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# Method for the biological monitoring of hexahydrophthalic anhydride by the determination of hexahydrophthalic acid in urine using gas chromatography and selected-ion monitoring

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## ABSTRACT

A method for the determination of hexahydrophthalic acid, a metabolite of hexahydrophthalic anhydride, in human urine has been developed. The urine was worked-up by liquid–solid extraction, esterified with boron trifluoride–methanol, and analysed by capillary gas chromatography and selected-ion monitoring. Hexadeuterium-labelled hexahydrophthalic acid was used as the internal standard. The precision was 4% at 0.7 μg/ml and 5% at 0.07 μg/ml. The recovery of the acid for the overall method was 101% at 0.07 μg/ml of urine (with a coefficient of variation of 4%) and 95% at 0.7 μg/ml (coefficient of variation 2%). The limit of detection was 20 ng/ml urine.

## INTRODUCTION

Hexahydrophthalic acid (HHP acid) has been identified as the major metabolite of hexahydrophthalic anhydride (HHPA) [1,2]. HHPA, which is used as a hardener in epoxy-resins, is an irritant to the eyes and to the mucous membranes in the respiratory tract [3] and has also been found to be a sensitizing agent at very low exposure levels [4]. Of workers exposed to air levels of HHPA between 5 and 10 $\mu g/m^3$, 33% had immoglobulin E (IgE) antibodies to a conjugate between HHPA and human serum albumin, and 38% were skin-prick-test positive to the same conjugate. HHPA is thought to cause occupational asthma and allergic rhinitis [5].

Recently, methods for the determination of HHPA in air have been described [6]. However, biological monitoring has some advantages over the air-sampling procedure, *e.g.* air-sampling methods do not, in contrast to the biological sampling methods, compensate for different breathing rates during different body work. Moreover, if respiratory protective devices are being used, a biological monitoring method is virtually essential for the estimation of the exposure.

Methods for the determination of HHP acid in urine have been described. In one procedure, liquid–liquid extraction of the urine, esterification with boron trifluoride–methanol and analysis by gas chromatography–mass spectrometry

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(GC–MS) was used [1]. The procedure of the other method included liquid–liquid extraction of the urine, esterification with trichloroethanol, and analysis by GC with electron-capture detection [2].

The aim of the study was to develop and evaluate a method for the determination of HHP acid in urine. A more efficient clean-up procedure than for the earlier methods was desirable. The detection limit for HHP acid in urine should correspond to an air concentration of less than 5 $\mu g/m^3$. It was also desirable to improve the precision compared with the earlier methods. In the present method, two liquid–solid extractions were used followed by derivatization with boron trifluoride–methanol and analysis by selected-ion monitoring (SIM). The solid sorbents used in the liquid–solid extractions were silica-bonded octadecylsilyl and trimethylaminopropylsilyl.

## EXPERIMENTAL

### *Apparatus*

A Shimadzu GCMS-QP 1000 EI/CI quadrupole mass spectrometer (Shimadzu, Kyoto, Japan), connected to a Shimadzu GC-9A gas chromatograph equipped with a split–splitless injection system SPL-G9 and a Shimadzu autosampler (AOC-9), was used. The capillary column was connected directly into the ion source. The chromatograms obtained were evaluated by the MS-PACK 200 software from Shimadzu, using a Compaq 20e computer.

For phase separation, sedimentation and drying of clean-up columns, a Sigma 3E-1 centrifuge (Sigma, Harz, Germany) was used. A Vac Elut SPS 24 (Analytichem International, Harbor, CA, USA) connected to an aspirating pump was used for the liquid–solid extractions.

### *Columns*

The analytical column used was a fused-silica capillary column (30 m × 0.25 mm I.D.) coated with a DB-5 stationary phase with a film thickness of 0.25 $\mu m$ (J&W Scientific, Folsom, CA, USA). The clean-up columns used were Bond Elut LRC (Analytichem International) with silica-bonded octadecylsilyl ($C_{18}$) and trimethylaminopropylsilyl (SAX) as sorbents (500 mg).

### *Chemicals*

Methanol and toluene were from LabScan (Dublin, Ireland), boron trifluoride (14% in methanol) was from Sigma (St. Louis, MO, USA) and HHPA (>98%), sodium bicarbonate, sodium acetate, sodium hydroxide, sodium sulphate, formic acid, hydrochloric acid, acetic acid, sulphuric acid, dichloromethane, tris-(hydroxymethyl)aminomethane, platinum on active charcoal and maleic anhydride were from Merck (Darmstadt, Germany). Hexahydrophthalic acid dimethyl ester (HHP acid DME) was from SyntElec (Lund, Sweden), and deuterium-labelled butadiene ($^2H_2C=C^2H–C^2H=C^2H_2$) from MSD Isotopes (Merck Frosst Canada, Montreal, Canada).

*Synthesis of hexadeuterium-labelled HHP acid*

Maleic anhydride (4 g) was partly dissolved and partly suspended in 15 ml of toluene. The mixture was cooled to −50°C, and 1 l of gaseous deuterium-labelled butadiene was condensed and then slowly gasified and bubbled into the mixture with vigorous stirring. The mixture was allowed to return slowly to ambient temperature, at which it was kept for 24 h. The mixture was evaporated to dryness using a rotating evaporator. To the residue were added 100 ml of Millipore-filtered water at 3°C. After 30 min the tetrahydrophthalic anhydride (THPA) precipitate was separated by filtration, and dissolved in 50 ml of Millipore water at 25°C. The THPA–water mixture was allowed to react (hydrolysis) for 24 h and was then acidified with sulphuric acid to pH 1 and shaken with four portions of 50 ml of dichloromethane. The combined dichloromethane extracts were evaporated to dryness. The residue was dissolved in 50 ml of glacial acetic acid, to which *ca.* 50 mg of platinum on active charcoal were added. The tetrahydrophthalic acid was hydrogenated by shaking the mixture with hydrogen for 20 h. The mixture was then filtered, and the filtrate was evaporated to dryness. The [$^2H_6$]HHP acid residue was used without any further clean-up.

