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

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Abstract

Propylene oxide (PO) is an important industrial compound and a directly acting mutagen. Human exposure to PO can be monitored by the determination of haemoglobin adducts. An immunoassay that quantifies the N-terminal adduct N-(2-hydroxypropyl)valine in whole haemoglobin was developed and its potential usefulness as a tool for biologically monitoring occupational exposure was demonstrated. Analytical reliability was confirmed in a comparative study with GC-MS (range 3.7–992 nmol g^{-1} haemoglobin (Hb), correlation coefficient 0.99, $n = 10$). The assay has been configured as a competitive enzyme-linked immunosorbent assay to facilitate the rapid throughput of samples. The assay employs a whole blood matrix and has a working range of 2–250 pmol g^{-1} Hb. It does not appear to be affected by structurally similar metabolites and has been used to determine adducts in human blood samples. The first results in potentially exposed workers indicate the assay's high potential usefulness in routine occupational biomonitoring of exposure to PO.

Keywords: *Propylene oxide, haemoglobin adducts, immunoassay, biological monitoring.*

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Introduction

Propylene oxide (PO) is an important industrial compound. Its major use is in chemical synthesis where it is used in the manufacture of polyethers, propylene glycol, dipropylene glycol and glycol ethers. Polyethers are the primary component of polyurethane foams. PO is also used in the fumigation of foodstuffs and as a sterilant by the healthcare industry. PO occurs in car exhausts and cigarette smoke (US Environmental Protection Agency 1987).

Since PO has a vapour pressure of 445 mmHg at 20°C, occupational exposure due to inhalation can occur (Merck Index 1989). In addition, dermal exposure may occur during the production, storage, transport and use PO. PO is classified by the International Agency for Research on Cancer (1994) as a possible (2B) human

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carcinogen and as a result of this there is a need to monitor exposure to this chemical in the work place.

The current occupational exposure limits (OEL, 8-h TWA) in the UK (maximum exposure limit, MEL) and USA (permissible exposure limit, PEL) are 5 ppm (12 mg m^{-3}) and 2.5 ppm (6 mg m^{-3}), respectively (ACGIH 1998, UK Health and Safety Executive 1999). To comply with limit values of 5 and 2.5 ppm exposures must be, for most of the time, well below these concentrations. Methods sensitive enough to measure below 2.5 ppm are therefore required.

The measurement of haemoglobin adducts as a tool for determining human exposure to carcinogenic substances was recently reviewed by Boogaard (2002) and Törnqvist et al. (2002). The Modified Edman Degradation Procedure (Törnqvist et al. 1986) used to determine N-alkylvalines is a sensitive analytical method allowing the determination of N-(2-hydroxypropyl)valine adducts in non-occupationally exposed populations.

Adducts formed by the reaction of haemoglobin with small molecules such as propylene oxide are chemically stable and do not effect the erythrocytes average life span of approximately 126 days to 4 months (Britton et al. 1991). Using the Modified Edman Degradation procedure, Boogaard et al. (1999) investigated adduct levels in potentially exposed workers and found a strong correlation between PO adduct concentration and time-integrated exposure demonstrating the usefulness of adduct determination in occupational exposure monitoring and health surveillance programmes.

Furthermore, Boogaard et al. have calculated biological exposure limits (BELs) corresponding to an average level of exposure to an OEL over 4 months, 5 days $week^{-1}$ and 8 h day^{-1}. For OELs of 5 and 2.5 ppm, the BELs were 6400 and 3200 pmol N-(2-hydroxypropyl)valine g^{-1} globin, respectively.

Unfortunately, the determination of haemoglobin adducts by the Modified Edman Degradation Procedure requires a complex and time-consuming work-up of samples before analysis and the use of sophisticated and expensive analytical equipment. These factors limit the usefulness of the method, particularly in large-scale screening programmes.

The use of immunoanalytical methods for the detection of biomarkers offers the prospect of an alternative approach (Van Welie et al. 1992). The introduction of simple, rapid and cost-effective immunoassays for determining haemoglobin adducts should allow the introduction of routine screening programmes.

