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Method for analysis of methyltetrahydrophthalic acid in urine using gas chromatography and selected ion monitoring

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Abstract

A method for the determination of methyltetrahydrophthalic acid (MTHP acid), a metabolite of methyltetrahydrophthalic anhydride (MTHPA) in human urine, was developed. The investigated MTHP acid was obtained by hydrolysis of a commercial MTHPA mixture, composed of three major isomers. These were synthesized and identified as 3-methyl-Δ^4-tetrahydrophthalic anhydride, 4-methyl-Δ^4-tetrahydrophthalic anhydride and 4-methyl-Δ^3-tetrahydrophthalic anhydride. The urine was worked up by a liquid–solid extraction technique using C_{18} sorbent columns. Esterification was performed with methanol and boron trifluoride. The derivative in toluene was analyzed with capillary gas chromatography and selected ion monitoring. Deuterium-labeled MTHP acid was used as internal standard. The intra-assay precision for the overall method was between 4 and 8% in the range 3–110 ng/ml and the inter-assay precision was between 4 and 7% in the range 30–110 ng/ml. The total recoveries of the MTHP acid at 19 and 190 ng/ml were 94 and 97%, respectively. The total detection limit for the three isomers was <6 ng/ml. Analysis of urine from a worker exposed to MTHPA makes it reasonable to assume that the method may be used for biological monitoring of MTHPA exposure.

1. Introduction

Organic acid anhydrides are reactive chemicals with a widespread use in the chemical industry. They have been used for more than 50 years [1], mainly as intermediates of various manufactured products, such as plasticizers, alkyds and unsaturated polyester resins, phenolphthalein and other dyes, and as curing agents for epoxy resins [2,3]. Methyltetrahydrophthalic anhydride (MTHPA) is one important acid anhydride which is mainly used as a hardener in epoxy resins. There are many isomeric forms of MTHPA. MTHPA is also referred to as methylcyclohexene-1,2-dicarboxylic anhydride in the literature.

MTHPA, like other organic acid anhydrides, is an irritant to the eyes and to the mucous membranes in the respiratory tract [1]. It also causes occupational asthma and allergic rhinitis and it is a sensitizing agent at very low exposure levels [4–7]. Methods for determination of MTHPA in air has been published [8]. However, biological monitoring has some advantages over air sampling procedures. For example, biological sampling methods, in contrast to air sampling methods, compensate for individual variations such as different breathing rates during different body work. Moreover, biological monitoring is pos-

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sible even when protective devices are being used. It has previously been shown that hexahydrophthalic acid (HHP acid) is the main metabolite of hexahydrophthalic anhydride (HHPA) [9–11]. Since MTHPA and HHPA are chemically closely related compounds, it can be assumed that methyltetrahydrophthalic acid (MTHP acid) is a metabolite of MTHPA. No method for determination of MTHP acid in urine has been described. However, there are descriptions of methods for similar acids in urine [9,10,12,13].

The aim of this study is to develop and evaluate a method for determination of MTHP acid in human urine which can be used for biological monitoring of exposure to MTHPA. The present method uses a double liquid–solid extraction procedure with a silica bonded octadecylsilyl (C_{18}) phase followed by derivatization with methanol and boron trifluoride. Analysis is performed by gas chromatography–mass spectrometry (GC–MS) and selected ion monitoring (SIM).

2. Experimental

2.1. Apparatus

For the quantitative analyses, a VG Trio 1000 quadrupole mass spectrometer (Fisons, Manchester, UK) connected with a Carlo Erba 8065 GC equipped with an A200S autosampler (Carlo Erba, Milan, Italy) was used. For determinations of relative concentrations of anhydride or ester in mixtures, a Varian 3500 GC system (Varian, Palo Alto, CA, USA) equipped with a Varian flame ionization detection (FID) system was used. The clean-up columns was connected to a VacElut SPS 24 (Analytichem International, Habor City, CA, USA) which in turn was connected to an aspirating pump. For phase separations and drying of the clean-up columns, a Sigma 3E-1 centrifuge (Sigma, Harz, Germany) was used.

2.2. Columns

The analytical column was a fused-silica capillary column (30 m × 0.25 mm I.D.) with a DB-5 stationary phase and a film thickness of 0.25 μm (J & W Scientific, Folsom, CA, USA). For the clean-up procedure C_{18} Bond Elut LRC columns (500 mg; Analytichem International) were used.

