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Quantification method of human hemoglobin adducts from hexahydrophthalic anhydride and methylhexahydrophthalic anhydride

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Abstract

A method was developed for the determination of human hemoglobin (Hb) adducts from hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA). The procedure includes lysis of erythrocytes, dialysis of the Hb-solution followed by acid hydrolysis. The released hexahydrophthalic (HHP) acid and methylhexahydrophthalic (MHHP) acid were purified using a combined liquid–liquid and solid-phase extraction procedure followed by derivatization with pentafluorobenzyl bromide. The derivatives were analyzed using GC–MS in negative ion chemical ionization mode with ammonia as moderating gas. As internal standards, deuterium-labeled HHP and MHHP acids were used. The detection limits were 0.3 pmol/g Hb for HHP acid and 0.9 pmol/g Hb for MHHP acid. The between-day precisions for HHP acid were 18% at 2 pmol/g Hb and 10% at 13 pmol/g Hb. For MHHP acid, the precision was 27% at 2 pmol/g Hb and 14% at 22 pmol/g Hb. The method was applicable for analysis of Hb adducts from workers occupationally exposed to HHPA and MHHPA.

Keywords: Hemogloblin; Hexahydrophthalic anhydride; Methylhexahydrophthalic anhydride

1. Introduction

Organic acid anhydrides (OAAs) are commonly used compounds in industry. Many OAAs are strong sensitizers which induce the formation of specific immunoglobulin (Ig) E and IgG antibodies in a large fraction of exposed workers [1]. Hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA) are known to be particularly strong sensitizers [2].

It is commonly believed that the reaction of the OAAs to proteins is an important step in the pathomechanism behind their sensitizing potential. Thus, studies of protein adducts of these compounds may provide information on the induction of type-1 allergies. However, there are only few reports of protein adducts of allergenic compounds [3–10]. Methods for the determination of adducts of OAAs to proteins have previously been published. Jönsson et al. [4] described a method for analysis of methyltetrahydrophthalic anhydride adducted to lysine in collagen in guinea pig lung. A method for determination of HHPA adducted to Hb has also been briefly described by Jönsson et al. [8].

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In 1974, Ehrenberg et al. [11] suggested that protein adducts could be used for assessment of cumulated exposures to carcinogenic compounds. Since then, many papers have reported that the levels of adducts of a number of carcinogens may be used as biomarkers of cumulated exposures during weeks or months [12]. Thus, methods for determination of adducts of OAAs may, in addition to pathomechanistic studies, also be used for biological monitoring of exposure. Methods for biological monitoring of HHPA and MHHPA by analysis of free hexahydrophthalic (HHP) and methylhexahydrophthalic (MHHP) acids in plasma and urine has been described in previous studies [13–15]. However, the half-lives of these metabolites are short and the methods only reflect the exposure during the last day. Since Hb adducts have a potential to show on exposures during the last 120 days, methods for analysis of these OAA adducts would therefore be of great importance, e.g., in dose–response studies.

The aim of this study was to develop and evaluate a method for simultaneous determination of the Hb adducts from both HHPA and MHHPA.

2. Experimental

2.1. Apparatus

The gas chromatography–mass spectrometry (GC–MS) system consisted of a VG Trio 1000 quadrupole MS (Fisons, Manchester, UK) connected to a Carlo-Erba 8065 GC equipped with an A200S autosampler (Carlo-Erba, Milan, Italy). The analytical column was a fused-silica capillary column (40 m×0.18 mm I.D.) with a DB-5 MS stationary phase and a film thickness of 0.18 μm (J&W Scientific, Folsom, CA, USA). For the purification of HHPL, a Hewlett-Packard 1050 LC system was used. The column was from Jones Chromatography (5 μm Apex octadecyl, 25 cm×10 mm I.D.). For the solid-phase extraction (SPE) procedure, C_{18} Bond Elut columns (100 mg; Varian, Palo Alto, CA, USA) were used. The SPE columns were connected to a Cerex SPE processor (Varian) operated at 100 kPa of nitrogen gas. The Hb conjugate was purified using an Amicon ultrafiltration cell equipped with an Omega 30 kDa filter (Amicon, Denver, MA, USA). A Sigma 3E-1 centrifuge (Sigma, Deisenhofen, Germany) was used for phase separations.

2.2. Materials

Dialysis tubing was from SpectraPore (Spectrum, Houston, TX, USA) with a volume of 2.0 ml/cm and a cut off at 12–14 kDa. Blood was collected in 10-ml Venoject blood sampling tubes (Terumo Europe, Leuven, Belgium) containing sodium heparin.

