BIOMARKERS, VOL. 9, NO. 6 (NOVEMBER–DECEMBER 2004), 407–417

Taylor & Francis
healthsciences

Development of a competitive immunoassay for the determination of N-(2-hydroxyethyl)valine adducts in human haemoglobin and its application in biological monitoring

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Received 16 August 2004, revised form accepted 12 November 2004

Ethylene oxide (EO) is an important industrial compound and a directly acting mutagen. Human exposure to it can be monitored by the determination of haemoglobin (Hb) adducts. An immunoassay that quantifies the N-terminal adduct N-(2-hydroxyethyl)valine in whole blood was developed and its potential usefulness as a tool for biologically monitoring occupational exposure demonstrated. Analytical reliability was confirmed in a comparative study with gas chromatography-mass spectrometry (range 0.040–589 nmol g^{-1} Hb, correlation coefficient 0.98, $n = 10$). The assay was configured as a competitive enzyme-linked immunosorbent assay to facilitate the rapid throughput of samples. The assay uses a whole blood matrix and has a working range of 10–10 000 pmol N-(2-hydroxethyl)valine g^{-1} Hb. The assay does not appear to be affected by structurally similar metabolites and has been used to determine adducts in human blood samples. The first results from potentially exposed workers indicate the assay might be a powerful tool for the routine occupational biomonitoring of EO exposure.

Keywords: ethylene oxide, haemoglobin adducts, immunoassay, biological monitoring.

Introduction

Ethylene oxide (EO) is an important industrial compound. It is generally produced for chemical synthesis and is used in the manufacture of glycols, glycol ethers and surfactants. It is also used as a sterilant by the healthcare industry where the potential for extensive human exposure exists (Koda *et al*. 1999). EO is classified as a human carcinogen by the International Agency for the Research on Cancer (IARC) and, as a result, there is a need to monitor exposure to this compound in the workplace to check for compliance with exposure control measures.

The current occupational exposure limits (8-h TWA, time weighted average) in the UK (maximum exposure limit, or MEL) and USA (permissible exposure limit, or PEL) are 5 ppm (9 mg m^3) and 1 ppm (1.8 mg m^3), respectively. To comply with a limit of 1 ppm 8-h TWA, exposures must be, for most of the time, well below this value. Methods sensitive enough to measure below 1 ppm are therefore needed.

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2004 Taylor & Francis Group Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500400024974

Exposure to EO can be determined by air monitoring. However, the estimation of exposure by air monitoring is complicated if exposure is variable and occurs intermittently (Boogaard *et al*. 1999). Moreover, air monitoring estimates the potential rather than the actual exposure an individual receives. These problems can be overcome by biomonitoring — the determination of biomarkers of exposure such as DNA and protein adducts.

Inhalation is the major route of entry for EO into the body. From the lungs, EO is rapidly distributed throughout the body tissues where it alkylates macromolecules such as DNA and haemoglobin (Hb) (Ehrenberg *et al*. 1974). The determination of N-terminal valine adducts in Hb has enabled the development of biomonitoring programmes for EO (Van Sittert and Van Vliet 1994).

The adduct N-(2-hydroxyethyl)valine appears to be chemically stable and does not affect the life span of the erythrocytes (Britton *et al*. 1991). Exposure dose measured by the determination of the adduct concentration is therefore integrated over the average life span of the erythrocyte, which is approximately 126 days (4 months).

Boogaard *et al*. (1999) demonstrated good correlation between N-(2-hydroxyethyl)valine adducts and time-integrated airborne exposures in workers exposed to low levels of EO. From these correlations, they determined a biological exposure limit (BEL) that corresponded to an average level of exposure to an OEL over 4 months, 5 days/week, 8 h/day. For OELs of 1 and 5 ppm (8-h TWA), the corresponding BELs are 6.8 and 34.8 nmol N-(2-hydroxyethyl)valine g^{-1} Hb, respectively.

The determination of N-(2-hydroxyethyl)valine by the modified Edman degradation procedure has shown the presence of adducts in non-occupationally exposed populations. However, the determination of N-(2-hydroxyethyl)valine by the modified Edman degradation procedure requires a relatively complex work-up of samples before analysis and the use of sophisticated analytical equipment. These and other factors limit the usefulness of the method, particularly in large-scale screening programmes.