Recently, an immunoassay that quantifies N-(2-hydroxyethyl)valine in whole blood has been described (Ball et al. 2004) and its potential usefulness as tool for monitoring occupational exposure to ethylene oxide demonstrated. The development of an immunoassay for the determination of the haemoglobin adduct N-(2-hydroxypropyl)valine would greatly facilitate PO exposure monitoring. This paper describes the production of a polyclonal antiserum that recognizes N-(2-hydroxypropyl)valine and the development of a simple, rapid and cost-effective immunoassay for determining occupational exposure to PO.

Materials and methods

Polyclonal antibodies were raised to N-(2-hydroxypropyl)valine in the form of the N-terminal heptapeptide from the alpha chain of human haemoglobin. Both the

N-(2-hydroxypropyl)heptapeptide and the non-modified analogue were prepared by chemical synthesis. Due to its low molecular weight, and therefore limited antigenicity, the adducted heptapeptide was coupled to carrier protein before immunization.

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

L-valine methyl ester hydrochloride (3.19 g, 0.019 moles) was dissolved in dry methanol (40 ml) and neutralized by the slow addition of 3 ml sodium methoxide (pH 8). The mixture was reacted for 20 min with stirring under an atmosphere of dry nitrogen. PO (1.1 g, 1.28 ml, 0.019 moles) was added and the mixture was reacted at 45°C with continuous stirring for 5 days.

The reaction was monitored by TLC, using silica gel 254F plates (0.2 mm thick, 20 × 5 cm) and 1:1 petroleum ether 30–60°:diethyl ether as the mobile phase. Plates were developed using either iodine vapour or 4% phosphomolybdic acid in ethanol. TLC demonstrated two major reagents in the reaction mixture–valine methyl ester ($R_f = 0.40$) and N-(2-hydroxypropyl)valine methyl ester ($R_f = 0.67$).

Salt was removed from the reaction mixture by filtration and the methanol was removed from the reaction mixture by rotary evaporation. To ensure complete removal of the salt, the reaction mixture was suspended in ethyl acetate and filtered again. The ethyl acetate was removed by rotary evaporation.

The reaction products were separated by silica gel chromatography. The reaction residue was dissolved in mobile phase (7 ml) of mixed solvent 1:2 petroleum ether 30–60°:diethyl ether and loaded on to a silica gel column (Kieselgel Merck Type 9385, 230–400 mesh 60A, 14 × 6.5 cm). Fractions (20 ml) were collected from the column and the position of the reaction products determined by TLC.

Fractions containing the N-(2-hydroxypropyl)valine methyl ester were pooled and the solvent removed by rotary evaporation.

The N-(2-hydroxypropyl)valine methyl ester was dissolved in 20 ml distilled water and stirred at 45°C for 3 days. After hydrolysis the water was removed by freeze drying to give a white solid (1.1 g, 33% yield).

Mass spectrometry (m = 118) and NMR studies (shift (ppm) 0.90 (d, 3H, $\mathbf{CH_3}CHCH_3$), 0.96 (d, 3H, $CH_3CH\mathbf{CH_3}$), 1.2 (d, 1H, $CH_3\mathbf{CH}$), 2.2 (m, 1H, $CH_3\mathbf{CH}CH_3$), 2.8–3.0 (dd, 1H, H of $\mathbf{CH_2}$), 3.1–3.2 (dd, 1H, H of $\mathbf{CH_2}$), 3.5 (d, 1H, **CH**NH), 4.0 (m, 1H, **CH**OH), 4.8 (s, 3H, $\mathbf{CH_3}CH$)) are consistent with a pure product with the structure of N-(2-hydroxypropyl)valine.

Peptide synthesis

Heptapeptide and N-(2-hydroxypropyl)heptapeptide containing the amino acid sequence found at the N-terminus of the alpha chain of human haemoglobin were prepared by solid-phase peptide synthesis. Heptapeptide was prepared according to Ball et al. (2004).

N-(2-hydroxypropyl)Val-Leu-Ser-Pro-Ala-Asp-Lys was synthesized by the addition of N-(2-hydroxypropyl)valine to the growing peptide chain. The adducted heptapeptide was purified by HPLC (column; C18, 4.6 mm × 25 cm. Solvent A: 0.1% TFA in water. Solvent B: 0.1% TFA in acetonitrile. Gradient: 1–30% B in 30 min. Detection:

UV 230 nm. Flow: 1.5 ml min^{-1}). HPLC purification yielded a product of 83% purity.