2.3. Chemicals

Hydrochloric acid and sodium chloride were from Arcos Organics (Geel, Belgium; analytical-reagent grade quality). Ammonium hydrogen carbonate was from BDH (Poole, UK). Di-sodium hydrogenphosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$; ACS reagent grade), tetrabutylammonium hydrogen sulfate (TBA; 97%), pentafluorobenzyl bromide (PFBBr), potassium chloride (ACS reagent grade), potassium di-hydrogen phosphate trihydrate ($KH_2PO_4 \cdot 3H_2O$; ACS reagent grade) and tri-sodium phosphate dodecahydrate ($Na_3PO_4 \cdot 12H_2O$; ACS reagent grade) were from ICN (Costa Mesa, CA, USA). Dichloromethane, ethyl acetate, hexane, methanol and toluene were of analytical grade; acetonitrile was of HPLC quality and were all from LabScan (Dublin, Ireland). MHHPA (>95%) was from Ciba-Geigy (Basel, Switzerland). Acetic acid, dioxane, HHPA (>98%) and sodium hydroxide were from Merck (Darmstadt, Germany). Hb, Nα-*tert*.-BOC-L-lysine and trifluoroacetic acid (TFA) were from Sigma (St. Louis, MO, USA). The synthesis of $^2H_6$-labeled HHP acid and $^2H_8$-labeled MHHP acid was performed in the Department's laboratory and assisted by Synthelec (Lund, Sweden) as previously described in Ref. [16]. The water was purified using a Millipore (Bedford, MA, USA) purification system.

2.4. Reagents and buffers

The 0.13 *M* PFBBr solution was prepared by adding 200 μl PFBBr to 10 ml of dichloromethane. This solution was prepared fresh daily. The 0.1 *M* TBA solution was prepared by adding 3.39 g TBA, 1.79 g $Na_2HPO_4 \cdot 12H_2O$, and 1.90 g $Na_3PO_4 \cdot 12H_2O$

to 100 ml water. Phosphate buffered saline (PBS) was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.3 g $Na_2HPO_4 \cdot 12H_2O$ and 0.2 g $KH_2PO_4 \cdot 3H_2O$ in 1 l water. The Drabkins reagent kit for determination of total Hb was from Sigma.

2.5. Synthesis

2.5.1. Hb–HHPA conjugate

Human Hb (200 mg) was dissolved in 0.1 *M* $NaHCO_3$ (67 ml). HHPA (27.6 mg) dissolved in 1.0 ml dioxane was slowly added to the Hb solution at room temperature. Thereafter, the solution was stirred for 18 h at 4°C. The conjugate was purified from free HHP acid and other low-molecular-mass chemicals via ultrafiltration and the buffer was exchanged by washing five times with 0.1 *M* NH_4HCO_3. The last solution was freeze dried and the conjugate was kept at −20°C until use.

2.5.2. Synthesis of Nε-hexahydrophthaloyl-L-lysine (HHPL)

Nα-*tert*.-BOC-L-lysine (0.5 g) was dissolved in water (30 ml). HHPA (0.6 g) was dissolved in 1 ml acetonitrile and slowly added to the water solution. The pH of the solution was kept at 7–9 by addition of 5 *M* NaOH. The solution was evaporated and the *tert*.-BOC protective group was removed using 20 ml 1 M HCl in acetic acid. The solution was kept at room temperature for 15 min and evaporated. The HHPL was then purified using liquid chromatography (LC). The sample was dissolved in 0.1% TFA in water and 1.0 ml was injected through a loop. The mobile phase was 0.1% TFA for 1 min with a flow of 2 ml/min followed by a linear gradient to 32 min of 0.07% TFA in acetonitrile. HHPL was collected and immediately evaporated. No HHP acid was found by LC–UV detection in the sample after the purification. Standards of the dried HHPL was prepared in 0.02 *M* NH_4HCO_3 and kept at −20°C until use.

2.6. Exposure

The workers investigated were employed in a plant manufacturing electrical capacitors. They were exposed to either HHPA, MHHPA or both. The device was mechanically fixed and electrically insulated by epoxy resins using HHPA or MHHPA as hardeners. The air levels in the plant, monitored by personal sampling, were up to 500 μg HHPA/m^3 and 100 μg MHHPA/m^3. The HHPA and MHHPA exposure of the occupationally non-exposed population can be expected to be so low that this will not contribute to measurable adduct levels.