The use of immuno-analytical methods for the detection of biomarkers offers the prospect of an alternative approach (Van Welie *et al*. 1992). In clinical chemistry, rapid, antibody-based, user-friendly tests have been the key to large-scale screening programmes. Recently, an immunoassay that quantifies urinary *S*-phenylmercapturic acid, a benzene-specific biomarker, has been developed and its usefulness as a screening tool for monitoring occupational exposure to benzene demonstrated (Aston *et al*. 2002).

The determination of N-(2-hydroxyethyl)valine by immunoassay should facilitate the implementation of EO biomonitoring screening programmes. Wraith *et al*. (1988) described a radio-immunoassay for the determination of human exposure to EO, quantifying the concentration of N-(2-hydroxyethyl)heptapeptide after trypsin hydrolysis from the alpha chain of purified globin. However, like the modified Edman degradation procedure, this assay also possessed a tedious sample work-up before analysis.

The optimum, therefore, would be the quantification of EO-adducted valine in whole Hb. The production of antibodies that recognize N-(2-hydroxyethyl)valine

in whole Hb would greatly facilitate assay development allowing the development of a simple, rapid and cost-effective screening tool.

The paper describes the development of an immunoassay for the determination of N-(2-hydroxyethyl)valine in whole blood and discusses its potential application as a screening tool for monitoring occupational exposure to EO.

Materials and methods

Polyclonal antibodies were raised to N-(2-hydroxyethyl)valine in the form of the N-terminal heptapeptide from the alpha chain of human Hb. Both the N-(2-hydroxyethyl)heptapeptide and the non-modified analogue were prepared by chemical synthesis. Due to their low molecular weight and, therefore, limited antigenicity, the adducted heptapeptide was coupled to carrier protein before immunization.

Synthesis of N-(2-hydroxyethyl)valine by the reaction of ethylene oxide with L-valine methyl ester hydrochloride

Briefly, L-valine methyl ester hydrochloride (7.57 g, 0.045 moles) was dissolved in dry methanol (40 ml) and cooled on ice. The mixture was neutralized by the slow addition of 45 ml 1 M sodium methoxide (0.045 moles) with cooling and reacted for 20 min with stirring under an atmosphere of dry nitrogen. EO was bubbled through the mixture, containing L-valine methyl ester, until 7.3 g (0.15 moles) had been added. The mixture was reacted at room temperature with continuous stirring.

The reaction was monitored by thin-layer chromatography (TLC) using silica gel 254F plates (0.2 mm thick, 20 × 5 cm) and 1:1 dicloromethane:acetonitrile as the mobile phase. Plates were developed using iodine vapour or 4% phosphomolybdic acid in ethanol. TLC demonstrated the presence of three products: valine methyl ester ($R_f = 0.28$), N-(2-hydroxyethyl)valine methyl ester ($R_f = 0.46$) and the disubstituted N,N-*bis*-(2-dihydroxyethyl)valine methyl ester ($R_f = 0.28$).

After 3 days, the reaction was complete. Salt was removed by filtration (Buchner scinter funnel, size 3) and the methanol was removed from the reaction mixture by rotary evaporation. (20°C and 60 mmHg).

The reaction products were separated by silica gel chromatography. The reaction residue was dissolved in mobile phase (7 ml) of a mixed solvent 1:1 acetonitrile:dichloromethane and loaded on to a silica gel column (Kieselgel Merck Type 9385, 230–400 mesh 60A, 7 × 9 cm). Fractions (20 ml) were collected from the column and the position of the reaction products determined by TLC.

Fractions containing the N-(2-hydroxyethyl)valine methyl ester were pooled and the solvent removed by rotary evaporation (30°C and 100 mmHg).

The N-(2-hydroxyethyl)valine methyl ester was dissolved in 20 ml distilled water and stirred at 45°C for 3 days. Hydrolysis of the N-(2-hydroxyethyl)valine methyl ester gives N-(2-hydroxyethyl)valine. After hydrolysis, the water was removed by freeze drying to give a white solid (1.63 g, 31% yield).

High-performance liquid chromatography (HPLC) analysis gave a single product with a retention time of 21 min. Mass spectrometry ($m+1 = 118$) and nuclear magnetic resonance (NMR) studies (shift (ppm) 0.99 (d, 3H, $\mathbf{CH_3}CHCH_3$), 1.05 (d, 3H, $CH_3CH\mathbf{CH_3}$), 2.1–2.3 (m, 1H, $CH_3\mathbf{CH}CH_3$), 3.15 (t, 2H, $\mathbf{CH_2}OH$), 3.5 (dd, 1H, **CH**COOH), 3.8 (t, 2H, $\mathbf{CH_2}NH$)) are consistent with a pure product with the structure of N-(2-hydroxyethly)valine.

