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Determination of hexahydrophthalic acid and methylhexahydrophthalic acid in plasma after derivatisation with pentafluorobenzyl bromide using gas chromatography and mass spectrometric detection

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Abstract

A method for the simultaneous determination of hexahydrophthalic acid (HHP acid) and methylhexahydrophthalic acid (MHHP acid) in human plasma was developed. The procedure was a rapid, single step extractive derivatisation with pentafluorobenzyl bromide as the derivatisation agent. The formed pentafluorobenzyl esters were analysed by gas chromatography–mass spectrometry in negative ion chemical ionisation mode with ammonia as the moderating gas. Deuterium-labeled HHP acid and MHHP acid were used as internal standards. The detection limit was 0.4 ng/ml for HHP acid (m/z 153) and 0.3 ng/ml for MHHP acid (m/z 365). The within-day precision of the method was between 2 and 3% and the between-day precision was between 3 and 12%. The overall recovery was between 65 and 83%. A comparison between HHP acid determinations with a previous and this method showed that the methods gave similar results. The method was applicable for analysis of plasma from occupationally exposed workers.

Keywords: Hexahydrophthalic acid; Methylhexahydrophthalic acid

1. Introduction

Hexahydrophthalic anhydride (HHPA) and methylhexahydrophthalic anhydride (MHHPA) are important, industrially used organic chemicals. Like many other organic acid anhydrides, HHPA and MHHPA are irritating to the eyes and to the mucous membranes in the respiratory tract [1]. They also cause occupational asthma and allergic rhinitis and are sensitising agents, even at extremely low exposure levels [2–4]. Thus, there is a need for sensitive methods for the determination of HHPA and MHHPA in work environments.

Methods for the determination of HHPA and MHHPA in industrial air have been described [5–7]. However, biological monitoring has some advantages over air monitoring. For example, it is normally easier to collect biological samples than air samples. Moreover, biological sampling methods compensate for individual variations such as different breathing rates.

Jönsson and Skerfving [8] showed that hexahydrophthalic acid (HHP acid; Fig. 1) is the main metabolite of HHPA. Using a similar analogy, it is reason-

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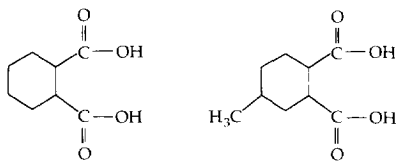

Fig. 1. Chemical structures of hexahydrophthalic acid (HHP acid) and methylhexahydrophthalic acid (MHHP acid).

able to assume that methylhexahydrophthalic acid (MHHP acid; Fig. 1) is a major metabolite of MHHPA, since HHPA and MHHPA are chemically similar compounds. Moreover, Pfäffli et al. [9] found MHHP acid in urine from workers exposed to MHHPA. Thus, there are reasons to investigate if HHP acid and MHHP acid in biological samples may be used for biological monitoring of exposure to HHPA and MHHPA.

Methods for the determination of HHP acid and MHHP acid in urine [9–11] and of HHP acid in plasma [8] have been developed. Jönsson and Skerfving [8] showed a close correlation between air levels of HHPA and the excretion of HHP acid in urine from experimentally exposed volunteers and an even better correlation between the air levels and the concentration in plasma for the same subjects. Moreover, there was a close correlation between HHPA in air and HHP acid in urine from exposed workers [12,13].

We now present a new method for the simultaneous determination of HHP acid and MHHP acid in plasma. The new method is considerably faster and more sensitive than the earlier method for HHP acid. The method is based on a direct two-phase derivatisation procedure using pentafluorobenzyl bromide (PFBBr) as the derivatisation reagent and tetrabutylammonium hydrogen sulfate (TBA) as the counter-ion. The derivatives are analysed by gas chromatography (GC) and negative ion chemical ionisation–mass spectrometry (NICI–MS).

