagulant using γ-radiation-sterilised syringes and needles. The use of disposables sterilised by γ-irradiation is essential as the traces of EO that are absorbed to the plastic surfaces in EO-sterilised materials will react with the Hb in the blood which lead to systematic erroneous high background concentrations of HOEtVal. The time of blood sampling is not critical.

2.2.2.2. Storage. The blood samples can be stored at 4°C for a maximum of 24 h. Preferably, however, erythrocytes are isolated immediately after blood collection by the procedure described below. The washed erythrocytes may be stored frozen at −80°C for several weeks without decomposition of the N-terminal valine adducts.

2.2.3. Analytical phase

2.2.3.1. Isolation of globin. Erythrocytes were precipitated from the whole blood samples by centrifuging, washed twice with isotonic saline, suspended in deionised water and subjected to four cycles of freezing at −80°C and thawing at 37°C. The lysed cell suspension was centrifuged to remove cell debris (6000 *g*, 90 min, room temperature). The supernatant may be stored frozen at −80°C in polypropene tubes. The supernatant was mixed with 6 vol of 50 m*M* HCl in 2-propanol and the cell debris was removed by centrifugation. The globin was precipitated from the supernatant by addition of ethyl acetate, collected by centrifugation and subsequently washed with fresh ethyl acetate, then with *n*-pentane, and finally dried in a desiccator under vacuum overnight [49]. The globin was not subject to dialysis as described in the original method. The dried globin samples may be stored at −80°C until further analysis.

2.2.3.2. Modified Edman degradation. The dry globin (approx. 200 mg) was dissolved in methylformamide at a concentration of approximately 33 mg/ml, and following addition of the appropriate internal standard, either d_4-HOEtVal, d_6-HOPrVal or d_6-HOButVal (approximately 10 pmol/g globin), the pH of the sample was adjusted to 6.6–7.0 by addition of 1 *M* NaOH. For calibration, known amounts of the calibration standard solution were added to control globin prior to neutralisation. After the addition of derivatisation reagent (PFPITC, 14 μl/100 mg) the samples were left over night in a shaking mixer in the dark at room temperature. In order to complete the derivatisation the samples were finally warmed at 45°C for 90 min. Purified water was added to the samples (2 ml/100 mg), which were subsequently extracted twice with diethyl ether (4 ml/100 mg). The combined ethereal phases were evaporated to dryness by a gentle stream of N_2 and the residue was redissolved in toluene. The toluene solution was subsequently extracted with 2-ml aliquots of purified water, 0.1 *M* aqueous sodium carbonate solution (twice) and with water again. The toluene extract was evaporated to dryness at ±50°C in a waterbath using a gentle stream of N_2. After evaporation of the organic phase, the dried samples may be stored at −20°C for up to at least 4 weeks.

2.2.3.3. Gas chromatography–mass spectrometry (GC–MS). Dried samples were redissolved in toluene (approximately 50 μl) and kept in the dark at 4°C until analysis. Samples from persons exposed to ethylene or EO only, were analysed by a GC–MS method based on the modified Edman degradation as developed by Törnqvist and co-workers [31] and modified as described previously [41,43]. For persons exposed to relatively high concentrations of propylene or PO, resulting in HOPrVal concentrations higher than 10 pmol/g globin, the same GC–MS method was used. For lower HOPrVal con-