*Storage*

Urine samples were stored without any pretreatment at −20°C until analysis.

*Preparation of standards*

Standard solutions of HHP acid were prepared by hydrolysis of 100 mg of HHPA in 25 ml of aqueous 0.01 *M* sodium hydroxide to form the sodium salt of HHP acid. This was further diluted in aqueous sodium hydroxide to appropriate concentrations. Urine standards containing HHP acid were prepared by spiking blank urine with 200 μl of portions of the alkaline aqueous standard solutions. The urine sample standards were worked-up and analysed together with the urine samples as described below.

To determine the recovery of the method, standard solutions of HHP acid DME were prepared in toluene: 100 mg of HHP acid DME were dissolved in 25 ml of toluene. The solution was then further diluted in toluene to appropriate concentrations.

*Work-up procedure*

*Hydrolysis.* To 4 ml of urine, in 13-ml test-tubes with PFTE screws-caps, were added 200 μl of a solution containing 2.5 μg of deuterium-labelled HHP acid per ml of 0.01 *M* sodium hydroxide and 4 ml of 2.0 *M* hydrochloric acid. The hydrolysis was performed overnight at 100°C and then cooled to room temperature. Then 3 ml of 1.5 *M* NaOH were added, and the pH was checked and adjusted to between 0.5 and 2 with 2.0 *M* hydrochloric acid or 1.5 *M* sodium hydroxide. The precipitate was removed by centrifugation at 1500 *g* in 10 min.

*Liquid–solid extraction.* $C_{18}$ Bond Elut columns were conditioned by passing 5

ml of methanol followed by 10 ml of 0.1 *M* hydrochloric acid through the columns. The columns were not allowed to dry out between or after the conditioning steps. Then 10 ml of the hydrolysed and centrifuged urine samples were passed through the columns, resulting in the trapping of HHP acid. A washing step were performed using 10 ml of 0.1 *M* hydrochloric acid–methanol (95:5, v/v). The columns were dried by air suction for 5 min, and the HHP acid was eluted with 3 ml of 0.1 *M* Tris buffer (pH 8.8).

The SAX Bond Elut ion-exchange columns were conditioned with 5 ml of formic acid–methanol (50:50, v/v), 5 ml of methanol, 10 ml of 1.0 *M* sodium acetate and 10 ml of water. The columns were not allowed to dry out between or after the conditioning steps. The HHP acid eluates were then applied and trapped on the ion-exchange column. Washing was performed with 5 ml of 0.01 *M* Tris buffer (pH 8.2)–methanol (70:30, v/v). The columns were dried by centrifugation at 1500 *g* for 10 min and then by air-suction for 5 min. Elution of the HHP acid were performed with 3 ml of formic acid–methanol (50:50, v/v) and the eluate was sampled in 13-ml test-tubes with PFTE screw-caps. The sample was evaporated to dryness at 40°C under a stream of dry nitrogen gas. A sample-processing station was used for the simultaneous treatment of several samples. A vacuum was maintained at 0.5 kPa below the atmospheric pressure, except for the elution of the SAX columns (0.4 kPa). To dry the columns a full vacuum was employed.

*Derivatization.* To the dry residue containing the HHP acid, 1 ml of methanol and 2 ml of 14% boron trifluoride in methanol were added. Esterification was performed overnight at 70°C. After cooling to room temperature, 4 ml of saturated sodium bicarbonate solution and 2 ml of toluene were added. The solution were shaken for 15 min and then centrifuged at 1500 *g* for 3 min. The toluene phase, containing the HHP acid DME, was separated and dried over anhydrous sodium sulphate. The samples were stored in a freezer until analysis.

*Analysis*

Samples were injected with a splitless technique at 250°C. The split exit valve was kept closed for 1 min after the injection, and the typical injected volume was 4 μl. The initial column temperature was 110°C for 2 min. After elution of the solvent, the temperature was increased at 20°C/min to a final temperature of 290°C. The HHP acid DME derivatives eluted after 3.3 min at 136°C. The GC–MS interface and ion source were held at 200°C. The mass spectrometer was in the electron-impact (EI) mode at 70 eV. The carrier gas was helium at a pressure of 1.0 kg/cm$^2$. The fragment ions *m/z* 140 and 169 of the HHP acid DME and *m/z* 145 and 175 of the internal standard were monitored. Duplicate determinations with triplet injections were made.

In the case of a high concentration of HHP acid in the urine (greater than 1.0 μg/ml), the urine samples were diluted four times with water before the work-up procedure.

## RESULTS AND DISCUSSION

### *Choice of internal standard*

Methods for the analysis of biological samples often involve laborious work-up procedures, making the choice of the internal standard important. Deuterium-labelled compounds have been used as internal standards [7–9]. The obvious choice of internal standard was the deuterium-labelled HHP acid. This was not commercially available but was synthesized in our laboratory. [$^2H_6$]HHP acid has several advantages as the internal standard. It has almost identical physical and chemical properties to HHP acid, and therefore behaves similarly to HHP acid in the work-up procedure. Moreover, the chromatographic properties of the deuterium-labelled HHP acid DME ([$^2H_6$]HHP acid DME) are almost identical with those of HHP acid DME. Finally, deuterium-labelled HHPA and [$^2H_6$]HHP acid are not expected to be found in the environment as interferences.