2.7. Collection of blood samples

Blood samples were collected from an antecubital vein in the Venoject blood sampling tubes. One 10-ml tube is needed for duplicate determinations of the adduct levels. After sampling, the blood was allowed to cool to room temperature and then centrifuged at 1500 *g* for 10 min. The plasma were separated and the erythrocytes were washed three times with an 0.9% sodium chloride solution and then transferred to polyethylene test tubes. The samples were added with an equal volume of water and then stored at −20°C until use.

2.8. Preparation of standards

Hb solutions used for standards were from expired blood samples taken from a blood bank. The erythrocytes were washed three times with 0.9% NaCl and lysed by addition of an equal volume of water and freezing. Thereafter, the solutions were dialysed four times against PBS during seven days. The Hb content was 100–130 g/l.

Solutions of HHP and MHHP acids were prepared by hydrolysis of the corresponding anhydrides in 0.01 *M* NaOH. Solutions of desired concentrations were then prepared by further dilutions in 0.01 *M* NaOH. The standards were prepared by addition of 100-μl aliquots of these solutions to 3.0 ml of the blank Hb-solutions from the blood bank in amounts of 0.2, 0.4, 0.5, 1.0, 2.0, 5.0, 10.0 and 19.9 pmol HHP acid, and of 0.2, 0.4, 0.5, 0.9, 1.8, 4.6, 9.2 and 18.4 pmol MHHP acid.

2.9. Determination of Hb

The Hb content in the dialysed samples was determined using a quantitative colorimetric method. A 20-μl aliquot of the samples was added to Drabkins solution and the absorbance of cyano-

methemoglobin was measured at 540 nm. The between-day precisions of the method were given at concentrations 53, 80 and 161 g/l and the coefficients of variation (C.V.s) according to Sigma Diagnostics (procedure No. 525) were 0.4, 1.0 and 0.4%, respectively.

2.10. Work-up procedure for Hb adducts

The levels of HHPA and MHHPA bound to Hb were determined as follows. The frozen erythrocytes were thawed, frozen and thawed again. The cell debris were removed by centrifugation at 18 000 *g* for 30 min. The samples were dialysed against four changes of PBS during seven days. Aliquots of the dialysed samples were taken for determination of the Hb concentration. The samples (3 ml containing 50–150 g Hb/l) were added with 3.0 ml 0.1 *M* HCl and 100-μl aliquots of an internal standard solution containing 5.6 pmol $^{2}H_{6}$-labeled HHP acid and 5.2 pmol $^{2}H_{8}$-labeled MHHP acid. The adducts were hydrolysed from the Hb at 100°C for 90 min. After the hydrolysis, the samples were extracted two times with 6 ml ethyl acetate. The samples were shaken for 1 min and centrifuged for phase separations at 1500 *g* for 10 min. Occasionally, a gel was formed in the ethyl acetate extraction stage. This was broken by gently shaking the tube followed by centrifugation for 10 min. The combined ethyl acetate phases were evaporated to dryness in a flow of nitrogen gas and heating. The dry residues were dissolved in 5 ml 0.1 *M* HCl. C_{18} Bond Elut columns were conditioned by passing 5 ml of methanol followed by 2 ml of 0.1 *M* HCl through the columns. The samples were then added to the columns, which resulted in the trapping of HHP and MHHP acids. As a washing step, 2 ml of 0.1 *M* HCl with 10% methanol was added. The columns were then dried by the nitrogen gas flow for 1 min. Thereafter, the samples were eluted into test tubes using 2 ml 0.1 *M* HCl with 40% methanol. The eluates were evaporated to dryness in a nitrogen gas flow and the residues were derivatized by addition of 250 μl of 0.1 *M* TBA and 250 μl of 0.13 *M* PFBBr. The samples were vortexed and placed in an ultrasonic bath for 90 min. Then, 2 ml of hexane was added and the samples were vortexed and centrifuged for phase separation for 1 min at 1500 *g*. The test tubes were placed in a freezer at −20°C until the aqueous phases were frozen. Thereafter, the organic phases were poured into other test tubes and evaporated in a dry nitrogen gas flow. The dry residues were dissolved in 150 μl of toluene and transferred to auto-sampler injection vials.

2.11. Analysis of Hb adducts

The samples were analyzed by GC–MS. Samples were injected with a splitless injection technique. The injector temperature was kept at 300°C, the injection volume was 2.0 μl, and the split exit valve was kept closed for 0.5 min after the injection. For the analysis of di-pentafluorobenzyl hexahydrophthalate (PFB-HHP), the initial column temperature was 100°C for 1 min. The temperature was thereafter increased by 20°C/min to 340°C and maintained at that level for 3 min. For the analysis of di-pentafluorobenzyl methylhexahydrophthalate (PFB-MHHP) the initial column temperature was 110°C for 1 min. The temperature was then gradually increased by 10°C/min to 330°C. The MS interface was at 320°C and the ion source at 200°C. The column carrier gas was helium at a pressure of 150 kPa. The MS was in the negative ion chemical ionization (NICI) mode with ammonia as moderating gas. Selected ion monitoring of PFB-HHP was performed at m/z 153 while m/z 159 was chosen for the internal standard. For PFB-MHHP m/z 365 was chosen while m/z 373 was used for the internal standard.