2. Experimental

2.1. Apparatus

The mass spectrometer used for the quantitative analyses was a VG Trio 1000 quadrupole MS (Fisons, Manchester, UK) connected to a Carlo-Erba 8065 GC equipped with an A200S auto-sampler (Carlo-Erba, Milan, Italy). The analytical column was a fused-silica capillary column (30 m×0.25 mm I.D.) with a DB-5 MS stationary phase and a film thickness of 0.25 μm (J&W Scientific, Folsom, CA, USA). A Sigma 3E-1 centrifuge (Sigma, Harz, Germany) was used for phase separations.

2.2. Chemicals and reagents

HHPA (>98%; $C_8H_{10}O_3$; MW 154 g/mol), disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) and TBA were from Merck (Darmstadt, Germany), PFBBr was from Aldrich (Gillingham, UK), trisodium phosphate dodecahydrate ($Na_3PO_4 \cdot 12H_2O$) and sodium hydroxide were from Janssen (Geel, Belgium), hexane and toluene were from LabScan (Dublin, Ireland), $^2H_6$-butadiene and $^2H_8$-isoprene were from Cambridge Isotope Laboratories (Cambridge, MA, USA). MHHPA (>95%; $C_9H_{12}O_3$; MW 168 g/mol) was from Ciba-Geigy (Basel, Switzerland). Di-pentafluorobenzyl hexahydrophthalate (PFB-HHP; >95%; $C_{22}H_{14}O_4F_{10}$; MW 532 g/mol) and di-pentafluorobenzyl methylhexahydrophthalate (PFB-MHHP; >95%; $C_{23}H_{16}O_4F_{10}$; MW 546 g/mol) were from Synthelec (Lund, Sweden) and were synthesised from HHPA and MHHPA.

The 0.13 *M* PFBBr solution was prepared by adding 200 μl of 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 of TBA, 1.79 g of $Na_2HPO_4 \cdot 12H_2O$ and 1.90 g of $Na_3PO_4 \cdot 12H_2O$ to 100 ml of water.

2.3. Synthesis

2.3.1. $^2H_6$-labeled HHP acid

Maleic anhydride (2.9 g) was dissolved in 20 ml of toluene and cooled in an acetone–dry ice bath. A 1-l volume of gaseous $^2H_6$-labeled butadiene was condensed by cooling in the acetone–dry ice bath and 17 ml of cooled toluene were added. This solution was then slowly added to the maleic anhydride solution. The mixture was stirred while slowly returning to room temperature overnight and thereafter it was evaporated to dryness. The product

was hydrolysed in boiling water overnight and the water was then evaporated. The resulting tetrahydrophthalic acid was hydrogenated to $^2H_6$-labeled HHP acid in glacial acetic acid and hydrogen gas with platinum as the catalyst. After filtration and evaporation, the product was used without any further clean-up.

2.3.2. $^2H_8$-labeled MHHP acid

Maleic anhydride (1.2 g) was dissolved in 10 ml of toluene. The mixture was cooled in an ice-bath and 1.0 g of $^2H_8$-labeled isoprene was added slowly. The mixture was stirred and kept in the ice-bath for 2 h and thereafter refluxed for 3 h and evaporated to dryness. The product was hydrolysed in boiling water overnight and the water was then evaporated. The resulting methyltetrahydrophthalic acid was hydrogenated to $^2H_8$-labeled MHHP acid in glacial acetic acid and hydrogen gas with platinum as the catalyst. After filtration and evaporation, the product was used without any further clean-up.

The hydrogenations were performed by Synthelec (Lund, Sweden).

2.4. Sampling and storage

Blood samples were collected from HHPA- and MHHPA-exposed subjects and from unexposed controls. In the exposed subjects, the blood was collected after the end of an 8-h exposure period. The blood was drawn from an antecubital vein and collected in 10 ml Venoject blood sampling tubes (Terumo Europe, Leuven, Belgium) containing sodium heparin. After sampling, the blood was allowed to cool to room temperature and then centrifuged at 1500 *g* for 10 min. The plasma were stored in 10 ml polyethylene test tubes at −20°C until analysis. All subjects gave their informed consent.