Preparation of N-(2-benzyloxyethyl)valine

Sodium hydride (234 mg, 7.8 moles) was dissolved in 2 ml dry DMF and cooled to 4°C under a dry nitrogen atmosphere. N-(2-hydroxyethyl)valine (250 mg, 1.56 moles) was dissolved in dry dimethyl formamide (DMF) (6 ml), cooled to 4°C under a dry nitrogen atmosphere and added dropwise with stirring to the sodium hydride solution. The reaction was allowed to equilibrate to room temperature. After the release of hydrogen had ceased, benzyl bromide (0.4 ml, 3.4 moles) was added and reacted overnight with continuous stirring at room temperature. The reaction was monitored by TLC.

TLC was performed using silica gel 254F plates (0.2 mm thick, 20 × 5 cm) and 1:4 methanol:chloroform as the mobile phase. Plates were developed using iodine vapour. Three compounds were observed in the reaction mixture. The largest compound was N-(2-benzyloxyethyl)valine ($R_f = 0.28$).

When the reaction was complete, excess sodium hydride was removed by the careful dropwise addition of distilled water until effervescence stopped. DMF was removed by high-vacuum rotary evaporation. The solid residue was taken up in distilled water (8 ml) and extracted with diethyl ether (2 × 5 ml). The aqueous layer was freeze dried. N-(2-benzyloxyethyl)valine was purified from the reaction mixture by flash chromatography on silica gel.

The reaction mixture was suspended in 1:4 methanol:chloroform (5 ml) and loaded on to a silica gel (Merck, 230–400 mesh, 40 × 2 cm). The column was eluted with 1:4 methanol:chloroform and

fractions (5 ml) collected. Peak fractions of N-(2-benzyloxyethyl)valine were identified by TLC, pooled and rotary evaporated to dryness. N-(2-benzyloxyethyl)valine was recrystallized from boiling water to give a white solid. NMR (shift (ppm) 1.03 (d, 3H, $\mathbf{CH_3}CHCH_3$), 1.08 (d, 3H, $CH_3CH\mathbf{CH_3}$), 2.2 (m, 1H, $CH_3\mathbf{CH}CH_3$), 3.2 (m, 2H, CH_2N), 3.4 (d, 1H, **CH**COOH), 3.73 (m, 2H, $\mathbf{CH_2}O$), 4.58 (dd, 2H, $ArCH_2$), 7.35 (m, 5H, ArH)).

Peptide synthesis

Heptapeptide and N-(2-hydroxyethyl)heptapeptide, containing the amino acid sequence found at the N-terminus of the alpha chain of human Hb, were prepared by solid-phase peptide synthesis. Peptides were synthesized from the carboxylic acid terminus of the molecule. N-(2-hydroxyethyl)valine adducted peptides were prepared by the addition of N-(2-benzyloxyethyl)valine to the developing sequence. After cleavage from the solid support, the protecting group was removed by catalytic hydrogenolysis. Deprotection was performed overnight in a Parr hydrogenator with a 10% palladium carbon catalyst at 50 psi. The catalyst was removed by filtration. Heptapeptide and adducted heptapeptide were purified by HPLC (column; C18, 4.5 mm $\times$ 15 cm, solvent A: 0.1% trifluoracetic acid (TFA) in water, solvent B: 0.1% TFA in acetonitrile, gradient: 0–60% B in 30 min, detection: ultraviolet light 230 nm, flow: 1.5 ml min^{-1}). The purities of the hepapeptide and N-(2-hydroxyethyl)heptapeptide were 90 and 98%, respectively.

The molecular weights of the products were determined by time of flight mass spectroscopy and were consistent with the assigned structures (heptapeptide MW 728 and N-(2-hydroxyethyl)heptapeptide MW 772).

Protein conjugation

N-(2-hydroxethyl)heptapeptide was conjugated to bovine serum albumin (BSA) and horse serum albumin (HSA).