The molecular weight of the product was determined by time of flight mass spectroscopy and found to be consistent with the assigned structure (N-(2-hydroxpropyl)heptapeptide MW 786).

Amino acid analysis of the N-(2-hydroxypropyl)heptapeptide (Asp 1.05; Ser 0.87(Ser degrades during acid hydrolysis); Pro 1.02; Ala 0.93; Leu 0.98; Lys 1.02; adducted Nal n/d) confirms the identity of the product.

Protein conjugation

N-(2-hydroxypropyl)heptapeptide was conjugated to bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH).

BSA and KLH were dissolved in distilled water to give 10 and 5 mg ml^{-1} solutions, respectively. N-(2-hydroxypropyl)heptapeptide was dissolved in distilled water (10 mg ml^{-1}). To 8.25 mg BSA (1.3×10^{-7} moles) and KLH was added 2.5 mg (3.2×10^{-6} moles) of N-(2-hydroxypropyl)heptapeptide. 1-Ethyl-3-diamino-(3-dimethylaminopropyl)carbodiimide (EDC) was dissolved in distilled water (160 mg ml^{-1}) and added immediately to the protein–heptapeptide 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 12 000–14 000 daltons) and extensively dialysed against PBS. Protein concentrations were determined from OD readings at 280 nm. After coupling, the concentrations of the BSA and KLH conjugates were approximately 7.0 and 3.0 mg ml^{-1} respectively (recoveries of 90–95%).

Peptide–protein conjugates were stored at −70°C until use. The KLH conjugates were used for immunization. BSA conjugates were used for coating ELISA plates during antibody screening and assay development.

Antibody production

Immunization protocol. Polyclonal antibodies were produced in rabbits. Primary immunizations (day 1) and secondary immunizations (day 21) were given subcutaneously. Each animal received 100 μg KLH N-(2-hydroxypropyl)valine conjugate in 200 μl of a 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-hydroxypropyl)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-hydroxypropyl)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-reactivities. N-(2-hydroxypropyl)heptapeptide, N-(2-hydroxyethyl)heptapeptide and heptapeptide standards were prepared in PBS.

The 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-hydroxypropyl) 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 were 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) was 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, Raleigh Court, Crawley, UK).

Haemoglobin analysis

PO-adducted haemoglobin was prepared by the reaction of human haemoglobin with PO overnight at 37°C. PO was reacted with haemoglobin in the ratio of 100:1 moles. Haemoglobin standards were prepared by the dilution of adducted haemoglobin (in non-adducted haemoglobin). Antibody binding of N-(2-hydroxypropyl)heptapeptide was assessed in competitive binding studies.

Whole blood (24 ml) containing approximately 3.8 g (56 µmol) haemoglobin was reacted with 10 µl (200 µmol) PO overnight at 37°C. Unreacted PO was allowed to evaporate and the N-(2-hydroxypropyl)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 microtitre plates coated with 2 µg ml^{-1} adducted haemoglobin (in carbonate/bicarbonate buffer, pH 8.6, overnight at 4°C). The assay was developed using R2 (bleed 4) diluted 1:8000 in PBS/Tween (0.2%).

Whole blood analysis

Samples ($n = 10$) containing high adduct concentrations were prepared by the dilution of the PO adducted blood in the blood of a non-occupationally exposed individual. N-(2-hydroxypropyl)valine adduct concentration was determined using the Modified Edman Degradation Procedure (Boogaard et al. 1999).

Samples ($n = 18$) of whole blood from potentially exposed workers were collected from the antecubital vein into a Vacutainer™ containing K_3 EDTA 15% as anticoagulant (about 7 ml). Samples were collected using syringes and needles sterilized with gamma-radiation. Samples were stored refrigerated at 4°C. Studies in this laboratory show samples stored in this way are stable for at least 6 months. Samples were analysed by both ELISA and the Modified Edman Degradation Procedure.

The ELISA was performed using six standards (2–250 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin in a whole blood matrix), two quality controls and 22 samples in duplicate per assay plate. Calibration plots were developed using antiserum R2 (bleed 4) diluted 1: 8000 in PBS/Tween (0.05%).

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

Results

Polyclonal antisera that recognize conjugated N-(2-hydroxypropyl)heptapeptide were produced in rabbits. Antibody titre, the antiserum dilution at which 50% of the maximum binding occurs, is shown in Table I. Rabbit 2 (R2) gave the greatest antibody titre (1:1500).