2.5. Preparation of standards

Standard solutions of HHP acid and MHHP acid were prepared from the corresponding anhydride. Usually about 50 mg of anhydride were hydrolysed in 25 ml of 0.01 *M* NaOH. Solutions of desired concentrations were prepared from this solution by further dilution in 0.01 *M* NaOH. Plasma standards containing 1, 3, 9, 17, 28, 34 and 67 ng of HHP acid and MHHP acid/ml were prepared by adding 50 μl aliquots of these standard solutions to blank plasma.

For determining the recovery, standard solutions of PFB-HHP and PFB-MHHP were prepared in toluene. About 50 mg of the esters were dissolved in 25 ml of toluene. The solution was then further diluted in toluene to 2, 6, 11, 14, 29, 59 and 88 ng PFB-HHP/ml and 1, 5, 9, 12, 25, 50 and 75 ng PFB-MHHP/ml.

2.6. Work-up and the derivatisation procedure

Plasma (250 μl) was transferred to 13 ml test tubes with PTFE screw caps and 100 μl of an internal standard solution containing 14 ng of $^2H_6$-labeled HHP acid and 14 ng of $^2H_8$-labeled MHHP acid were added. Then, 250 μl of the 0.1 *M* TBA solution and 250 μl of the 0.13 *M* PFBBr solution were added to the samples. The test tubes were sealed, vortex-mixed and agitated for 90 min in an ultrasonic bath containing only sufficient water to cover ca. 10 mm of the test tubes. Thereafter, 2 ml of hexane were added and the samples were shaken for 15 min. The samples were centrifuged for phase separation for 10 min at 1500 *g* and then placed in a freezer at −20°C until the aqueous phase had frozen. The organic phase was transferred to another test tube and then evaporated to dryness in a stream of nitrogen gas. The residues were dissolved in 0.5 ml of toluene, transferred to auto-sampler injection vials and stored at −20°C until analysis.

2.7. Analysis

Samples were injected using a splitless injection technique. The injector temperature was 300°C and the syringe needle was heated in the injector for 10 s before injection. The injection volume was 2 μl and the split exit valve was kept closed for 0.5 min after the injection. The initial column temperature was 100°C for 1 min. The temperature was then increased by 15°C/min to 320°C, where cooling started immediately. The MS interface was at 320°C and the ion source at 200°C. The column carrier-gas was helium at a pressure of 70 kPa. The MS was in the negative ion chemical ionisation (NICI) mode with ammonia as the moderating gas. Selected-ion monitoring (SIM) of PFB-HHP was performed at *m/z* 153 and

351 while m/z 159 and 357 was chosen for the deuterium-labeled PFB-HHP. For PFB-MHHP, m/z 365 was used while m/z 373 was chosen for the deuterium-labeled PFB-MHHP. Peak area ratio measurements were used for the determinations.

3. Results and discussion

3.1. Internal standards

The identity of the internal standards was confirmed by mass spectrometry [11]. The isotopic purity of the deuterium labeling was studied using SIM. The HHP acid fragment at m/z 153 was 0.1% of the fragment at m/z 159 for $^2H_6$-labeled HHP acid. The MHHP acid fragment at m/z 365 was 0.2% of the fragment at m/z 373 for $^2H_8$-labeled MHHP acid.

3.2. Stability

Twelve plasma samples from HHPA-exposed volunteers containing between 1 and 8 ng of HHP acid/ml were analysed by the method described by Jönsson and Skerfving [8]. The samples were then stored at −20°C for 3.5 years and then analysed again by the same method. There was no significant difference in the determinations and, thus, HHP acid seems to be stable in plasma during storage in a freezer. Six plasma samples from MHHPA-exposed subjects containing between 2 and 12 ng of MHHP acid/ml were analysed by the present method. The samples were analysed on two different occasions with seven months between the analyses. No differences in the results were obtained and, thus, MHHP acid also seems to be stable in plasma during storage in a freezer.

Samples and standards containing 0–40 ng/ml HHP acid or MHHP acid were worked-up and derivatised. Analysis of these directly after derivatisation and after four days at room temperature showed the same results. Thus, it seems that samples can be stored for some time before the analysis.