3. Results and discussion

3.1. Dialysis

Blank Hb-solution (3.0 ml) was spiked with 100 pmol of HHP acid and 92 pmol MHHP acid. The samples were then dialysed against PBS for one, two, three and seven days. The PBS was changed once daily. The HHP and MHHP acids levels were close to the detection limit already after one day of dialysis, and below the detection limit after two days. However, a dialysis time of seven days was chosen to assure an efficient removal of low molecular compounds which otherwise could interfere with the analytical matrix.

3.2. Hydrolysis

3.2.1. Hydrolysis of HHPL

OAAs have been extensively used in protein chemistry due to their ability to modify free ε-amino groups of lysine and terminal α-amino groups [17]. Little modification of other amino acid residues has been found. Thus, HHPL was synthesised, and it was investigated at what conditions this conjugate was hydrolysed. The HHPL (18 ng/ml) was treated with HCl at concentrations of 0.05, 0.1, 3 and 6 *M*. The solutions were hydrolysed at 100°C for 1, 10 and 30 h. In this, as well as in the following hydrolysis experiments, the internal standards were added before the hydrolysis. Already after 1 h at 0.05 *M*, 113% of the HHP acid from the HHPL was recovered. After 30 h, the same amount of HHP acid was also recovered. The recovery was slightly lower at 0.1 and 1 *M*. At 6 *M*, 88% of the HHP acid was recovered after 30 h. In an additional experiment, a solution at 0.05 *M* HCl was hydrolysed at 50°C for 1, 10 and 30 h. Under these conditions, 10 h of hydrolysis was required to obtain the same amount of HHP acid as compared with hydrolysis at 100°C and 0.05 *M* HCl at 1 h.

3.2.2. Hydrolysis of Hb–HHPA conjugate

A conjugate between Hb and HHPA was synthesised in order to study the hydrolysis of HHPA from Hb. The Hb–HHPA conjugate was hydrolysed with HCl at various concentrations of acid and temperatures to determine the ideal hydrolysis conditions.

In a first set of experiments, the Hb–HHPA conjugate (1.0 μg) was dissolved in water and hydrolysed at the HCl concentrations of 0.05, 0.1, 1 and 6 *M*. The solutions were hydrolysed at 100°C for 0, 0.2, 1, 4 and 19 h. The maximum yield of free HHP acid was obtained using 0.05 *M* for 19 h (Fig. 1). After 1 h at 0.05 *M*, the amount of the HHP acid recovered was 88% of the yield after 19 h of hydrolysis. The recovery was slightly lower at 0.1 and 1 *M*. At 6 *M* HCl, only 68% HHP acid was recovered after 19 h. Thus, the conditions of the hydrolysis of the Hb–HHPA conjugate seemed to be in accordance with those for HHPL.

In a second set of experiments, the Hb–HHPA conjugate (1.0 μg) was dissolved in blank Hb-solution at the HCl concentrations of 0.05, 0.1 and 1 *M*. The solutions were hydrolysed at 100°C for 1, 10 and 30 h. The maximum yield of free HHP acid was obtained using 0.05 *M* HCl for 30 h. After 1 h at 0.05 *M*, 94% of the HHP acid was recovered compared with hydrolysis for 30 h. The recovery was slightly lower at 0.1 and 1 *M*. In addition, a solution was hydrolysed with 0.05 *M* HCl at 50°C. Under these conditions, 30 h of hydrolysis gave a recovery of 94% HHP acid as compared to the hydrolysis at 100°C and 0.05 *M*. The maximal amount of hydrolysed HHP acid from the samples in Hb-solution was 86% compared with that of hydrolysis in water.