Competitive inhibition studies show all the antiserum contained antibodies that recognize exogenous N-(2-hydroxypropyl)heptapeptide (Table I). Calibration plots developed over the range 0.1–100 pmol N-(2-hydroxypropyl)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 R2 gave the lowest limit of detection (0.2 pmol N-(2-hydroxypropyl)valine ml^{-1}).

None of the antiserum cross-reacted with either heptapeptide or N-(2-hydroxyethyl) heptapeptide in the measuring range of the assay. Cross-reactivity did occur when heptapeptide and N-(2-hydroxyethyl)heptapeptide were present in large molar excess. Antiserum R2 demonstrated the greatest relative specificity. Antiserum 2 showed no cross-reactivity with non-adducted heptapeptide (up to 100 000 pmol ml^{-1}). The IC_{50} values, the standard concentration that reduces binding by 50% in an ELISA, with N-(2-hydroxypropyl)valine N-(2-hydroxyethyl)valine, was 10 and 1100 pmol ml^{-1}, respectively.

In competitive binding studies, all four antisera could distinguish between adducted and non-adducted human haemoglobin. Immunoassays can be performed using either PO adducted haemoglobin or N-(2-hydroxypropyl)heptapeptide conjugate to coat the assay wells.

Adducted haemoglobin prepared by the reaction of human blood with propylene oxide contained 5.8 μmol N-(2-hydroxypropyl)valine g^{-1} globin. All the antisera recognized N-(2-hydroxypropyl)valine in a whole blood matrix.

Several problems associated with assaying whole blood have been overcome and an immunoassay that performs in a whole-blood matrix has been realized. An ELISA, developed with antiserum R2 (bleed 4), had a measuring range of 2–250 pmol

Table I. Characterization of an N-(2-hydroxpropyl)heptapeptide antiserum obtained from a sheep immunized with KLH N-(2-hydroxpropyl)heptapeptide conjugate (see the Materials and methods for details).

		% Inhibition	
Rabbit	Titre	N-(2-hydroxypropyl) heptapeptide*	Heptapeptide**
1	1:390	53%	0%
2	1:1500	82%	3%
3	1:40	54%	0%

*100 pmol/ml N-(2-hydroxypropyl)heptapeptide
**100 000 pmol/ml heptapeptide

N-(2-hydroxypropyl)valine g^{-1} haemoglobin (Figure 1). Samples of whole blood ($n=10$) containing between 3.7 and 992 nmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin were determined by immunoassay and the Modified Edman Degradation Procedure. The correlation coefficient between the two methods was 0.99 (Figure 2).

Samples of blood from potentially exposed workers contained N-(2-hydroxypropyl)valine adducts. Results obtained by ELISA (range 4–14 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin, median = 8, $n=18$) and the Modified Edman Degradation Procedure (range 0–18 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin, median = 10, $n=18$) are in good agreement. The results suggest no endogenous components of blood are recognized by the antibody or interfere with antibody-analyte binding. The low adduct concentrations in these samples confirm that no significant exposures have occurred.

With-in assay percentage coefficients of variation (CV) of 3.5–5.5% were obtained over the concentration range 10.3 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin (SD 0.5, $n=11$) to 48.5 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin (SD 2.6, $n=12$). Between-assay percentage coefficients of variation for two blood samples were 10.6% (mean = 8.4 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin, SD = 0.9, $n=5$) and 6.2% (mean = 51 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin, SD = 3.2, $n=6$,) respectively.

Discussion

The ACGIH (1998) and IARC (1994) have classified PO as an animal carcinogen (3A) and a possible (2B) human carcinogen, respectively. Although several authoritative bodies have evaluated the genotoxicity of PO and concluded that there is no evidence, or insufficient evidence, for the carcinogenic effects of PO in humans pressure remains nevertheless to lower the OELs for PO.