### *Synthesis of [$^2H_6$]HHP acid.*

The full or partial synthesis of HHP acid has been described by several authors [10–14]. The procedure used was a Diels–Alder reaction between maleic anhydride and hexadeuterium-labelled butadiene. The resulting deuterium-labelled THPA was hydrolysed to tetrahydrophthalic acid, and finally hydrogenated to [$^2H_6$]HHP acid. The recovery of [$^2H_6$]HHP acid in the described procedure was poor (*ca.* 10%). The probable reason for this was an unsatisfactory absorption of the gasified butadiene in the toluene mixture. It was therefore necessary to remove the unchanged maleic anhydride from the deuterium-labelled THPA. This could be done by hydrolysing the maleic anhydride in water at 3°C to the water-soluble maleic acid. THPA reacts very slowly in cold water, and the solid phase containing the THPA could therefore be removed by filtration.

### *Storage and hydrolysis*

No degradation of HHP acid was found in urine samples spiked with 0.42 or 4.2 μg HHP acid per ml, even after storage in 4°C for six months.

The hydrolyses of 4 ml of spiked urine containing 700 ng/ml HHP acid and of urine samples from HHPA workers containing 900 ng/ml with the addition of 4 ml of 2 *M* hydrochloric acid were studied. Hydrolysis was performed at 100°C and the hydrolysis time was studied up to eight days. The internal standard was added after the hydrolysis. No loss of HHP acid was observed for the spiked urine samples. No change in the HHP acid concentration was observed for the HHPA workers urine (Fig. 1). It is therefore probable that HHP acid is excreted in unconjugated form, which is in agreement with previous reports [2]. In principle, no hydrolysis step is therefore necessary. Initial attempts to analyse unhydrolysed urine with the work-up procedure described resulted in a yellow toluene solution, which destroyed the capillary column on injection. Hydrolysis overnight eliminated these problems. When the hydrolysis was performed as

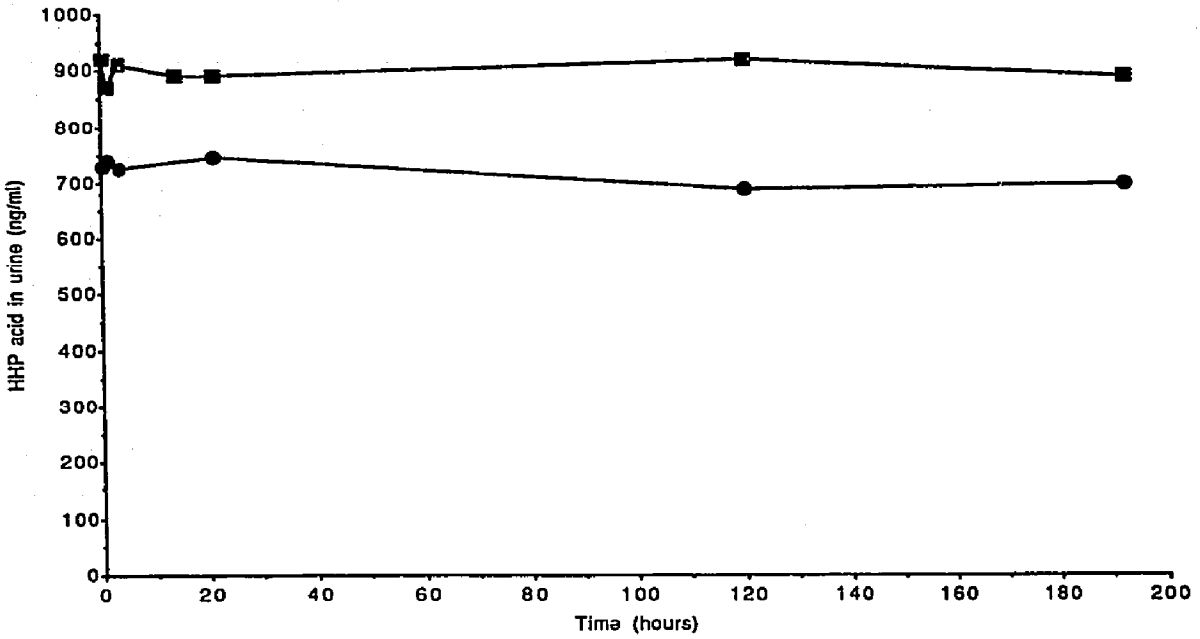


Fig. 1. Concentration of HHP acid in worked-up urine *versus* hydrolysis time. (●) HHP acid-spiked urine, (■) urine from a worker, exposed to HHPA.

above, but with 6 ml of 6 *M* hydrochloric acid added to 4 ml of urine, *ca.* 10% of the HHP acid was lost in three days both from spiked urine and from workers' urine. Hydrolysis using 6 ml of 10 *M* sodium hydroxide added to 4 ml of urine resulted in severe loss of HHP acid.

*Work-up procedure*

Work-up of dicarboxylic acids from urine samples has been performed by liquid–liquid extraction techniques [1,2,15,16]. However, these techniques often give insufficient clean-up, and they are generally time-consuming when several samples need to be treated. The liquid–solid extraction technique, especially in the work-up of biological samples with complex matrices such as urine and blood, has been used frequently the recent years, and has several advantages. Ion-exchange columns can be used, to give high recoveries of ionic compounds. Strong anion-exchange (SAX) columns with silica-bonded trimethylaminopropyl as the sorbent have been used for the work-up of dicarboxylic acids [17,18] at the μg/ml level. Initial attempts to use strong anion-exchange columns when determining HHP acid in urine from exposed workers resulted in insufficient clean-up of the sample. The hydrolysis step had to be excluded owing to the relatively high ion strength otherwise obtained.

A technique based on the combination of a $C_{18}$ column and a SAX column has been used for the determination of a monocarboxylic acid in urine, 4-methyl-3-methoxymandelic acid [19]. The detection limit was at the low ng/ml level.