centrations and for the analysis of HOButVal following exposure to BD a more sensitive method was applied [42,47,50]. For the simultaneous quantification of HOEtVal, HOPrVal and HOButVal the following GC method with tandem MS spectrometry using negative chemical ionisation (GC-NECI-MS–MS) was developed. Aliquots of 1 μl of the samples in toluene were injected 'cold-on-column', on a J&W DB5MS fused-silica capillary column (dimensions: 30 m×0.25 mm Ø, 0.25 μm film thickness) in a Carlo Erba SFC 3000 or a Varian 3400 GC. Carrier gas was He at a flow-rate of 1 ml/min. The initial oven temperature was 80°C. After 1 min, the temperature was increased at a rate of 30°C/min to 190°C followed by an increase by 3°C/min to 320°C. Either a VG Quattro I or a Finnigan TSQ700 triple quadrupole MS was used for detection in negative ion electron capture ionisation mode. Methane was used as moderator gas and Ar as collision gas at a source temperature of 120°C and a source pressure of 5 Torr. Ions of the pentafluorophenylthiohydantoin (PFPTH) derivatives were used for detection using multiple reaction monitoring (MRM) of the following ions: m/z 368→320 (fragments from [d_6]-HOPrVal-PFPTH) and m/z 362→318 (fragments from HOPrVal-PFPTH) for PO adducts and at m/z 374→304, 318 (HOButVal-PFPTH) and m/z 380→304, 320 ([d_6]-HOButVal-PFPTH) for BMO adducts.

2.2.3.4. Calibration, calculation and quality control. Calibration curves were prepared using control globins spiked with known quantities of HOEtVal (0–1800 pmol/g globin), HOPrVal (0–500 pmol/g globin), or HOButVal (0–50 pmol/g globin) which were derivatised using the modified Edman degradation procedure described above. The peak area of the PFPTH-derivatives increased linearly with concentrations of N-terminal valine adducts in globin over the entire range for the selected concentrations. Quantification of the N-terminal valine adducts in globin were based on the internal deuterated reference standards. For calibration the peak areas of the PFPTH-derivatives were compared with the peak areas of the corresponding deuterated analogues, while a correction was made to take into account the difference in response of the unlabelled and deuterated adducts obtained from the calibration samples. The absolute amounts of the three N-terminal valine adducts in the globins used for internal standards were determined by comparison of the peak areas of their respective PFPTH-derivatives with the corresponding peak areas of the PFPTH derivatives of radiolabelled N-terminal valine adducts in the reference globins which were accurately determined based on the specific radioactivity [6,31,42].

Before each use the GC–MS–MS apparatus was carefully tuned and the sensitivity checked by measuring the signal-to-noise ratio of the m/z 272 peak in a selected ion chromatogram following injection of 1 pg octafluoronaphthalene. If the sensitivity was satisfactory (i.e., signal-to-noise ratio >10), the system was calibrated and the performance checked (1) by analysis in full scan mode of a small amount (70 pg) of the synthetic calibration standard of HOButVal (the standard with the lowest response in the mass spectrometer); selected ion chromatograms of m/z 374 and 380 should have intensities ranging from 10^5 to 10^6, (2) by analysis of 70 pg of the HOButVal synthetic standard in product ion full scan mode; product ion spectra should contain the ion pairs (380, 320) and (374, 318), and (3) by analysis of 0.7 pg of the HOButVal synthetic standard in MRM mode; signal-to-noise ratios for HOButVal and [d_6]-HOButVal should both be ≥ 5. Each day at least two quality control samples for each analyte under study, a high and a low concentration sample, were analysed. The concentration of these quality control samples was assigned as the mean of 15 separate determinations. The analytical results of a series were accepted if the deviations in both quality control samples were less than 5% of the assigned value. For each batch of samples that was analysed a series of calibration standards was run randomly spread through the samples of the batch. About 10% of all samples were duplicate or triplicates of the same sample. About 20% of all samples were quality control samples. All quality control data were checked to ensure run-to-run reproducibility and stability of calibration.

3. Results

3.1. Analytical methodology

3.1.1. Personal air monitoring

Details on the analytical sensitivity, specificity,

precision and accuracy for personal air monitoring of EO, PO and BD have been reported and discussed elsewhere [6,42,47]. The sampling strategy used for the air monitoring was of paramount importance. Poor correlations were found between Hb adduct concentration in a spot blood sample and ambient air concentrations if the air monitoring was done on a limited number of days, i.e., 1–3 days during the entire shift, which were deemed representative by the occupational hygienist [42]. Good correlations between Hb adduct levels in a spot blood sample and ambient air concentration were found if either very frequent air monitoring was applied, i.e., approximately 10 full-day shift measurements over a period of 2 months [6,47]. Good correlations were also found between the increase of Hb adduct concentration in a certain, relatively short, period of time and the cumulative exposure in this period determined by continuous air monitoring over this period [42,50].