BSA (10 mg ml^{-1}), HSA (10 mg ml^{-1}) and N-(2-hydroxyethyl)heptapeptide (10 mg ml^{-1}) were dissolved in distilled water. To 8.25 mg BSA (1.3×10^{-7} moles) and HSA was added 2.5 mg (3.2×10^{-6} moles) of N-(2-hydroxyethyl)heptapeptide with mixing. 1-Ethyl-3-diamino-(3-dimethylaminopropyl) carbodiimide (EDC) was dissolved in distilled water (160 mg ml^{-1}) and added immediately to the protein-peptide solutions (12.2 mg, 6.4×10^{-7} moles) with stirring. The mixtures were reacted for 90 min at room temperature then overnight at 4°C with continuous stirring. Protein conjugates were transferred to a Pierce Filtration unit (MW cut-off 12–14 000 daltons) and extensively dialysed against phosphate-buffered saline (PBS). Protein concentrations were determined from optical density readings at 280 nm. After coupling, the concentrations of the BSA and HSA conjugates were approximately 3.0 mg ml^{-1}, respectively (recoveries of 90–95%).

Immunization protocol

Polyclonal antibodies were produced in rabbits ($\times$4). Primary immunizations (day 1) and secondary immunizations (day 21) were given subcutaneously. Each animal received 100 μg HSA N-(2-hydroxyethyl)valine conjugate in 200 μl 50:50 v/v conjugate adjuvant emulsion. Primary immunizations were prepared in Freund's complete adjuvant, secondary immunizations were prepared using Freund's incomplete adjuvant. Rabbits were bled from the ear (about 20 ml) on day 28. Serum was separated from whole blood by centrifugation (2200 rpm for 5 min).

Evaluation of anti-N-(2-hydroxethyl)valine heptapeptide response

Antibody titres were determined by the preparation of antiserum titration curves (1:10–1:51 200 in PBS/Tween 0.05%) using microtitre plates coated with 1 μg ml^{-1} BSA N-(2-hydroxyethyl)heptapeptide conjugate (in carbonate/bicarbonate buffer, pH 8.6, overnight at 4°C). Competitive enzyme-linked immunosorbent assays (ELISAs) were optimized in terms of conjugate coat and antiserum dilution and used to determine the relative antibody affinities and cross-reactivity. N-(2-hydroxyethyl)heptapeptide, N-(2-hydroxypropyl)heptapeptide and heptapeptide standards were prepared in PBS.

ELISA was carried out by adding 50 μl standard and 50 μl antiserum diluted in PBS/Tween (0.2%) to the central 60 wells of a 1 μg ml^{-1} BSA-N-(2-hydroxyethyl)heptapeptide conjugate-coated plate. The plate was sealed with a plastic film and incubated overnight at room temperature. The contents of the wells were emptied, washed five times with saline/Tween (0.05%) wash solution and shaken dry. To each well was added 100 μl anti-rabbit immunoglobulin phosphatase-conjugated antibody diluted 1:1000 in PBS/Tween (0.05%). The plate was covered and incubated for a further 2 h at room temperature. The plate was washed and dried, as above, and 100 μl *p*-nitrophenyl phosphate (1 mg ml^{-1}) in Tris buffer (0.2 M) were added to each well. In the presence of phosphatase, this enzyme substrate turns from a colourless to a yellow solution. After 30 min, the absorbance (405 nm) was measured using a V_{max} microtitre plate reader (Molecular Devices, Crawley, UK).

Haemoglobin analysis

EO-adducted human Hb was prepared by the reaction of human Hb with EO overnight at 37°C. The adduct concentration was determined using the modified Edman degradation procedure (Boogaard *et al*. 1999). Antibody binding of N-(2-hydroxyethyl)heptapeptide in whole Hb was investigated by ELISA. Hb (1 mg ml^{-1} in PBS) calibration plots containing 10–10 000 pmol N-(2-hydroxyethyl)valine g^{-1} Hb were developed using antisera R4 diluted 1:120 000 in PBS/Tween (0.05%).

Samples of whole blood from potentially exposed workers were collected from the antecubital vein into a Vacutainer™ containing K_3 ethylenediamine tetra-acetic acid (EDTA) 15% as anticoagulant (about 7 ml). Samples were collected using syringes and needles sterilized with gamma-radiation.

Erythrocytes were isolated by centrifugation (10 min, 1000*g*, 20°C) and washed three times by suspension in PBS (15 ml). The washed cells were resuspended in distilled water (15 ml) and subject to four cycles of freezing at −70°C and thawing. The lysed suspension was centrifuged at 50 000*g* for 90 min at room temperature to remove cell debris. The supernatant was dialysed against distilled water (5 × 1 litre) and the Hb isolated by freeze-drying. In a comparative study, N-(2-hydroxyethyl)valine concentration was determined by ELISA and the modified Edman degradation procedure.