This technique was initially tested at our laboratory and satisfactory clean-up of the sample was obtained. The technique was therefore investigated in more detail for the determination of HHP acid in urine.

The $C_{18}$ columns gave a good retention of HHP acid, and sample volumes much higher than 10 ml of urine could be used without breakthrough. Negligible sample losses (less than 1%) were observed when 10 ml of methanol–water (5:95, v/v) were used as the washing solution. However, when the methanol concentration was increased to 10%, losses of greater than 10% were observed. The elution of HHP acid could be performed either with 1.5 ml of methanol or with 3 ml of 0.1 *M* Tris buffer (pH 8.8). Elution with 0.1 *M* sodium acetate buffer (pH 6.4) was not successful. The elution using Tris buffer were chosen because it was possible to use the eluate without pretreatment on the SAX column. Initial attempts to use $C_{18}$ columns in the work-up procedure without any further clean-up, by performing the elution with methanol, resulted in a yellow–brown toluene solution which, when injected, destroyed the analytical column.

The SAX columns were conditioned with 1 *M* aqueous sodium acetate solution. The counter-ion of the trimethylaminopropyl-based sorbent was thus changed from chloride to acetate. The acetate ion has lower affinity for the sorbent than chloride and is therefore more easily exchanged. Furthermore, conditioning with acetate solution rendered the sorbent alkaline and thus more ready to adsorb the salt of the HHP acid. The ionic strength after conditioning with the acetate solution was too high, and the column was therefore washed with 10 ml of water before the sample was applied. Water was used because the use of a solution with an ionic strength as low as 0.05 *M* Tris buffer (pH 8.8) resulted in a decreased recovery. Negligible breakthrough (less than 5%) of HHP acid was observed with the acetate-conditioned column.

The optimum composition of the sample to be applied to the SAX column was investigated. Solutions tested were 0.1 *M* sodium dihydrogenphosphate buffer (pH 6.3), 0.1 *M* Tris buffer (pH 8.8) and 0.2 *M* Tris buffer (pH 8.8). Both the sodium dihydrogenphosphate buffer and 0.2 *M* Tris buffer showed a considerable breakthrough (greater than 20%) compared with the 0.1 *M* Tris buffer. The breakthrough also increased with the applied volume. With 5 ml of 0.1 *M* Tris buffer, the breakthrough was 2.5% and with 10 ml it was 5%.

Washing the SAX columns with solutions with ionic strength higher than a 0.01 *M* Tris buffer and volumes more than 5 ml resulted in breakthrough. The methanol content in the washing solution could be as high as 30% before considerable losses occurred.

The SAX columns were dried after the washing step by centrifugation and thereafter by air-suction for 5 min. The laborious drying step was performed because the subsequent derivatization procedure with boron trifluoride–methanol is known to be sensitive to moisture. The sample was eluted from the SAX columns with methanol–formic acid, because the eluates could readily be evaporated to dryness.