3.2. Biomonitoring

3.2.1. HOEtVal

The limits of detection and quantification for HOEtVal in Hb could not reliably be determined since these values are well below the average background concentration of HOEtVal in Hb of persons not occupationally exposed to ethylene or EO, which is approximately 20 pmol/g globin in non-smokers [42]. In five out of 1883 samples of non-smoking operators the concentration of HOEtVal was below 5 pmol/g globin, but measurable without analytical difficulty. The within-series variation coefficient was 3.7% at 168 pmol HOEtVal/g globin and 3.1% at 811 pmol HOEtVal/g globin ($n=4$). The between-series variation coefficient was 6.5% at 101 pmol HOEtVal/g globin, 2.4% at 416 pmol HOEtVal/g globin, and 2.6% at 673 pmol HOEtVal/g globin ($n=6$, over a period of 15 months).

3.2.2. HOPrVal

The limit of detection for HOPrVal in Hb was 0.1 pmol/g globin. The limit of quantitation was dependent on the matrix of the globin sample. Due to matrix interference, the concentration of HOPrVal could not be accurately assessed in 287 out of 1515 samples of operators and was reported as less than 5 pmol/g globin. In 379 out of these 1515 samples, however, concentrations below 5 pmol HOPrVal/g globin could be accurately measured, and in 64 of these samples even concentrations as low as 1 pmol HOPrVal/g globin could be assessed. The within-series variation coefficient was 6.7% at 12 pmol HOEtVal/g globin and 3.4% at 127 pmol HOEtVal/g globin ($n=4$). The between-series variation coefficient was 7.8% at 22 pmol HOPrVal/g globin, 5.4% at 63 pmol HOPrVal/g globin, and 4.8% at 137 pmol HOPrVal/g globin ($n=6$, over a period of 15 months). The use of d_6-HOPrVal as internal standard in stead of d_4-HOEtVal resulted in slightly (5.6%), but significantly higher values.

3.2.3. HOButVal

The limit of detection for HOButVal was 0.05 pmol/g globin and was dependent on the matrix of the globin sample. The limit of quantitation was 0.1 pmol HOButVal/g globin. The within-series variation coefficient was 11.5% at 0.3 pmol HOButVal/g globin and 3.14% at 29.8 pmol HOButVal/g globin ($n=4$). The between-series variation coefficient was 19.2% at 1.0 pmol HOButVal/g globin ($n=6$). The use of d_6-HOButVal in stead of d_4-HOEtVal had a profound influence on the analytical results: the values were increased by a factor 5, which was explained by a much smaller response factor of d_6-HOButVal in the mass spectrometer compared to d_4-HOEtVal.

3.3. Correlation of biomonitoring with airborne concentrations

3.3.1. EO and PO

For both EO and PO, the increment in Hb adducts in the blood of the exposed operators during the study, expressed as HOEtVal and HOPrVal, respectively, correlated strongly with the measured concentrations of EO and PO in these operators, expressed as the total airborne concentration of EO or PO in milligrams per cubic metre per hour during the study. From Fig. 1, in which these relations are graphically depicted, it is obvious that EO forms more adducts than PO, which is probably due to its higher reactivity towards electrophiles [16,17], and which reflects its higher mutagenicity compared to PO [19,20].