Whole blood analysis

Whole blood (24 ml) containing approximately 3.8 g (56 μmol) Hb was reacted with 10 μl (200 μmol) EO overnight at 37°C. Unreacted EO was allowed to evaporate and the N-(2-hydroxyethyl)valine adduct concentration was determined using the modified Edman degradation procedure (Boogaard *et al*. 1999).

Calibration plots were prepared by the serial dilution of the adducted whole blood in the blood from a non-occupationally exposed volunteer. Immediately before immunoassay, these whole blood standards were diluted (1:3) in distilled water. The lysed matrix was transferred (50 μl) to the wells of a BSA-conjugate coated microtitre plate and the assay developed using R1 diluted 1:10 000 in PBS/Tween (0.2%).

ELISA validation

Blood samples were collected from both workers ($n = 18$) potentially exposed to EO in a chemical manufacturing plant and non-occupationally exposed non-smokers ($n = 20$). Samples were also prepared by the dilution of the EO adducted blood in the blood of a non-occupationally exposed individual ($n = 10$). Aliquots of these samples were analysed by both ELISA and the modified Edman degradation procedure.

ELISA was performed using eight standards (range 10–10 000 pmol g^{-1} Hb), two quality controls and 20 samples in duplicate per assay plate. The assay was developed as previously described using R1 diluted 1:10 000 in PBS/Tween (0.2%).

Inter- and intra-assay reproducibility were determined by the repeat analysis of blood samples containing high and low concentrations of N-(2-hydroxyethyl)valine, respectively.

Results

Antisera dilution profiles from rabbits immunized with HSA N-(2-hydroxyethyl)heptapeptide conjugate demonstrated the presence of antibodies that recognize conjugated BSA N-(2-hydroxyethyl)heptapeptide coupled to the microtitre plate wells. Antibody titre, the antiserum dilution at which 50% of the maximum binding occurs, is shown in table 1. Rabbit 4 (R4) gave the greatest antibody titre (1:120 000).

Competitive inhibition studies show all the antisera contained antibodies that recognize exogenous N-(2-hydroxyethyl)heptapeptide (table 1). Calibration plots developed over the range 0.01–10 000 pmol N-(2-hydroxyethyl)heptapeptide ml^{-1} demonstrate a dose-dependent effect on antiserum binding. An inverse relationship was observed between adduct dose and the signal generated in the ELISA. Under the conditions employed, calibration plots developed with antisera R4 gave the lowest limit of detection (0.01 pmol N-(2-hydroxyethyl)valine ml^{-1}) (figure 1).

Table 1. Titre and relative affinity of antisera from rabbits immunized with HSA-N-(2-hydroxyethyl)heptapeptide conjugate. The assay was performed using microtitre plates coated with BSA-adducted heptapeptide as described in the Materials and methods.

Rabbit	Titre	Inhibition adducted heptapeptide (%)*
1	1:50 000	55
2	1:3000	43
3	1:10 000	42
4	1:120 000	81

*100 pmol ml^{-1} N-(2-hydroxyethyl)heptapeptide.

None of the antisera cross-reacted with either heptapeptide or N-(2-hydroxypropyl)heptapeptide in the measuring range of the assay. Cross-reactivity did occur when heptapeptide and N-(2-hydroxypropyl)heptapeptide were present in a large molar excess. Antiserum R1 and R4 demonstrated the greatest relative specificity when compared with structurally similar compounds. The IC_{50} values, the standard concentration that reduces binding by 50% in an ELISA, are shown in table 2.

Adducted Hb, prepared by the reaction of human Hb with EO, contained 2847 nmol adduct g^{-1} globin. Similar results were obtained in competitive binding studies using either N-(2-hydroxyethyl)heptapeptide BSA conjugate or whole adducted Hb to coat the assay wells.

A calibration curve developed with antisera R4 and whole adducted Hb diluted in PBS (1 mg ml^{-1}) had a measuring range of 10–10 000 pmol N-(2-hydroxyethyl)valine g^{-1} Hb (figure 2). Ten Hb samples containing between 120 and 8500 pmol N-(2-hydroxyethyl)valine g^{-1} Hb were determined by immunoassay and the modified Edman degradation procedure. The correlation coefficient between the two methods was 0.97.