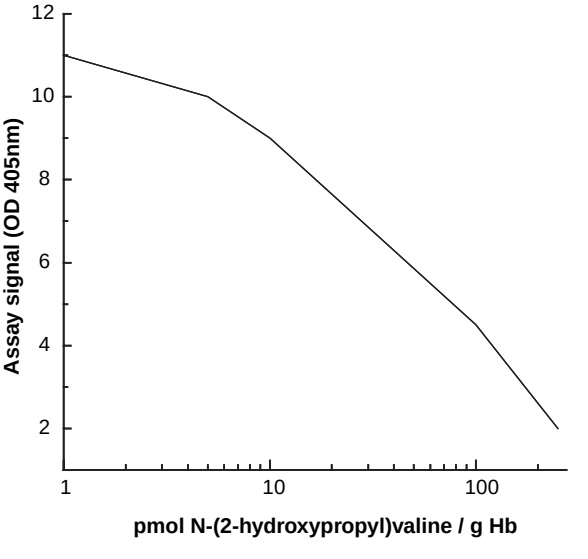

Figure 1. Calibration plot showing pmol N-(2-hydroxpropyl)valine per g haemoglobin developed by ELISA in a whole blood matrix as described in the Materials and methods.

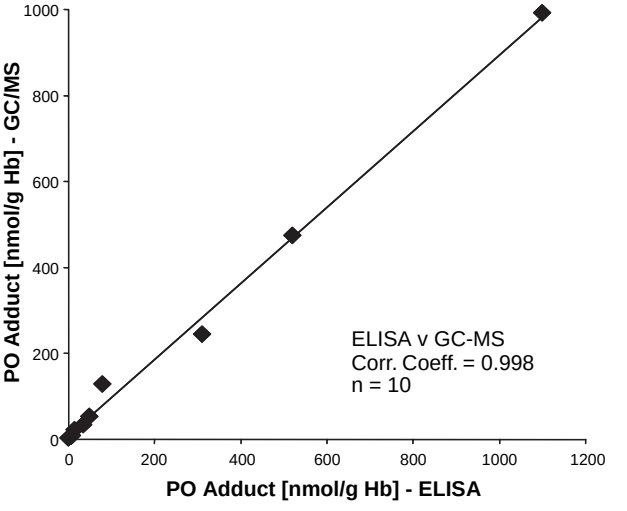

Figure 2. Immunoassay v GC/MS correlation and scatter diagram. Blood samples ($n = 10$) containing N-(2-hydroxpropyl)valine adducts were analysed using the immunoassay and GC/MS described in the Materials and methods. Comparison of the data yielded a coefficient of correlation of 0.99.

The determination of haemoglobin adducts has proved a useful tool for measuring human exposure to electrophilic chemicals or chemicals which during metabolism give rise to electrophilic species. Quantification of the N terminal valine adduct N-(2-hydroxypropyl)valine using the Modified Edman Degradation Procedure has allowed the measurement of adducts in non-occupationally exposed individuals (Törnqvist 1991, Van Sittert & Van Vliet 1994).

The strong correlation between personal air-monitoring data and haemoglobin adducts has allowed the setting of BELs equivalent to OELs (Boogaard et al. 1999). For an average level of exposure to an OEL of 5 ppm over 4 months, 5 days week^{-1}, 8 h day^{-1}. The corresponding BEL is 6400 pmol N-(2-hydroxypropyl)valine g^{-1} globin.

Unfortunately, the Modified Edman procedure involves a laborious and complex work-up of samples before analysis and the methodology requires a relatively sophisticated and expensive analytical laboratory. These factors limit the usefulness of the method, particularly in large-scale screening programmes.

By contrast, antibodies allow the development of simple, rapid and cost-effective immunoassays. Immunogen design is a key step in the production of antibodies. To focus the immune response, heptapeptides containing the amino acid sequence found at the N-terminal of the alpha chain of human haemoglobin were prepared by solid-phase peptide synthesis. During solid-phase peptide synthesis the chain is grown from the C terminus, the N terminal valine been added in the final steps. However, the addition of N-(2-hydroxypropyl)valine was slow and the yields were low (less than 40%). This may be improved by the use of appropriate protection chemistry during synthesis.

Compounds of low molecular weight (< 1000 daltons) are not normally immunogenic. However, low molecular weight substances (haptens) can be rendered antigenic by coupling to carrier proteins such as albumins and haemocyanins — antibody specificity being generally directed to that part of the hapten molecule furthest from

the site of attachment to the carrier protein. Peptides were therefore coupled to carrier proteins through the free carboxylic acid at the C terminus of the molecule using 1-ethyl-3-diamino-(3-dimethylpropyl)carbodiimide.