The regression lines allow the calculation of the daily increment in Hb adducts when a person is

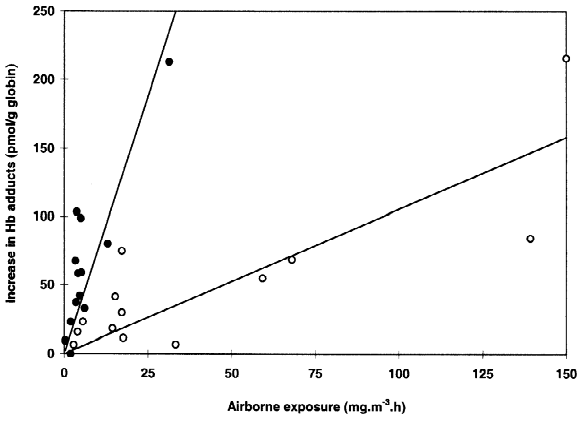

Fig. 1. Correlation between 8-h TWA respiratory exposure to EO and PO and the formation of Hb adducts. Airborne levels of EO and PO were determined daily over the entire shift during the study and the cumulative exposure over this period was calculated for each operator from his exposure data. Hb adducts of EO and PO were determined at the beginning and at the end of this period as HOEtVal and HOPrVal, respectively. The increase in blood levels of Hb adducts are given as function of the potential airborne exposure. Linear regression analysis showed highly significant correlations. For EO (closed circles): increment in Hb adducts (expressed as pmol HOEtVal/g globin) = 7.53 (SE 0.90) × airborne concentration of EO (expressed in mg m^{-3} h) ($P < 0.0001$; $r^2 = 0.618$; $n = 13$); and for PO (open circles): increment in Hb adducts (expressed as pmol HOPrVal/g globin) = 1.06 (S.E. 0.14) × airborne concentration of PO (expressed in mg m^{-3} h) ($P = 0.0004$; $r^2 = 0.672$; $n = 13$). When the curves were fitted with an intercept, small positive values were found that were not significantly different from zero, indicating that these values are most likely due to random error in the analyses and variations in natural background values.

continuously exposed to a particular concentration of EO or PO [37,38]. If this concentration is the occupational exposure limit, the corresponding biological exposure limit (BEL) can be calculated. The accumulation of stable Hb adducts in the blood during continuous exposure is the net result of daily increments (a) of the concentration of adduct, and daily losses due to removal of erythrocytes from the circulation. A steady state concentration of adducts (A_{ss}) will be attained upon continuous exposure to a particular concentration EO or PO for a period of time exceeding the average lifespan of the erythrocytes t_{er}, which is 126 days in humans. This steady-state concentration, A_{ss}, may be calculated from the average daily increment in adducts in 7-day weeks, a, by a simple equation: $A_{ss} = 0.5 \times t_{er} \times a$ [37,38]. For instance, from the slope of the regression line in Fig. 1 of 7.53 pmol g^{-1}/mg m^{-3} h, it can easily be calculated that exposure to the TLV (8-h TWA) for EO of 1.8 mg m^{-3} (1 ppm) [10] during a full 8-h shift results in a value for the increment a equal to $7.53 \times 1.8 \times 8 = 108$ pmol HOEtVal/g globin and substitution of this value in the formula gives a steady-state concentration A_{ss} of 6.83±0.82 (SE) nmol HOEtVal/g globin (95% confidence interval 5.01–8.67 nmol HOEtVal/g globin). Similarly, for the proposed TLV (8-h TWA) for PO of 4.4 mg m^{-3} (2 ppm) [10], a equals 40.7 pmol HOPrVal/g globin, and A_{ss} equals 2.56±0.34 (S.E.) nmol HOPrVal (95% confidence interval 1.81–3.30 nmol HOPrVal/g globin).

3.3.2. BD

For BD also a strong correlation with personal air monitoring results was found. Due to the different design of the study, which comprised more operators but less frequent personal air sampling, the data were processed and presented differently (Fig. 2). In the studies on EO and PO, the operators were monitored during the entire, but relatively short, period of special activities. The BD study was conducted during routine operation of the plants during a period of 2 months. Personal air sampling was randomly applied and considered representative for the entire period, but covered only 15% of the total duration of the study. The actual exposure concentrations varied over three orders of magnitude, and logarithmic transformation of the data was performed to make the variation between the low-exposed operators visible and balance the influence of the data points in the linear regression analysis.