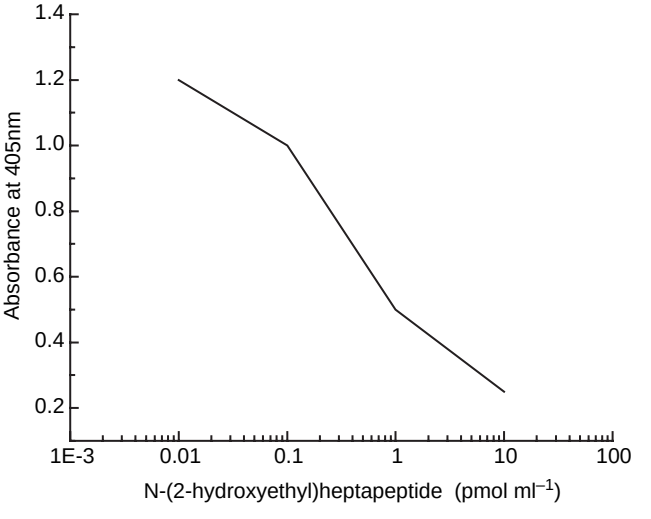

Figure 1. N-(2-hydroxyethyl)heptapeptide standard curve developed by ELISA as described in the Materials and methods. Ten standards were assayed in triplicate and the mean plotted. The coefficient of variation of the standard values was less than 5%.

Table 2. Charaterization of antisera raised against N-(2-hydroxyethyl)heptapeptide-HSA conjugate.

	IC^{50} (pmol ml^{-1})	
Rabbit	N-(2-hyroxyethyl) heptapeptide	Heptapeptide
1	8	>50 000
2	8	20 000
3	5	10 000
4	0.5	10 000

Using antisera R1, problems associated with a strong matrix effect were overcome and an immunoassay that performs in a whole blood matrix was realized. The ELISA has a measuring range of 10–10 000 pmol N-(2-hydroxyethyl)valine g^{-1} Hb. Samples of whole blood containing between 0.040 and 589 nmol N-(2-hydroxyethyl)valine g^{-1} Hb were determined by immunoassay and the modified Edman degradation procedure. The correlation coefficient between the two methods was 0.98 ($n = 10$) (figure 3).

All of the blood samples from non-occupationally exposed non-smokers contained N-(2-hydroxyethyl)valine adducts (range 14–43 pmol adduct g^{-1}, $n = 20$). The samples from workers ($n = 18$) potentially exposed to EO during chemical manufacture contained between 42 and 115 pmol N-(2-hydroxyethyl) valine g^{-1} Hb.

Percentage coefficients of variation (CV) of 5.4% (mean = 17, SD 0.9, $n = 10$) and 5.4% (mean = 149.9, SD 8.6, $n = 10$) were obtained for the with-in assay analysis of a low and high whole-blood sample. Intra-assay coefficients of variation for two blood samples were 24.6% ($n = 6$, mean = 14.5, SD = 3.6) and 8.4% ($n = 6$, mean = 442, SD = 37), respectively.

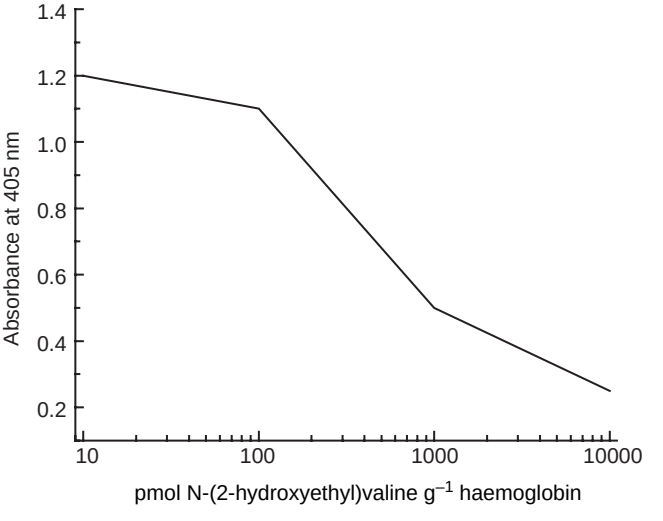

Figure 2. Determination of N-(2 hydroxyethyl)valine in whole haemoglobin. The assay was developed as described in the Materials and methods. Eight standards were assayed in duplicate and the means plotted. Determined values differed from the means by between 0.5 and 6%.