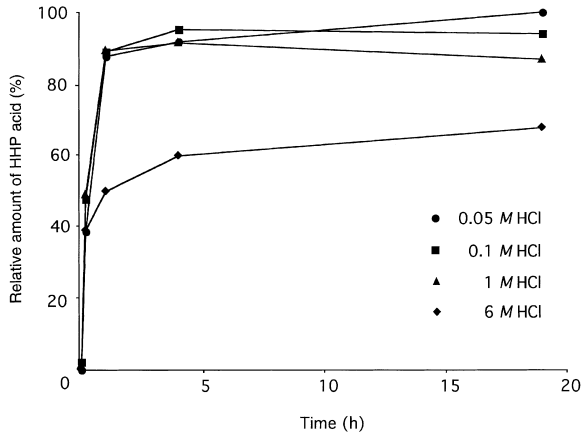

Fig. 1. Relative amount of free HHP acid after hydrolysis of water solutions containing 1 μg Hb conjugate of HHPA in different concentrations of hydrochloric acid. Each mark denotes one single determination.

3.2.3. Hydrolysis of Hb adducts from exposed workers

A Hb-solution, pooled from both HHPA and MHHPA exposed workers, was treated with 0.05, 1 and 6 *M* HCl. The solutions were hydrolysed at 100°C for 0, 1 and 24 h. The maximal amount of hydrolysed HHP acid from the pooled sample was 610 pg after 1 h at 1 *M* HCl. After 1 h at 0.05 *M* HCl, 91% of this was recovered, and after 1 h at 6 *M* HCl, the result was 89%. The maximal amount of hydrolysed MHHP acid from the pooled sample was 340 pg after 1 h at 1 *M* HCl. After 1 h at 0.05 *M* HCl, 95% of this was recovered and after 1 h at 6 *M*

HCl the result was 87%. After 24 h of hydrolysis, interfering compounds made the quantifications uncertain. However, it could be determined that there was no major increase in the levels of HHP and MHHP acids during prolonged hydrolysis times. The levels of HHP and MHHP acids were below the detection limits before the hydrolysis. Thus, the hydrolysis of HHP and MHHP acids from in vivo formed Hb adducts seemed to be similar to that of HHP acid from lysine. However, the results from the experiment using 6 *M* HCl may indicate some minor conjugation with amino acids forming more stable adducts that those from lysine. The results also indicate that the hydrolysis of MHHP acid is similar to that of HHP acid.

3.3. Work-up procedure

Several papers have been published on work-up and derivatization of HHP and MHHP acids in different biological matrices [13,16,18–20]. These were initially tried but found to give insufficient purifications for the determination of the extremely low levels of the adducts. Also, the approach to analyze the anhydride–lysine conjugate according to the method used for MTHPA [4] was initially tried but found to give a very high detection limit. A method aimed at analysis of adducts between HHPA and Hb which is similar to the one here described has been reported by Jönsson et al. [8]. However, the detection limits in that method were not sufficient for analysis of adducts with MHHPA or low levels of HHPA adducts. To improve the purity of the matrix in the present method, only chemicals of the highest purity were obtained when available. Also, in this method, a milder and shorter hydrolysis time was used. In addition, a new washing/elution procedure in the liquid–solid extractions was developed. The washing in this procedure was performed with 10% methanol in 0.1 *M* HCl, while the samples were eluted with 40% methanol in 0.1 *M* HCl. The whole work-up of the samples took place in a laboratory where neither HHPA nor MHHPA was handled. The use of PFBBr as derivatizing reagent has been shown to give excellent sensitivity for PFB-HHP and PFB-MHHP using NICI–GC–MS [16,20].

3.4. Derivatization

To test the time required to obtain maximum derivatization yield, a standard solution (100 μl) with 1.0 pmol of HHP acid and 0.9 pmol MHHP acid was added to 250 μl 0.1 *M* TBA and 250 μl of 0.13 *M* PFBBr. The samples were placed in an ultrasonic bath for 0.25, 0.5, 0.75, 1.0 and 1.5 h. In Fig. 2, the peak areas for PFB-HHP and PFB-MHHP are plotted against time. The maximum yield was reached after 0.75 h. Thus, in order to secure optimal derivatization for all samples, it was decided to use twice that time in the procedure.

3.5. Chromatography and mass spectrometry

The chromatographic behaviour of the pentafluorobenzyl esters was excellent. However, in the GC separation of the PFB-MHHP, a longer temperature program had to be used to separate the ester from an interfering compound. In Fig. 3, a chromatogram of a blank sample and a sample containing 1.0 pmol HHP acid is shown. Fig. 4 presents a chromatogram of a blank sample and a sample containing 0.9 pmol MHHP acid. The main fragmentations of PFB-HHP and PFB-MHHP in NICI–MS have been described by Jönsson and Lindh [16]. For PFB-HHP, both fragments gave sufficiently clean matrices, but