The regression line allows, again, to determine BEL values. Assuming constant working conditions during the study and the preceding period, the HB adducts in the blood of the operators are at steady state, and the BEL values can be read directly from the graph (Fig. 2). Corresponding to the current TLV (8-h TWA) of 2 ppm (4.4 mg m^{-3}) [10] is a BEL of 2.47 pmol HOButVal/g globin (95% confidence interval 1.85–3.18 pmol HOButVal/g globin).

4. Discussion

Risk assessment is based upon (1) hazard identification, (2) the establishment of a dose–response

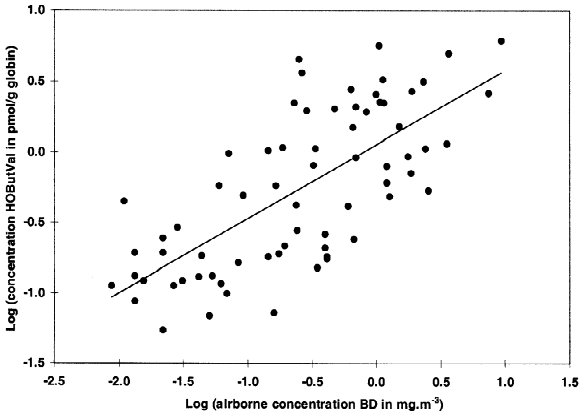

Fig. 2. Correlation between the average 8-h TWA respiratory exposure to BD during a period of 2 months and the formation of Hb adducts, expressed as the concentration of HOButVal in blood. A highly significant correlation ($P<0.0001$; $r^2=0.505$; $n=77$) was found, described by the following equation: log (concentration HOButVal, in pmol/g globin)$=0.527$ (SE 0.058)$\times$log (airborne concentration BD, in mg m^{-3})$+0.054$ (SE 0.043).

curve, and (3) determination of exposure. Although crucially important, determination of exposure is usually the least accurate parameter in risk assessment. Quite often industrial exposure assessment consists of estimates based on job descriptions in conjunction with air monitoring data from designated areas of the workplace. Such values often do not reflect individual worker exposures and peak exposures may easily be missed in such routine monitoring. As stated in the introduction, the determination of protein adducts, and especially stable Hb adducts, offers important advantages over air monitoring and other measurements of internal dose.

The methodology to determine adducts of EO to the N-terminal valine of Hb using GC–MS was rapidly adopted by the laboratory community and ring testing showed good correlations between the various laboratories that performed the assay [51]. Due to its high specificity and sensitivity GC–MS–(MS) analysis of adducts with the N-terminal valine of Hb following the modified Edman degradation proved also useful for determination of the internal dose of a number of other small reactive compounds, such as nitrogen mustards [52], epichlorohydrin [53], glycidol [54], aldehydes [55,56], acrylonitrile [57] and acrylamide [58], but also compounds that may readily form reactive metabolites in vivo such as isoprene and other alkenes [59–61].

By determination of specific Hb adducts, information is obtained on the chemical nature of the reactive intermediates, which may provide important information for risk assessment if more than one reactive intermediate is formed. This is, for instance, the case with BD, which forms at least two other reactive intermediates besides BMO, namely 1,2:3,4-diepoxybutane and 1,2-dihydroxy-3,4-epoxybutane. The genotoxic potencies of these three epoxide metabolites of BD vary enormously, and their relative rates of formation in experimental animals compared to humans may have a profound impact on the human health risk assessment of BD [6]. At present, however, it is not possible to differentiate between the adducts of 1,2:3,4-diepoxybutane and 1,2-dihydroxy-3,4-epoxybutane with the N-terminal valine of Hb, since they both form the same trihydroxybutyl adducts (THButVal) [6,62,63]. The group of Törnqvist proposed that 1,2:3,4-diepoxybutane would theoretically also form a specific, cyclic adduct on the N-terminus of valine, 2-(*N*-(3,4-dihydroxypyrrolidinyl)-3-methylbutanoic acid [64]. The modified Edman degradation methodology, however, cannot be applied to such an alkylated valine, as the tertiary amino group prevents its detachment. Hence, alternative methods are being developed and the first results look promising. In trypsin digests of the Hb of mice treated with 1,2:3,4-diepoxybutane, the N-terminal 2-(*N*-(3,4-dihydroxypyrrolidinyl)-3-methylbutyl heptapeptide was detected using liquid chromatography–electrospray ionisation mass spectrometry [65]. In human haemoglobin exposed to 1,2:3,4-diepoxybutane, elevated levels of 2-(*N*-(3,4-dihydroxypyrrolidinyl)-3-methylbutanoic acid could be detected following acid hydrolysis and subsequent derivatisation for GC–MS–MS [66]. These methods may also help to clarify the origins of the high background values of THButVal which were found in individuals without occupational exposure to BD [6,62,63].