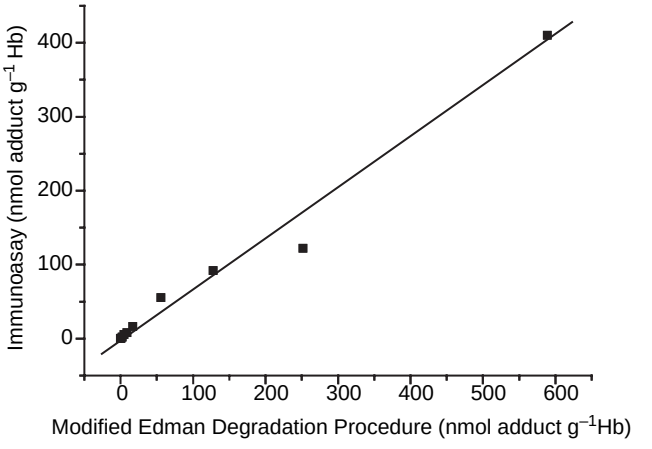

Figure 3. Immunoassay versus the modified Edman degradation procedure (GC-MS) correlation and scatter diagram. Blood samples ($n = 10$) containing N-(2-hydroxyethyl)valine adducts were analysed using the immunoassay and the modified procedure described in the Materials and methods. Comparison of the data yielded a coefficient of correlation of 0.98.

Discussion

The development of simple, rapid and cost-effective biomonitoring methods should enable the introduction of routine biomonitoring programmes and the construction of databases of exposure. Routine biomonitoring should allow the identification of trends and confirm that best working practice is being employed. Furthermore, timely reporting of results on employees involved in hazardous activities or accidental exposures will reassure individuals that safety procedures and personal protective equipment implemented to prevent exposure are effective.

The strong correlation between personal air monitoring data and Hb adducts in persons occupationally exposed to EO has allowed the setting of BELs equivalent to OELs (Boogaard *et al*. 1999). For an average level of exposure to an OEL 1 ppm (8-h TWA) over 4 months, 5 days/week, 8 h/day, the corresponding BEL is 6.8 nmol N-(2-hydroxyethyl)valine g^{-1} Hb.

Using equations developed by Granath *et al*. (1992) and Fennell *et al*. (1992), which account for steady-state exposures and the life-span of an erythrocyte, it is also possible to estimate the size of an acute exposure to EO. For example, Tates *et al*. (1995) estimated that workers had received accidental exposures of 29–444 ppm EO (adduct concentrations 1461–19 931 pmol N-(2-hydroxyethyl)valine g^{-1} globin) from a leaking pump in a chemical manufacturing plant.

The modified Edman degradation procedure developed by Tornqvist *et al*. (1986) is sensitive and specific allowing the determination of background adduct concentrations and has been the method of choice for researchers in this field including Van Sittert and Van Vliet (1994), Angerer *et al*. (1998) and Boogaard (2002). Unfortunately, the modified Edman degradation procedure involves a laborious and complex work-up of samples before analysis, and the analytical methodology requires a relatively sophisticated analytical laboratory. These and other factors limit the usefulness of the method, particularly in large-scale screening programmes.

By contrast, antibodies allow the development of simple, easy-to-perform immunoassays. Immunogen design is a key step in the production of antibodies with the characteristics required for the development of a sensitive and specific immunoassay. Immunization with EO-treated Hb induces antibodies that recognize both EO adducted and non-adducted Hb (unpublished data). To focus the immune response, heptapeptides containing the amino acid sequence that corresponds with the N-terminus of the alpha chain of human Hb were prepared by solid-phase peptide synthesis. (Concurrent studies suggest that immunization with adducted tripeptides induces a weak response in rabbits producing polyclonal antisera containing low-affinity antibodies; unpublished data.)

During solid-phase peptide synthesis, the amino acid chain is grown from the C-terminus. However, we could not couple N-(2-hydroxyethyl)valine during heptapeptide synthesis without protecting the hydroxy group as a benzyloxy derivative.

Compounds of low molecular weight (i.e. <1000) are not normally immunogenic, but can be rendered antigenic by coupling to carrier proteins. It is generally accepted that antibody specificity is greatest for that part of the molecule furthest from the carrier protein. N-(2-hydroxyethyl)heptapeptide was therefore coupled through the free carboxylic acid at the C-terminal to HSA.

Immunization with N-(2-hydroxyethyl)heptapeptide HSA conjugate induced antibodies that demonstrate a high affinity and specificity for exogenous N-(2-hydroxyethyl)heptapeptide. This allowed an ELISA with a limit of detection of 0.015 pmol N-(2-hydroxyethyl)heptapeptide ml^{-1} to be developed (figure 1).

Antisera that recognized exogenous N-(2-hydroxyethyl)heptapeptide could also recognize the N-(2-hydroxyethyl)heptapeptide sequence in purified whole human Hb (figure 2). Demonstrating antibodies can bind with high-affinity N-terminal amino acids sheltered in the central groove of the Hb.