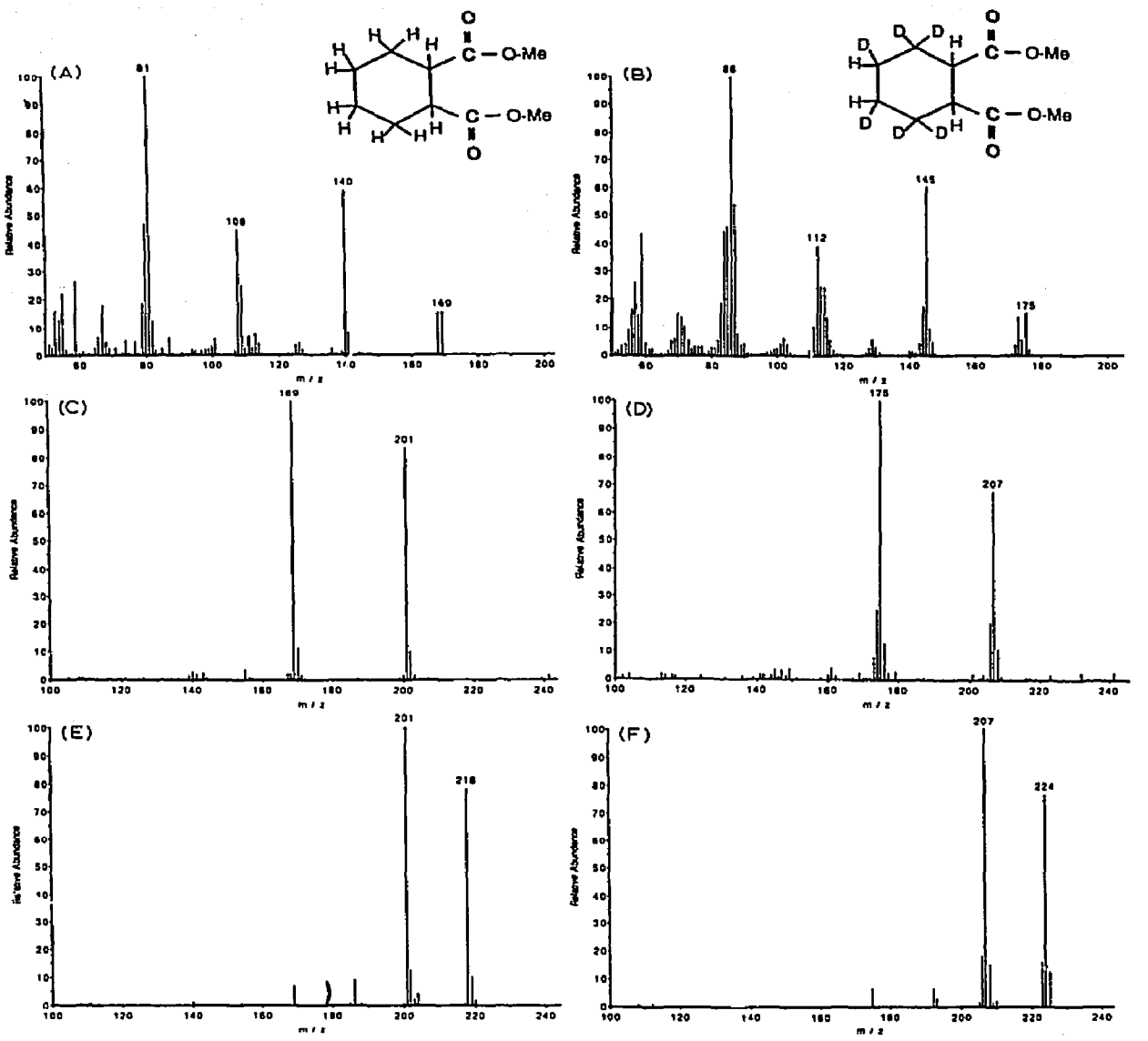


Fig. 2. Mass spectra of (A) HHP acid DME, EI mode (70 eV), (B) deuterium-labelled HHP acid DME, EI mode (70 eV), (C) HHP acid DME, CI mode (isobutane), (D) deuterium-labelled HHP acid DME, CI mode (isobutane), (E) HHP acid DME, CI mode (ammonia), and (F) deuterium-labelled HHP acid DME, CI mode (ammonia).

Both the $C_{18}$ and the SAX columns could be reused at least up to seven times following the described procedure. However, both types of column should be washed after each use with at least 5 ml of methanol.

*Mass spectrometry*

Mass spectra of HHP acid DME and [$^2H_6$]HHP acid DME of EI and chemical ionization (CI) with isobutane and ammonia are shown in Fig. 2. In the EI mode, no molecular ion was observed. The most abundant ions were *m/z* 81 (100%), 108

(35%), 140 (37%), 168 (10%) and 169 (9%) for HHP acid DME. For [$^2H_6$]HHP acid DME the most abundant ions were *m/z* 86 (100%), 112 (39%), 145 (60%), 173 (14%) and 175 (15%). Owing to interferences from the matrix, only the mass fragments *m/z* 140 and 169 for HHP acid DME were found suitable. The corresponding mass fragments of the labelled internal standard were *m/z* 145 and 175. However, the relative abundance of the *m/z* 145 fragment in the HHP acid DME spectra was 0.3%, and the relative abundance of the *m/z* 140 fragment in the [$^2H_6$]HHP acid DME spectra was 1.5%. A suitable concentration of the internal standard must therefore be chosen with regard to amount of HHP acid expected in the sample. At a concentration of 0.5 μg of [$^2H_6$]HHP acid per ml of urine, concentrations of HHP acid from the detection limit to over 1.0 μg/ml could be analysed without interferences.

CI with ammonia as the reagent gas gave the base peak (M + 1) *m/z* 201 for HHP acid DME and *m/z* 207 for [$^2H_6$]HHP acid DME. The relative abundances of the M + 18 fragments were 78 and 76%, respectively. When isobutane was used as the reagent gas the base peaks were *m/z* 169 for HHP acid DME and *m/z* 175 for the [$^2H_6$]HHP acid DME. The relative abundances of the M + 1 fragments were 78 and 68%, respectively. Comparison of the results using CI and EI showed four times higher sensitivity with EI at *m/z* 140.

Since the detection limit is determined by the matrix, four mass fragments were monitored simultaneously. This made it possible to ensure the peak purity of the HHP acid DME peak in the chromatogram. Using EI and plotting the quotient between the fragments *m/z* 140 and 169 for 36–1400 ng/ml HHP acid a virtually linear horizontal curve was obtained with the quotient of *ca.* 3.3 (Fig. 3). The coefficient of variation (C.V.) of the quotients of the curve was 3%. The quotient of the M + 1 and M + 18 fragments using CI with ammonia the quotient was *ca.* 1.4. Using isobutane and plotting the quotient of *m/z* 169 and 201, the quotient was *ca.* 1.3. The C.V. of the quotients of the two CI curves was *ca.* 5%.

*Chromatography*

Capillary GC and flame ionization detection (FID) resulted in a very complicated chromatogram. Initial attempts to separate the HHP acid DME from the matrix were unsuccessful. Selective detection using MS was therefore investigated in more detail. Chromatograms obtained using EI and CI with ammonia and isobutane for HHP acid-spiked urine are shown in Fig. 4. The chromatographic behaviour of the dimethyl esters was excellent. A fused-silica capillary column with a non-polar stationary phase with a film thickness of 0.25 μm was chosen, owing to the lower elution temperature and reduced column bleeding achieved. At least 1000 injections could be done without any noticeable degradation of the column. When investigating thirteen out of fifteen urine samples from persons not exposed to HHPA, it was demonstrated that interfering compounds at very low levels in the urine samples determine the detection limit of the method. In two urine samples, peaks eluting with the same retention time as the HHP acid DME