For HOEtVal also distinct background values in the Hb of individuals without any occupational exposure to EO were found. Smoking causes a significant elevation of the background concentrations of HOEtVal in the blood of not-occupationally exposed persons and the measured levels corresponded nicely with the number of cigarettes smoked.

It was estimated that smoking contributed between 7 and 11 pmol HOEtVal per cigarette smoked per day [29,67–70] and that exposure to ethylene through smoking might contribute up to 15% of all smoking-related cancers in Sweden [67]. For calculations of the additional cancer risk due to occupational exposure to ethylene or EO, the measured values of HOEtVal in smokers have to be corrected for their smoking patterns [2,31,42]. In non-smoking individuals without occupational exposure still an average background value of approximately 20 pmol HOEtVal/g globin was repeatedly reported for persons [2,42]. The most likely source of the background of HOEtVal is endogenously formed ethylene and EO from lipid peroxidation and bacterial activity in the gastro-intestinal tract [2,31]. It is highly unlikely, however, that the high background values measured for the THButVal in Hb are due to endogenously formed BD since background concentrations of HOButVal, originating from the primary epoxide of BD, are very low. In two different groups of 25 not occupationally exposed controls, median values of THButVal of 38 ± 13 (SD) and 79 (range 44–187) pmol/g globin were measured, whereas in two groups of 12 and 25 controls median values of HOButVal were only <0.1 (range <0.1–1.2) and 0.2 (range 0.1–1.0) pmol/g globin, respectively [6,63]. The origin of the background for THButVal is presently unknown. In blood samples from not-occupationally exposed controls, HOPrVal concentrations were usually below the detection limit (<5 pmol/g globin) [42]. Both for HOPrVal and HOButVal smoking behaviour did not significantly influence the HOPrVal levels.

From the very onset of the development of protein adduct determination as internal dose monitor, it has been suggested that Hb adducts might be used as a surrogate measure of DNA adducts because of the proportional reaction rates for the binding to Hb and DNA [27,31,71]. In general, Hb adducts were correlated with hepatic DNA adducts, and linear correlations for wide dose ranges were found following exposure to ethylene [7], EO [11,72] and PO [48]. Such relationships seem to confirm the assumption that Hb adducts may indeed be applied as a surrogate marker for DNA adducts and can thus be used to assess the internal dose of genotoxic compounds. More recently, however, it became evident that this assumption is an oversimplification since at a single blood concentration of an alkylating agent, and hence a single Hb adduct level, the DNA binding may vary considerably from one tissue to another. Due to differences in formation, persistence, repair, and chemical depurination, the relationship between DNA and Hb adducts of EO varied with length of exposure, interval since exposure, and tissue [73]. In mice exposed to 50 or 200 ppm BD (110 and 440 mg m^{-3}, respectively), very similar DNA adduct levels were found in lung and testes, but at 500 ppm (1100 mg m^{-3}) the adduct levels were significantly higher in testes than lung [74]. In a recent inhalation study with PO, a single value for HOPrVal was determined in blood, while large differences in DNA adduct levels, up to 43-fold, were found between various tissues [36]. This phenomenon may be explained by enzymatic repair of DNA adducts, which may differ for different tissues, and by instability due to chemical depurination of the DNA adducts on the N7-position of guanine (the major adduct following exposure to ethylene, EO, propylene, PO or BD), while Hb adducts are not subject to repair.