Calibration curves prepared with whole adducted Hb had a limit of detection of 0.022 pmol ml^{-1} N-(2-hydroxyethyl)valine. In a comparative study, Hb samples ($n=12$) whose N-(2-hydroxyethyl)valine concentration had been determined by gas chromatography-mass spectrometry (120–8504 pmol g^{-1} globin) were measured by immunoassay. The correlation between the two methods was 0.97. The results show a good agreement, demonstrating the validity of the approach — the measurement of N-(2-hydroxyethyl)valine adducts in whole Hb.

The determination of N-terminal adducted valine in whole blood would further facilitate sample preparation. By employing antisera R1, strong matrix effects were overcome (e.g. high non-specific binding) allowing blood samples to be analysed after dilution in distilled water. Calibration plots prepared in a whole blood matrix have a limit of detection of 10 pmol N-(2-hydroxyethyl)heptapeptide g^{-1} Hb, the value of the lowest standard.

Whole blood samples analysed by ELISA and the modified Edman degradation procedure over the concentration range 0.04–589 nmol N-(2-hydroxyethyl)valine g^{-1} Hb are in good agreement (correlation coefficient 0.98, $n=10$). Figure 3 suggests a possible low bias in the immunoassay data when compared with the results of the Edman degradation procedure. This suggestion could be due to the relatively few data points ($n=10$), especially at higher adduct concentrations, or

in the assignment of the standard values. Investigations are currently on going to substantiate this.

The inter- and intra-variance data indicate the repeatability of the ELISA over the assay conditions employed and show it is robust over its working range (10–10 000 pmol N-(2-hydroxethyl)valine g^{-1} Hb). When using a one-coated microtitre plate, the assay generates results on 20 samples, eight standards and two quality controls. At present, the assay possesses an overnight primary incubation, but this can be reduced allowing the assay to be completed in 4 h. Several assays can be carried out readily, in parallel, enabling the rapid throughput of a large batch of samples such as might be encountered in a screening programme.

Previously, sample preparation has required the collection of relatively large volumes (10–20 ml) of blood. Immunoassay of whole blood is performed on a small sample volume and in the future this might allow the sample (50–100 μl) to be taken by a finger prick. This will be the subject of a future investigation.

The determination of N-(2-hydroxyethyl)valine in whole blood by immunoassay has shown the presence of adducts in both non-occupationally exposed populations (14–43 pmol g^{-1} Hb) and potentially exposed (42–115 pmol g^{-1} Hb) workers, indicating that the occupational exposures are well below an OEL of 1 ppm.

Furthermore, the adduct concentrations are comparable with those previously reported in the literature. For example, in three studies, the average background for non-occupationally exposed non-smokers was between 20 and 52 pmol g^{-1} globin, increasing by 11 pmol g^{-1} globin per cigarette smoked/day (Tornqvist 1991). Angerer *et al*. (1998) measured workplace EO air concentrations (0.2–8.5 ppm) and adduct levels in regularly (5219–32 738 pmol g^{-1} globin) and occupationally (518–3321 pmol g^{-1} globin) exposed workers. Boogaard *et al*. (1999) determined the adduct concentration in samples collected from the operators in an ethylene glycol/glycol ether plant. The values recorded ranged from 12 to 320 pmol g^{-1} globin (median of 46 pmol g^{-1} globin), indicating little or no exposure had occurred.

The measuring range of the immunoassay and the increase in adduct concentration associated with occupational exposure to EO confirm the potential usefulness of the immunoassay as a biomonitoring tool. Using Boogaard *et al*.'s (1999) correlation of airborne exposure and adduct concentration, the immunoassay might then be used to determine average continuous exposures to EO of less than 0.01 ppm (8-h TWA, 5 days/week).

In conclusion, this paper suggests that a carefully designed hapten–protein conjugate and a judicious screening strategy can be used to produce antibodies that recognize N-terminal adducted valine in whole Hb. A sensitive and specific ELISA has been characterized and validated that may be used to monitor occupational exposure to EO. The development of a simple, rapid and cost-effective immunoassay should enable the introduction of routine screening programmes. The construction of databases should facilitate the comparison of exposures during different industrial processes and practices, and improve the assessment of human health risks. A field trial will now be undertaken.

Acknowledgements

Shell International Chemicals, Lyondell Chemical Co. and BASF AG supported the study financially. Thanks are also due for their interest, helpful suggestions and constructive involvement throughout the project.

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