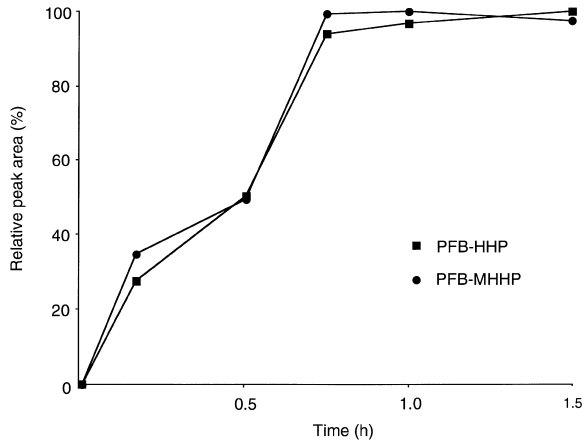

Fig. 2. The relative peak areas for di-pentafluorobenzyl hexahydrophthalate and di-pentafluorobenzyl methylhexahydrophthalate after various times of derivatisation. Each mark denotes one single determination.

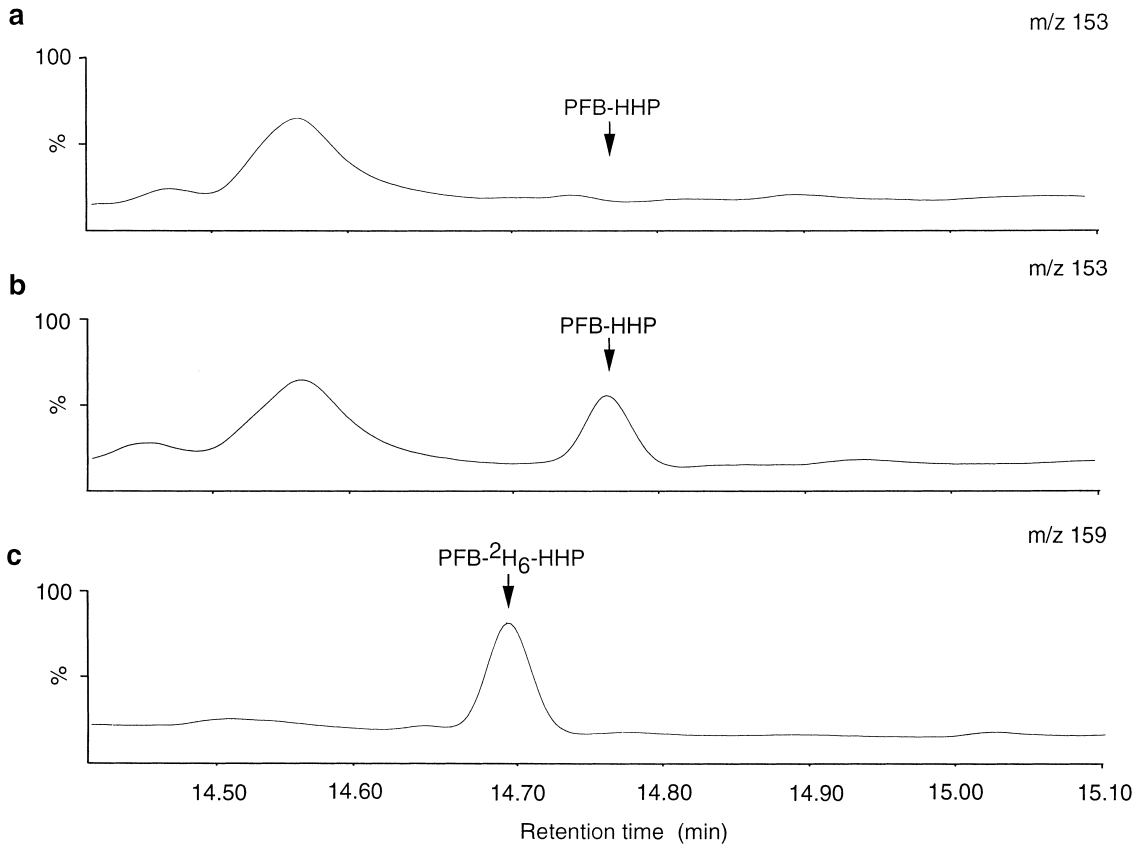

Fig. 3. Chromatograms of the fragment m/z 153 for HHP acid of (a) a sample from a non-exposed subject, (b) a single human sample containing 1.0 pmol HHP acid and (c) the fragment m/z 159 from the deuterium-labeled internal standard.

m/z 153 was slightly purer than m/z 351. For PFB-MHHP, the fragment m/z 365 was chosen since the fragment at m/z 167 was impossible to use due to an interfering compound.