In theory, the removal of Hb adducts is a zero-order process, only determined by the life-span of the erythrocyte, which in humans is approximately 126 days. In operators accidentally exposed to high concentrations of EO, indeed the concentration HOEtVal in Hb declined with an apparent half-life of 110 days [75]. This implies that time-integrated exposure to ethylene, EO, propylene, PO and BD over a period of several months can readily and reliably be assessed by determination of the concentration of their respective N-terminal valine adducts in a small blood sample from the exposed persons. As shown in the studies presented here, indeed highly significant correlations were found between the air concentrations of these chemicals measured in the breathing zone of the exposed operators and the corresponding Hb adduct levels in their blood. The slopes of the regression lines reflect the net result of relative reactivity [16,17] and rate of detoxification [76] and hence mutagenic potency of the compounds [19,20].

Due to the differences in stability and repair, the relationship between Hb and DNA adducts will be highly complex, unless daily exposure patterns are very similar for prolonged periods of time. Thus,

while Hb adducts provide an excellent measure of systemic exposure, they cannot easily be applied to assess the extent of DNA adduct formation associated with that exposure. Although counterintuitive at first glance, this is no limitation for risk assessment, since cancer risk is primarily associated with target dose, defined as the time integral of the concentration of the ultimate genotoxic agent [77]. So where DNA adducts, due to their variable rate of repair in different tissues and cell types, are difficult to correlate with the dose of the reactive intermediate, Hb adducts, due to their stability, are a perfect internal dose monitor and more suitable for *quantitative* risk assessments than DNA adducts [78].

So far, two types of models have been developed for quantitative risk assessment based on Hb adducts. The first model is based on the radiation-dose equivalence theory developed by Ehrenberg [79–81]. This theory is based on the concept that if the in vivo target dose of a carcinogen can be determined, then its cancer risks may be estimated by comparison with an agent for which the human cancer risks are known. Ehrenberg also proposed γ-radiation as the reference standard since it is the environmental factor for which the relationship between exposure dose and effects is best known. 'Radiation equivalent values' have been determined for a number of direct alkylating substances using a variety of genetic endpoints and biological systems [81–83]. The model has also been applied for the risk assessment of EO, using HOEtVal as dose monitor, and it could be concluded that occupational exposure below 1 ppm (1.8 mg m^{-3}) will not produce an unacceptable risk in humans [11]. Hb adducts may also prove useful in pharmacologically based toxicokinetic models, since they provide a direct link between experimental animals and humans, and may thus reduce the uncertainty associated with interspecies extrapolation [84].

The strong correlation between personal air monitoring data and Hb adduct levels in operators also allow the interpretation of Hb adduct concentrations in terms of airborne levels of EO, PO and BD and thus permit the setting of biological exposure limit (BEL) values equivalent to airborne OEL values. To the best of our knowledge, we have been the first, and so far the only, to propose experimentally based BEL values for exposure to PO and BD [6,42,50], but for EO several values for the BEL corresponding to an OEL (8-h TWA) of 1 ppm (10.1.8 mg m^{-3}) have been suggested. In 1989, Ehrenberg's group in Stockholm proposed a BEL of 2.4 nmol HOEtVal/g globin [85]. However, this value was not based on concomitantly determined air measurements but on estimations from recorded stationary and personal air measurements in combination with personal statements on work routines. Consequently, this value was considered to be uncertain by a factor of 3 (Ehrenberg, personal communication). In 1996, re-evaluation of the same dataset led to a BEL of 3.4 nmol HOEtVal/g globin [86], which was identical to the value we had proposed in 1994, based on a series of pilot studies [41]. In Germany, a BEL was established based on statistical extrapolations of experimental animal data [87]. A single human data point from a relatively high-level exposure to EO (approximately 4 ppm, or 7.2 mg m^{-3}) fitted well into this data set and it was estimated that 8-h TWA exposure to 1 ppm (1.8 mg m^{-3}) would yield approximately 4 nmol HOEtVal/g globin [88]. In the present study, a BEL of 6.2 nmol HOEtVal/g globin was found to correspond to a OEL (8-h TWA) of 1 ppm (1.8 mg m^{-3}).