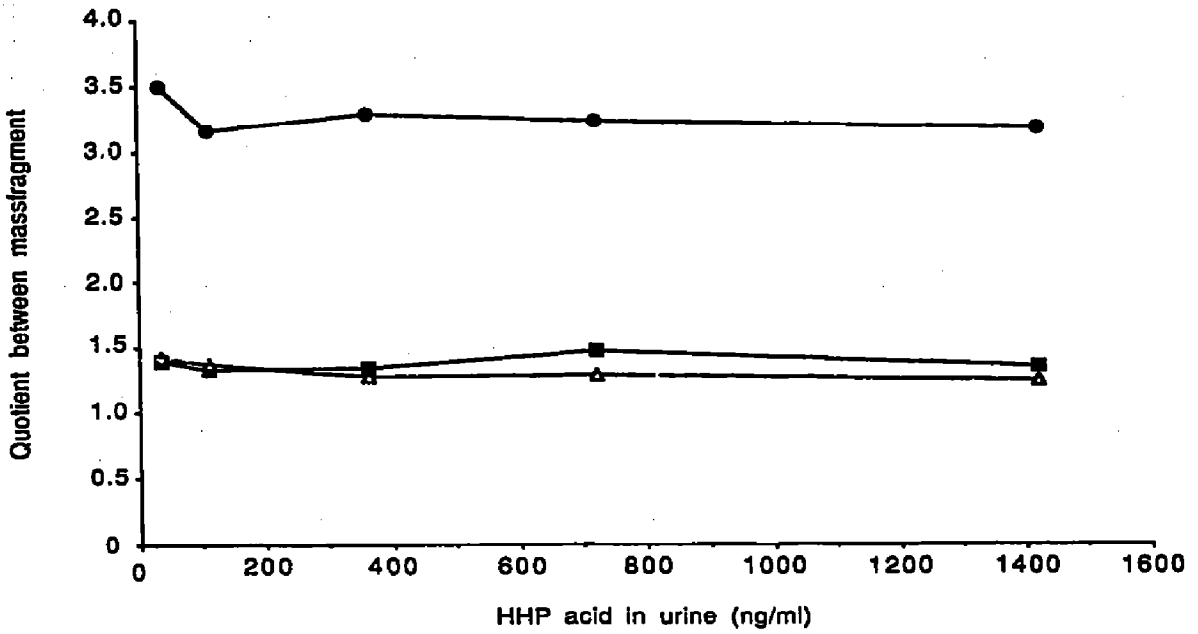


Fig. 3. Quotients of peak areas obtained using SIM between fragments from HHP acid DME: (●) EI mode (70 eV), monitored between *m/z* 140 and 169; (■) CI mode (ammonia), monitored between *m/z* 201 and 218; (△) CI mode (isobutane), monitored between *m/z* 169 and 201.

were obtained in the EI mode. These peaks were also observed using CI with ammonia or isobutane. It cannot be ruled out that the interfering peaks are derived from exposure to HHPA of an unknown origin.

*Quantitative analysis*

*Recovery.* The recovery for the overall method was investigated by working-up ten urine samples spiked with HHP acid containing 0.07 μg/ml and ten spiked urine samples containing 0.7 μg/ml without the addition of the internal standard. A 200-μl volume of toluene containing the internal standard [$^2H_6$]HHP acid DME was then added before the GC–SIM analysis. A comparison was made with dilute HHP acid DME standard solutions containing the same concentrations of the internal standard. Peak-area ratio measurements were used. The recovery at 0.07 μg/ml was 101% (C.V. 4%), and at 0.7 μg/ml the recovery was 95% (C.V. 2%).

*Precision.* Ten blank urine samples from ten different persons were spiked with three different amounts of HHP acid. The samples were then analysed according to the procedure. In Table I, the precision has been calculated with the external standard and the internal standard for *m/z* 140 and 169, and the mean of the concentrations was found at *m/z* 140 and 169. The best precision was found using the internal standard and the mean concentrations from both fragments.

*Calibration graphs.* Urine was spiked with different amounts of HHP acid, and the work-up procedure was then performed. For each concentration a single