3.3. Work-up and the derivatisation procedure

Numerous papers have been published on work-up and derivatisation of carboxylic acids in biological samples [14]. Liquid–liquid extraction techniques have frequently been used. However, these often give insufficient clean-up and are generally laborious. Recently, liquid–solid extraction techniques have been described for biological samples with complicated analytical matrices. Jönsson and Skerfving [8] used a technique based on the combination of columns with octadecylsilyl and trimethylaminopropylsilyl stationary phases for work-up of HHP acid from human plasma. These techniques provide high purifications but are still laborious and time-consuming when dealing with large quantities of samples, even if the sample through-put is better than that obtained with liquid–liquid extractions. Hachey et al. [15] have suggested the use of a fast and simple extractive derivatisation method for organic keto acids in plasma with PFBBr as the derivatisation agent. The concentrations of keto acids determined by this method were much higher than the levels of HHP acid or MHHP acid expected in plasma after occupational exposure to HHPA and MHHPA, respectively. However, Jönsson et al. [16] have reported excellent sensitivity for the pentafluorobenzyl esters of methyltetrahydrophthalic acid, a dicarboxylic acid that is chemically similar to HHP acid and MHHP acid. Thus, we decided to try this method for the determination of HHP acid or MHHP acid and found that, after some modification, the method worked well even for determinations in plasma from exposed workers. We did not obtain better detection limits when working-up plasma with octadecylsilyl liquid–solid extraction before the derivatisation compared to the presented method with direct extractive derivatisation.

3.4. Mass spectrometry

The mass spectra of PFB-HHP and PFB-MHP using NICI showed a very simple fragmentation with only two major fragments [11]. The base peak for PFB-HHP was m/z 153, while that for hexadeuterium-labeled PFB-HHP was 159. For PFB-MHHP, the base peak was m/z 167 and for octadeuterium-labeled PFB-MHHP it was m/z 175. This fragment is probably derived from a ring closure of the esters to the original anhydride. The second major fragment was for PFB-HHP m/z 351 and for the internal standard m/z 357. For PFB-MHHP, the corresponding fragment was at m/z 365

and that for the internal standard was at m/z 373. These fragments were ca. 25% of the base peak and were probably derived from the loss of one of the pentafluorobenzyl groups.

3.5. Chromatography

The chromatographic behavior of the pentafluorobenzyl esters was excellent. In Fig. 2, a chromatogram from a blank plasma and one from the same plasma spiked with 3 ng of HHP acid/ml are shown. Fig. 3 shows chromatograms from a blank plasma and from the same plasma spiked with 3 ng of MHHP acid/ml. In the chromatogram of PFB-HHP, both the mass fragments at m/z 153 and 351 gave sufficiently clean matrices and it seems that both fragments are equally applicable for the analyses. For PFB-MHHP, the fragment m/z 167 could not be used for quantification of lower concentrations because of an interfering compound. However, the fragment at m/z 365 was much cleaner and this was therefore chosen. All fragments from the internal