Immunization with N-(2-hydroxypropyl)heptapeptide coupled to carrier protein induced the production of high-affinity and high-specificity antibodies to N-(2-hydroxyproyl) heptapeptide (Table I). An ELISA that measures N-(2-hydroxypropyl) heptapeptide in the range of 0.2–120 pmol ml^{-1} has been developed. The assay shows no cross-reactivity with heptapeptide (cross-reactivity > 100 000 pmol ml^{-1}) and limited cross-reactivity with N-(2-hydroxyethyl)heptapeptide (100-fold molar excess) allowing an ELISA with a limit of detection of 0.2 pmol N-(2-hydroxypropyl) heptapeptide ml^{-1} to be developed.

Adducted haemoglobin was prepared by the reaction of haemoglobin with propylene oxide. Antibodies that appeared specific for the adducted peptides also bound the adducted haemoglobin in antibody binding studies, allowing ELISAs to be performed using either N-(2-hydroxpropyl)heptapeptide conjugate or PO-adducted haemoglobin to coat the assay wells.

The determination of N-terminal adducted valine in whole blood would further facilitate sample preparation. A number of matrix effects were overcome (e.g. high non-specific binding) by formulation of the assay buffers and blood samples can now be analysed after dilution in distilled water. Calibration plots prepared in a whole blood matrix have a limit of detection of 2 pmol N-(2-hydroxypropyl)heptapeptide g^{-1} haemoglobin the value of the lowest standard (Figure 1).

Whole blood samples analysed by ELISA and the Modified Edman Degradation Procedure over the concentration range 3.7–992 nmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin are in good agreement (Figure 2, correlation coefficient 0.998, $n = 10$). 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 (2–200 pmol N-(2-hydroxpropyl)valine g^{-1} haemoglobin). Using one coated microtitre plate, the assay generates results on 22 samples, six standards and two quality control samples. 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 through put of a large batch of samples such as may be encountered in a screening programme.

Due to the multi-stage sample preparation inherent in earlier methods for the determination of N-terminal adducted valines in haemoglobin, the analysis required the collection of relatively large volumes (10–20 ml) of blood. Determination by immunoassay allows the analysis to be performed on a small volume of sample (i.e. 10–100 μl). This provides the opportunity to simplify sample collection and increase the accessibility of the test. The collection of blood samples by finger prick will be the subject of a future investigation.

The determination of N-(2-hydroxypropyl)valine in whole blood, by immunoassay, has shown the presence of adducts in potentially exposed populations. The adduct concentrations, determined by immunoassay were between 4 and 14 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin (median = 8 pmol N-(2-hydroxypropyl)-valine g^{-1} haemoglobin, $n = 18$) (Table I) indicating that the occupational exposures are well below an OEL of 1 ppm. Results determined by GC-MS appear to be in good agreement (range 0–18 pmol N-(2-hydroxypropyl)valine g^{-1} haemoglobin, median = 10, $n = 18$).

Furthermore, the adduct concentrations are comparable with those previously reported in the literature. For example, the average background for non-occupationally exposed non-smokers was 1.9 pmol g^{-1} globin increasing by 2 pmol g^{-1} globin per ten cigarettes smoked per day (Törnqvist 1991). Van Sittert and Van Vliet (1994) determined the adduct concentration in samples collected from the operators in the manufacture and transport of PO. The averages recorded for workers in manufacture were 12 pmol g^{-1} globin (range 0–150) and in two loading operations were 4 pmol g^{-1} globin (range 0 –42) and 11 pmol g^{-1} globin (range 0–137), respectively.

The measuring range of the immunoassay and the increase in adduct concentration associated with occupational exposure to PO confirm the potential usefulness of the immunoassay as a biomonitoring tool. Employing Boogaard et al.'s (1999) BEL, the immunoassay may be used to determine average continuous exposures to PO of less than 0.01 ppm (8 h TWA, 5 days $week^{-1}$).

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-(2-hydroxypropyl)valine in whole haemoglobin. A sensitive and specific immunoassay has been characterized and validated that may be used to monitor occupational exposure to PO.

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. The construction of databases should facilitate the comparison of exposures during different industrial processes and practices, allow the identification of trends and confirm that best working practice is being employed. Furthermore, the 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. A field trial will now be undertaken.

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