3.6. Quantitative analysis

3.6.1. Calibration graphs

Data on calibration graphs of HHP and MHHP acids in spiked, dialysed and worked-up Hb solution in the range between 0.2 pmol and 19 pmol for HHP acid and in the range between 0.2 pmol and 18 pmol for MHHP acid are shown in Table 1. Simple linear regression was performed with the ratio between the compound and the internal standard peak-area responses as the dependent variable in the plotting of the graphs. The graphs were linear over the whole calibration range.

3.6.2. Detection limits

Blood was collected from ten volunteers who were presumed to be non-exposed to HHPA or MHHPA. The samples were worked-up and analyzed by the method described above. The detection limits were calculated, as reported by Miller and Miller [21], as the amounts corresponding to the peak area ratios with the same retention time as PFB-HHP and PFB-MHHP plus three-times the standard deviation of these. The detection limits for HHP acid were 0.3 pmol/g Hb (10 pg/sample) at m/z 153 and 1.7 pmol/g Hb (80 pg/sample) at m/z 351. For MHHP acid, the detection limit was 0.9 pmol/g Hb (40 pg/sample) for m/z 365. Since the exposure of the

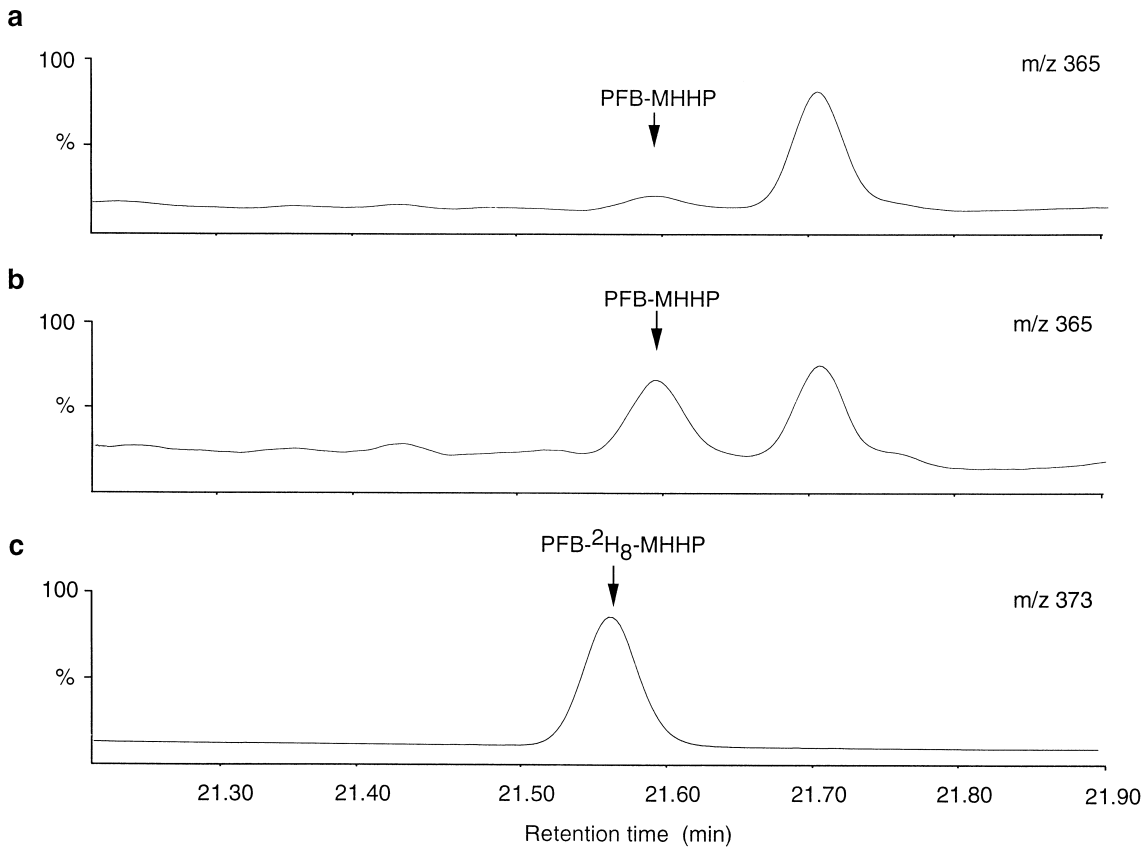

Fig. 4. Chromatograms of the fragment m/z 365 for MHHP acid of (a) a sample from a non-exposed subject, (b) a single human sample containing 0.9 pmol HHP acid and (c) the fragment m/z 373 from the deuterium-labeled internal standard.

occupationally non-exposed population not will contribute to measurable adduct levels, the detection limits of the method can be assumed to be equal to the upper reference limits in the non-exposed group.