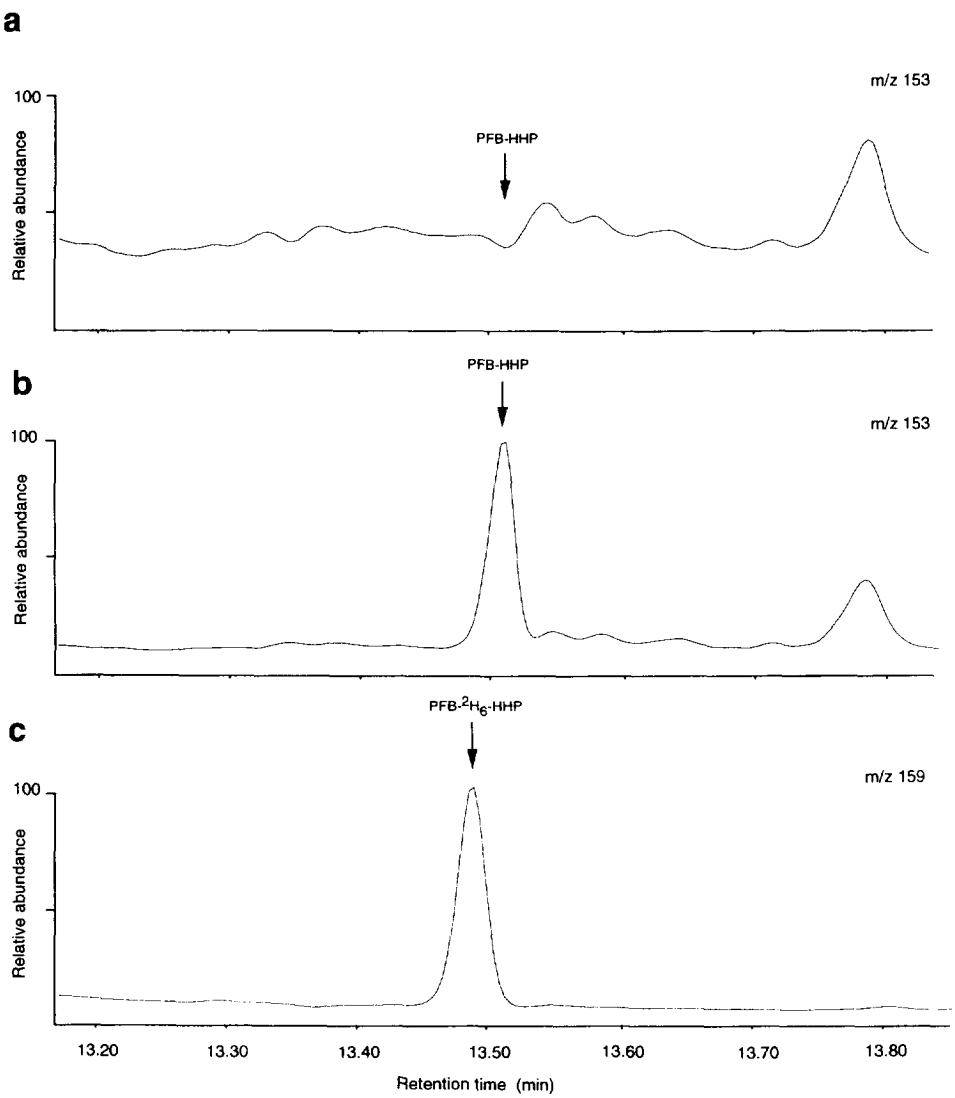

Fig. 2. Chromatograms, using the fragment at m/z 153 for HHP acid, of (a) a blank plasma sample and (b) the same plasma sample but spiked with 3 ng of HHP acid/ml. The third chromatogram (c) shows the fragment for the deuterium-labeled internal standard at m/z 159.