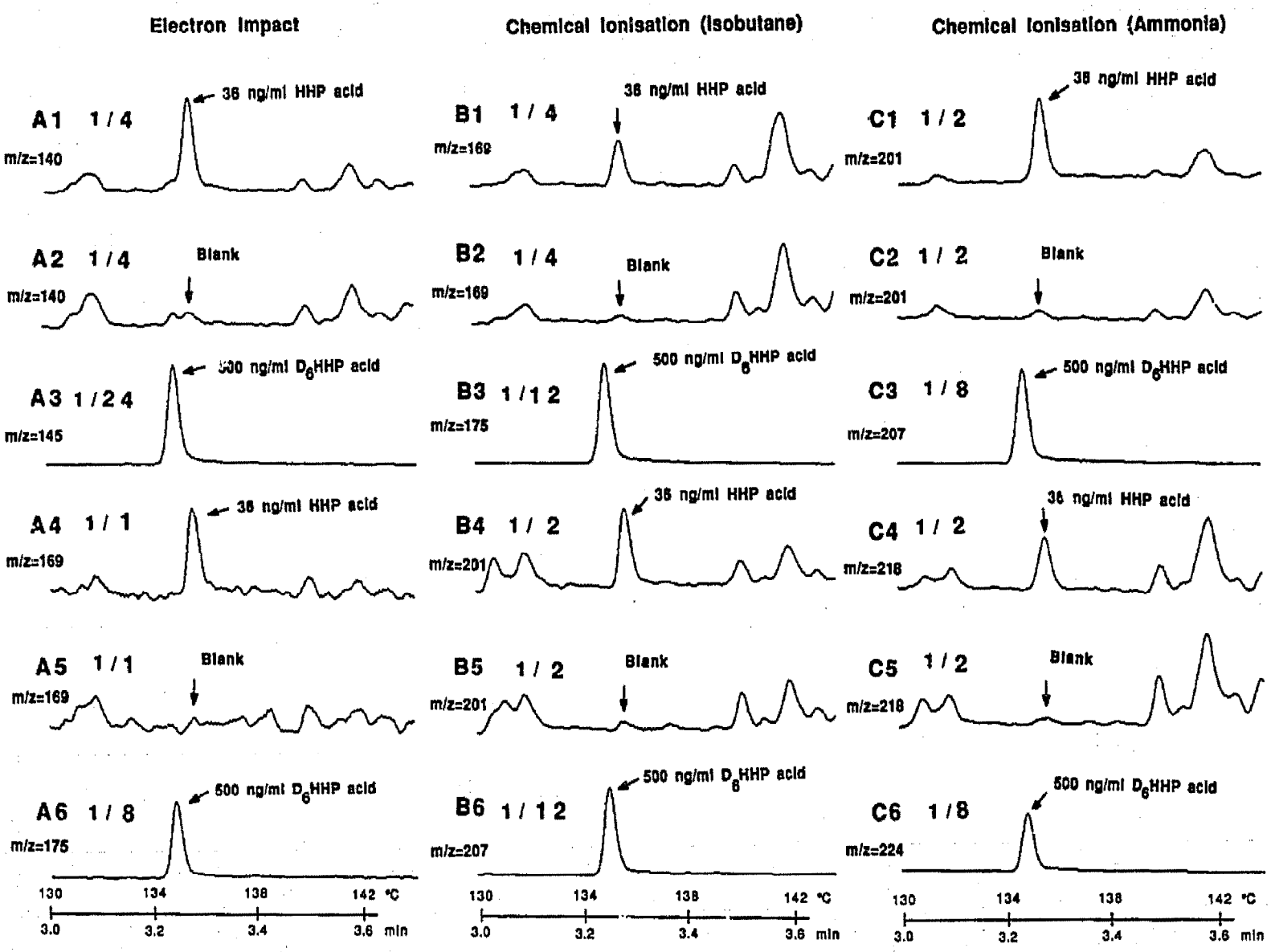


Fig. 4. SIM of urine samples spiked with HHP acid (36 ng/ml) and unspiked, with the addition of [$^2H_6$]HHP acid (500 ng/ml) as the internal standard, obtained in EI mode (A1–A6), in CI mode with isobutane (B1–B6), and in CI mode with ammonia (C1–C6). The mass fragmentograms in the figure are scaled (given in figure) with respect to the largest peak area (*m/z* 145 and monitoring 500 ng/ml [$^2H_6$]HHP acid).

TABLE I

PRECISION IN THE GC–MS ANALYSIS OF HHP ACID DME IN BLANK URINE SAMPLES SPIKED WITH THREE DIFFERENT AMOUNTS OF HHP ACID

| Concentration of HHP acid in urine (ng/ml) | Mass fragment | Coefficient of variation ($n$ = 10) (%) | |
|---|---|---|---|
| | | External standard | Internal standard |
| 700 | 140 | 12.5 | 4.0 |
| 700 | 169 | 12.1 | 3.9 |
| 700 | 140 + 169/2[a] | 12.3 | 3.6 |
| 70 | 140 | 19.0 | 5.4 |
| 70 | 169 | 19.7 | 5.6 |
| 70 | 140 + 169/2[a] | 19.2 | 4.8 |
| 20 | 140 | 25.5 | 21.0 |
| 20 | 169 | 29.4 | 22.9 |
| 20 | 140 + 169/2[a] | 27.0 | 21.1 |

[a] The mean of the concentrations found at $m/z$ 140 and 169 was used.

determination, with triple injections, was made. Calibration graphs in the investigated concentration range, 20–1400 ng/ml, were obtained using EI and CI with ammonia or isobutane as reagent gas. Linear calibration plots were obtained with correlation coefficients between 0.9996 and 0.9999. Peak-area measurements

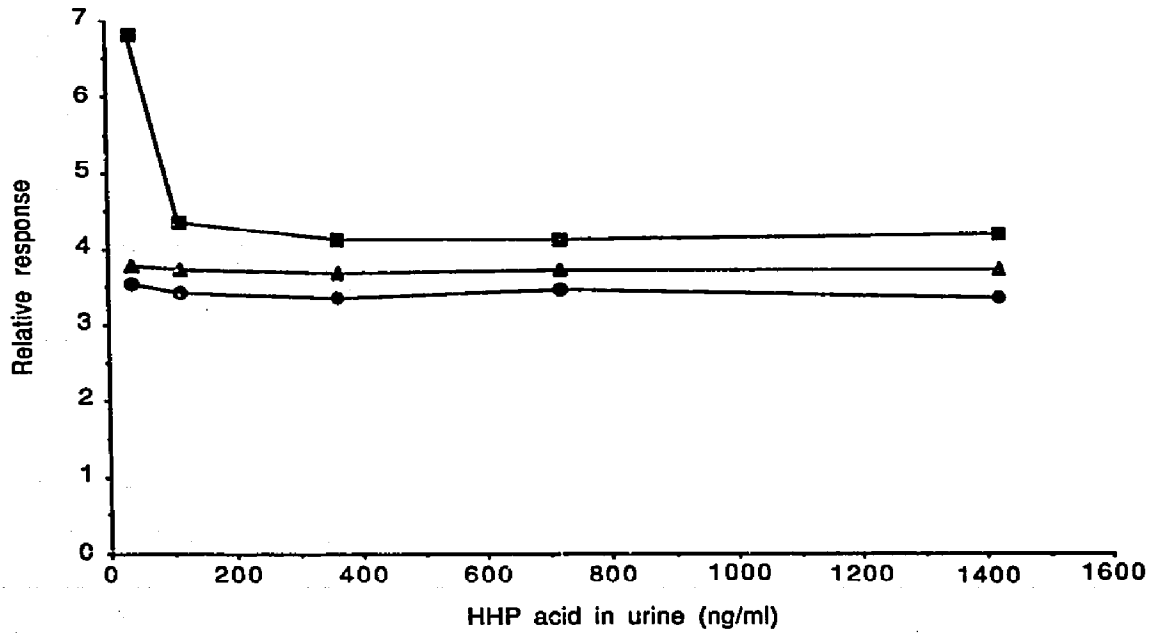


Fig. 5. Relative response (peak-area ratio to concentration) *versus* concentration of HHP acid in spiked urine: (●) EI mode (70 eV), monitored between $m/z$ 140 and 145; (■) CI mode (ammonia) monitored between $m/z$ 218 and 224; (▲) CI mode (isobutane) monitored between $m/z$ 201 and 207.