3.6.3. Precision

The between-day precisions were determined from duplicate analysis of blood samples from occupationally exposed workers. The C.V.s for HHP acid were 18% at 2 pmol/g Hb (n=19, range 0.7–6 pmol/g Hb) and 10% at 13 pmol/g Hb (n=24, range 6–25 pmol/g Hb). For MHHP acid the C.V.s were 27% at 2 pmol/g Hb (n=36, range 0.7–5 pmol/g Hb) and 14% at 22 pmol/g Hb (n=8, range 7–55 pmol/g Hb). Thus, despite the complicated work-up procedure, it is possible to analyze samples from occupationally exposed workers with a good precision.

Table 1
Data on calibration graphs for HHP and MHHP acids

	Mass fragments	Amount range (pmol)	Slope ($pmol^{-1}$)	Intercept	Correlation coefficient
HHP acid	153	0.2–20	0.192	0.007	0.997
HHP acid	351	0.2–20	0.191	0.168	0.995
MHHP acid	365	0.2–18	0.183	0.017	0.999

Table 2
Levels in air, urine and as Hb-adduct in six workers exposed to HHPA and MHHPA

Worker No.	Air levels		Urinary levels		Adduct levels	
	HHPA (μg/m^3)	MHHPA (μg/m^3)	HHP acid (nmol/mmol creatinine)	MHHP acid (nmol/mmol creatinine)	HHPA (pmol/g Hb)	MHHPA (pmol/g Hb)
1	2	9[a]	7	41[a]	0.3	1.4
2	4	14[a]	18	93[a]	<0.3	1.4
3	3	22	19	95	<0.3	2.3
4	48	9	450	37	16	<0.9
5	50	8	390	36	8.4	<0.9
6[b]	55	9	71	16	2.8	<0.9

[a] These results have previously been published in a correlation between air and urinary levels in a study of MHHPA exposed workers [15].
[b] This worker used a protection device during some operations.

3.7. Application

In Table 2, data on air levels of HHPA and MHHPA by personal sampling, urinary levels of HHP and MHHP acids (adjusted for creatinine) and HHPA and MHHPA Hb-adducts are shown. The air, urine and blood samples from each worker were collected on the same day. The results show that it is possible to analyze adduct levels in workers with a very low exposure to HHPA and MHHPA. Also, the results may indicate a correlation between the adduct levels and the exposure (determined by air sampling or by biological monitoring using urine samples). However, it must be emphasised that the air and urinary levels reflect the exposure solely for one day, while the adduct levels may provide information on the exposure during the last 120 days. On the other hand, a very close correlation between long-term exposure to HHPA and Hb–HHPA adduct levels has previously been found [8].

3.8. Considerations for using the method for biological monitoring

Criteria for an ideal biomonitoring assay have been described by Henderson et al. [22]. A biomarker of exposure is chemically specific, detectable in trace quantities, available by non-invasive techniques, inexpensive to assay and quantitatively relatable to a prior exposure regimen. The described method seems to fulfil these criteria reasonably well. The analyses are chemically specific and the detection limits are low enough to make it possible to analyze adducts from workers exposed to very low levels. Moreover, blood sampling must be considered to be less harmful. The assay is not inexpensive, but the use of GC–MS in the recent years has increased because these equipment now are available at rather moderate costs. On the other hand, equipment with the NICI-option may still be considered to be rather expensive. Moreover, the work-up procedure of the method seems to be rather laborious, but the fact that Hb-adduct levels reflect up to 120 days may actually make the method less time-consuming than other monitoring methods where a single analysis only reflects one day of exposure, e.g., urine analysis. For HHPA, it has been shown that the adduct levels are quantitatively relatable to the exposure. This remains to be established for MHHPA, but data in this paper indicate that this is also true for this anhydride. Moreover, monitoring of protein adducts, especially for sensitizing compound, must be considered as biomonitoring of effective dose (i.e., the dose reaching the target molecule). This may add another advantage to the presented method, compared to biomonitoring using urine sampling, which should be considered as a method monitoring the internal dose.

4. Conclusion

A method for determination of Hb adducts of HHPA and MHHPA by analysis of HHP and MHHP acids in hydrolysed protein solutions from erythrocytes has been described. The method has a high precision and the detection limits are sufficient for

determination of adducts in exposed workers even at low exposures. Thus, the method is applicable for biological monitoring of the exposure to HHPA and MHHPA. In addition, the method may be used in studies of the mechanisms behind development of allergies.

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