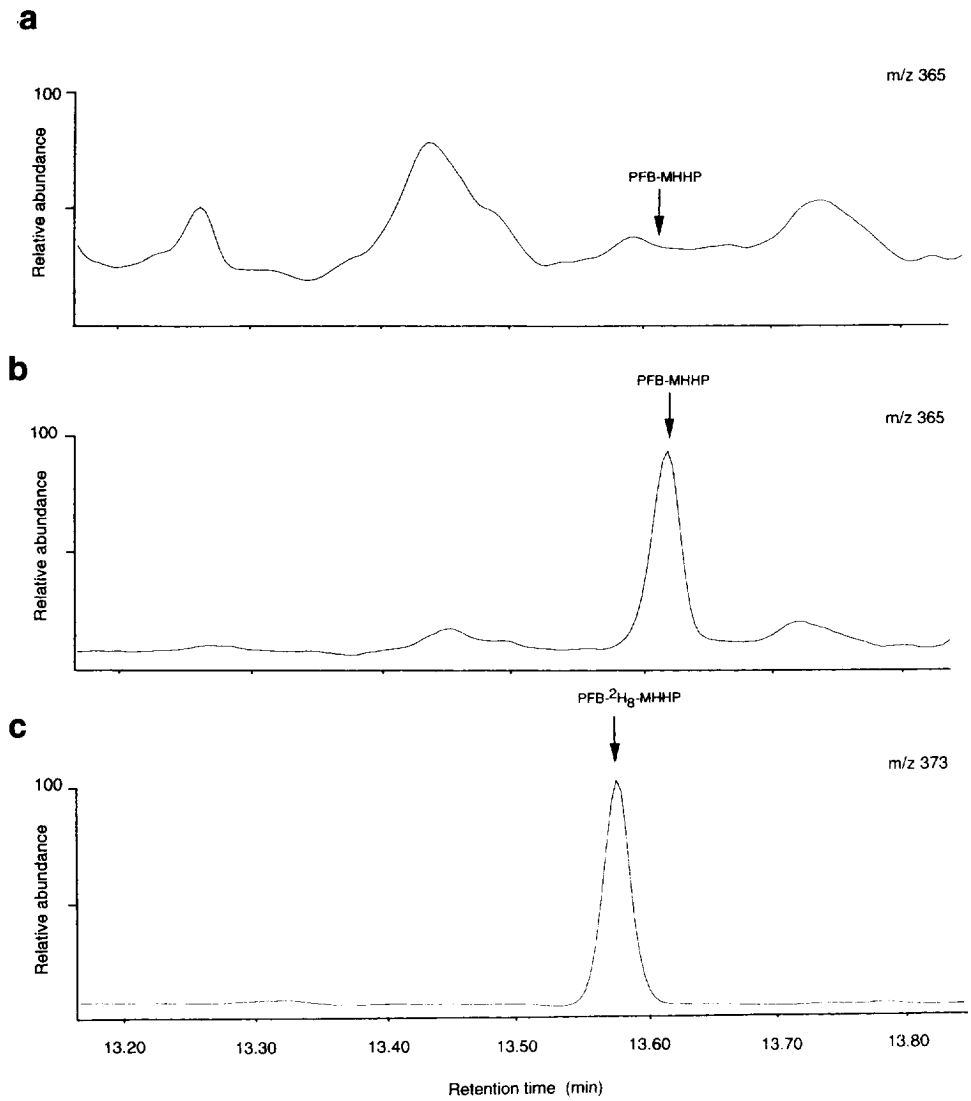

Fig. 3. Chromatograms, using the fragment at m/z 365 for MHHP acid, of (a) a blank plasma sample and (b) the same plasma sample but spiked with 3 ng of MHHP acid/ml. The third chromatogram (c) shows the fragment for the deuterium-labeled internal standard at m/z 373.

standards at m/z 159, 175, 357 and 373 were applicable for analysis. Thousands of injections have been done without any observed column aberrations.

3.6. Quantitative analysis

3.6.1. Calibration graph

Data on calibration graphs of HHP acid and MHHP acid in spiked and derivatised plasma in the concentration range between 1 and 67 ng/ml are shown in Table 1. Unweighted linear regression and the ratio between the compound and the internal standard peak-area responses were used in the computation of the graphs. The calibration graphs were linear over the whole range.

3.6.2. Detection limit

Plasma samples were collected from ten volunteers who were presumed to be unexposed to HHPA, HHP acid, MHHPA or MHHP acid. Internal standard

Table 1
Calibration graph for HHP acid and MHHP acid in plasma

	m/z	Concentration range (ng/ml)	Slope (ml/ng)	Intercept	Correlation coefficient
HHP acid	153	1–67	0.033	−0.008	0.999
HHP acid	351	1–67	0.035	0.008	0.999
MHHP acid	365	1–67	0.032	0.007	0.999

was added and the samples were derivatised and analysed according to the method described above. The detection limit was calculated, as reported by Miller and Miller [17], as the concentration 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 limit was 0.4 ng/ml plasma (*m/z* 153) and 0.5 ng/ml plasma (*m/z* 351) for HHP acid. For MHHP acid, the detection limit was 0.3 ng/ml plasma for *m/z* 365.