were preferred for calculation of the ratios because of the slightly better correlation coefficients achieved compared with peak-height measurement. The relative responses for the standard solutions are shown in Fig. 5 for EI with *m/z* 140 for HHP acid DME and *m/z* 145 for the internal standard, for CI with ammonia as reagent gas with *m/z* 218 for HHP acid DME and *m/z* 224 for the internal standard, and finally for CI with isobutane as reagent gas with *m/z* 201 for HHP acid DME and *m/z* 207 for the internal standard.

*Detection limit.* The detection limit was defined, according to Miller and Miller [20], as the concentration giving a peak-area ratio equal to the mean of the blank peak-area ratios plus three times the standard deviation in these. Fifteen blank urine samples, with a mean concentration of 12.7 m*M* creatinine, were analysed for the determination of the mean and standard deviation of the blank peak-area ratio. The detection limit for the GC–SIM method in the EI mode, with monitoring of the *m/z* 140 and 169 fragments, was *ca.* 20 ng/ml of urine. Using CI with ammonia or isobutane as the reagent gas, the detection limits were about twice as high. Furthermore, when ten of the urine samples were spiked with HHP acid to a concentration of 20 ng/ml, the standard deviations of the peak-area ratios of both the blank and the spiked urine samples were the same, which is in accordance with the Miller and Miller [20] definition. However, as described above, two of the blank urine samples analysed could possibly have contained HHP acid. When these two were omitted, the detection limit was 10 ng/ml.

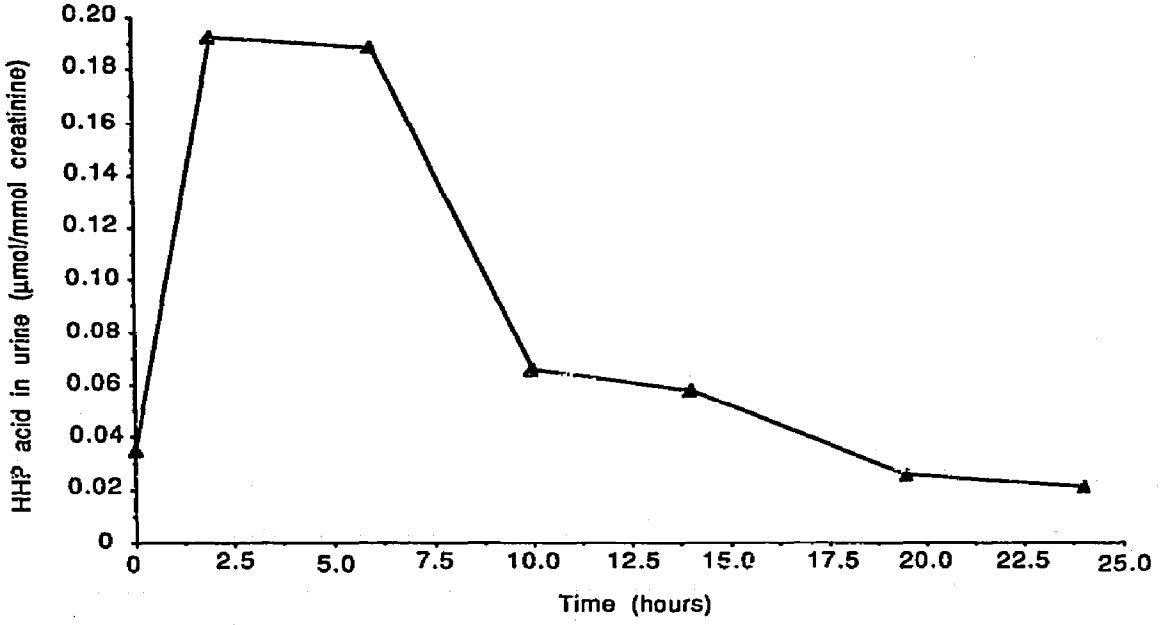


Fig. 6. Excretion of HHP acid in urine from a worker exposed to HHPA (8-h time-weighted average, 30 µg/m³) *versus* time. Urine samples were taken at 4-h intervals during the day and at 7-h interval overnight (triangles in the middle of the time intervals). The first urine sample represents the HHP acid concentration in the urine produced during 1 h before the working shift.

*Application.* Urine was sampled from a male worker exposed to an 8-h time-weighted average concentration of HHPA of 30 $\mu g/m^3$. All urine was collected during 24 h in 4-h samples during the day and 7-h samples during the night. Assuming an inhalation volume of 10 $m^3$ during the exposure period, more than *ca.* 85% of the inhaled dose was excreted in the urine as HHP acid. The ratio of HHP acid to creatinine is plotted against time for a 24-h period in Fig. 6. Extrapolation of the value from this worker makes it reasonable to assume that it is possible to monitor air concentrations of *ca.* 1–2 $\mu g/m^3$ HHPA.

## CONCLUSIONS

The GC–MS method was developed for the biological monitoring of occupational exposure to HHPA. The work-up of the urine samples with double liquid–solid extraction was demonstrated to be an effective clean-up procedure. The use of hexadeuterium-labelled HHP acid as the internal standard gave accurate and precise determinations. The limit of detection was sufficient for the determination of HHP acid in urine from exposed workers, thus making the method suitable for biological monitoring of HHPA.

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