There are several factors that may explain the differences in proposed BELs. The first is related to study design with regard to the determination of airborne concentrations. It is well known that air sampling is prone to introduce errors unless exposure patterns are highly homogeneous and constant in time, or unless large numbers of samples are collected. However, in general, exposure to EO consists of numerous small excursions, occurring at highly irregular patterns, from a very low exposure level. The numbers of samples collected are often too limited to reconstruct the exposure with a high degree of certainty. We found, for instance, poor correlations between airborne EO or PO and HOEtVal or HOPrVal levels in Hb in blood samples collected at the end of a 4-month period in which monthly personal air monitoring was applied during routine operations in operators of an EO-plant [42]. In contrast, as shown in the present study, highly significant correlations were found if personal air monitoring was applied during the complete period bridging the collection of blood samples for Hb

adducts measurements. The second factor is related to the use of standards. In our study, calibration was based on globin that had been isolated from blood treated with ^{14}C-labelled EO or PO. The N-terminal valine adduct concentrations in these globin samples were determined by scintillation counting after hydrolysis of the protein and separation of the modified amino acids by HPLC. Angerer and co-workers used a different method of calibration using a modified tripeptide as calibration standard and *N*-(2-ethoxyethyl)valine as internal standard. Modified tripeptide internal standards have also been used for the quantitative analysis of other Hb adducts [36, 57,62,89]. It is well known that the yield of derivatisation with the modified Edman degradation differs for free and protein-bound amino acids and is also dependent of the type of globin [51,89] which might explain the difference in results. A third factor that may influence absolute values of the results is the choice of internal standard. In our studies, we used the perdeuterated congeners of the analyte under study, [d_4]-HOEtVal, [d_6]-HOPrVal, and [d_6]-HOButVal, but standards with other isotopes than ^{2}H, such as ^{13}C or ^{15}N have also been used successfully, especially in methods using high-resolution mass spectrometry for detection [63]. In some studies, however, non-isotopically labelled compounds, such as *N*-(2-ethoxyethyl)valine [57,88] were used or [d_4]-HOEtVal was used as internal standard for the analysis of Hb adducts of compounds other than EO, such as PO [90], BD [4,91], assuming that there would be no difference in response factors in the mass spectrometer for the various PFPTH-derivatives. Although this is probably true for mass spectrometry in 'single ion monitoring' mode, our results showed that this assumption may be invalid when tandem mass spectrometry methods with 'multiple reaction monitoring' is being applied. During the development of this method, we observed that the concentration HOButVal was underestimated about five times if [d_4]-HOEtVal was used instead of [d_6]-HOButVal due to differences in the response factor in the MRM mode. In contrast, the concentration HOPrVal was only slightly (5.6%) underestimated when measured by GC–MS–MS using [d_4]-HOEtVal instead of [d_6]-HOPrVal as internal standard.

The determination of adducts with the N-terminal valine of Hb is a highly useful method for exposure control and risk assessment. The methodology is well validated and has proved highly reliable. Nevertheless, its routine application for exposure control is hampered by the fact that proper standards are not commercially available, that sophisticated equipment, such as GC–MS(–MS) is needed and that the analyses have to be performed by highly skilled technicians. In order to overcome these problems, which are inherent to the methodology rather than to the analytes themselves, we started the development of immuno-assays for HOEtVal and HOPrVal [92]. Easy to operate luminescence-based immunoassays have been developed and are currently being validated [93]. These test-kits should allow rapid, on-site determination of Hb adducts in finger-prick blood samples.

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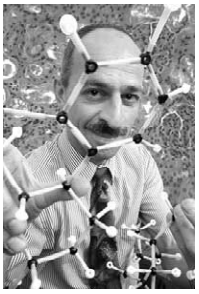

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