3.6.3. Recovery

The recovery for the overall method was investigated by working-up ten different plasma samples spiked with HHP acid and MHHP acid. Two sets of samples with the concentrations 6 and 60 ng/ml were tested. No internal standard was added before the work-up. However, prior to analysis, a toluene solution containing the internal standards, $^{2}H_{6}$-labeled PFB-HHP and $^{2}H_{8}$-labeled PFB-MHHP, was added. This internal standard was prepared by working-up water solutions of $^{2}H_{6}$-labeled HHP acid and $^{2}H_{8}$-labeled MHHP acid according to the method described above for plasma. Comparisons were made with standard solutions of PFB-HHP and PFB-MHHP in toluene with the same concentration of internal standard added. The result is shown in Table 2. The reason for the fairly high level of imprecision in the concentration at 6 ng/ml is that one sample (same for HHP acid and MHHP acid) was derivatised to a lesser extent than the others.

3.6.4. Precision

The within-day precision of the overall methods were determined by analysis of ten different plasma samples spiked with 6 and 60 ng/ml of HHP acid and MHHP acid, respectively. The coefficients of variation (C.V.) were determined for the ratio between the area for PFB-HHP, PFB-MHHP and their internal standards. The results are shown in Table 3.

The between-day precision were studied by analysing one plasma sample containing 6 ng of HHP acid and MHHP acid/ml and one plasma sample containing 60 ng of HHP acid and MHHP acid/ml, eight times over eight days. The C.V. for the determinations are shown in Table 3.

3.6.5. Comparison of methods

Ten plasma samples from HHPA-exposed subjects in the range 0–50 ng of HHP acid were determined by the method described by Jönsson and Skerfving [8] and by the present method (Table 4). The methods were compared using a paired *t*-test and there were no statistically significant difference between the two methods.

3.7. Application

A plasma sample from a worker exposed to 77 μg of HHPA/m^3 and 65 μg of MHHPA/m^3 (time-weighted average) was analysed by the method. The worker wore a protective device on some occasions

Table 2
Recovery of HHP acid and MHHP acid as pentafluorobenzyl esters after derivatisation

	Concentration (ng/ml)	Recovery (%)	Precision[a] (%)
HHP acid[b]	6	83	17
HHP acid[b]	60	82	12
MHHP acid[c]	6	65	18
MHHP acid[c]	60	78	5

Ten different, spiked plasma samples were used for each concentration.
[a]Given as coefficients of variation.
[b]Determined from the fragment at *m/z* 351.
[c]Determined from the fragment at *m/z* 365.

Table 3
Precision in the analysis of plasma samples spiked with different amounts of HHP acid and MHHP acid

Isomer	Fragment (*m/z*)	Concentration (ng/ml)	Within-day precision[a] (%)	Between-day precision[a] (%)
HHP acid	153	6	3	9
HHP acid	153	60	2	12
HHP acid	351	6	2	5
HHP acid	351	60	3	4
MHHP acid	167	60	3	6
MHHP acid	365	6	3	3
MHHP acid	365	60	2	4

[a]Given as coefficients of variation.

during the day. The levels in the sample were 8 ng of HHP acid/ml and 14 ng of MHHP acid/ml.

4. Conclusion

A simple and fast method for the simultaneous determination of HHP acid and MHHP acid in plasma has been described. The method has good precision and equally good recovery. The detection limits are sufficiently low for determinations of HHP acid and MHHP acid in plasma from exposed workers. The HHP acid concentrations determined by the method were similar to those determined by an earlier method. The method may be used for biological monitoring of exposure to HHPA and MHHPA.

Table 4
Comparisons between the concentrations of HHP acid in plasma from HHPA-exposed subjects determined by the present method and the method of Jönsson and Skerfving [8].

Concentrations by the present method (ng/ml)	Concentrations by the previous method (ng/ml)
0	0
2.0	1.8
4.8	4.8
4.9	4.9
5.6	5.6
6.2	6.5
6.7	6.7
8.7	9.2
51.0	45.9
48.1	49.2

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