2.3. Chemicals

Hydrochloric acid, isoprene, sodium hydrogencarbonate and sodium hydroxide were from Janssen Chemical (Geel, Belgium), 1,3-pentadiene (mixture of *cis* and *trans*) and 1,3-*trans*-pentadiene were from Aldrich (Gillingham, UK), mesityl oxide was from KEBO (Spånga, Sweden), methanol and toluene were from Lab-Scan (Dublin, Ireland), acrylic acid was from Merck (Darmstadt, Germany), acetyl chloride, bromine and sodium were from Riedel-de Haën (Seelze, Germany), boron trifluoride (14%) in methanol and tris(hydroxymethyl)aminomethane (Tris) were from Sigma (St. Louis, MO, USA) and dideuterium-labeled maleic anhydride was from MSD Isotopes (Merck Frosst Canada, Montreal, Canada). MTHPA of technical quality containing three isomers (HY 917; >95%) was from Ciba-Geigy (Basle, Switzerland) and methyltetrahydrophthalic acid dimethyl ester (MTHP acid DME; >95%) was from Syntes (Lund, Sweden) and synthesized from the technical MTHPA.

2.4. Synthesis

3-Methyl-Δ^4-tetrahydrophthalic anhydride (3-M-4-THPA) (Fig. 1)

Maleic anhydride (8.0 g) was dissolved in 50 ml toluene. The mixture was cooled in an ice-bath and 1,3-pentadiene (10.0 ml; mixture of *cis* and *trans*) was slowly added. The ice-bath was then removed, the mixture refluxed for 18 h, and thereafter evaporated to dryness. The residue was recrystallized from pentane [14,15].

4-Methyl-Δ^4-tetrahydrophthalic anhydride (4-M-4-THPA) (Fig. 1)

Maleic anhydride (8.0 g) was dissolved in 50 ml toluene. The mixture was cooled in an ice-bath and isoprene (10.0 ml) was slowly added. The ice-bath was then removed, the mixture was refluxed for 20 h, and thereafter evaporated to

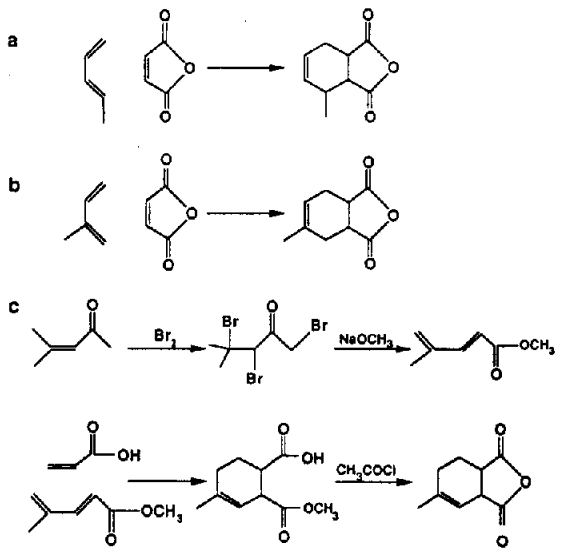

Fig. 1. General pathways in the synthesis of (a) 3-methyl-Δ^4-tetrahydrophthalic anhydride, (b) 4-methyl-Δ^4-tetrahydrophthalic anhydride and (c) 4-methyl-Δ^3-tetrahydrophthalic anhydride.

dryness. The residue was recrystallized from pentane [14,16].

4-Methyl-Δ^3-tetrahydrophthalic anhydride (4-M-3-THPA) (Fig. 1)

Bromine (58 g) was slowly added to mesityl oxide (28 ml) over a period of 40 min while the reaction vessel was chilled in an ice–salt bath. This product was used in a second step as soon as possible since the product, 1,3,4-tribromo-4-methyl-2-pentanone, is unstable in contact with air. A solution of sodium methoxide was prepared by reacting sodium (15 g) with methanol (280 ml). The 1,3,4-tribromo-4-methyl-2-pentanone was then slowly added to this solution while the vessel was chilled in an ice salt bath. The mixture was stirred for 2 h at room temperature and then added to 300 ml ice–water and extracted with 150 ml pentane. The pentane phase was dried, evaporated and the product was then vacuum distilled in the presence of hydroquinone and copper [17]. The product, methyl 4-methyl-2,4-pentadienoate (6 g), was mixed with acrylic acid (30 ml) and toluene (50 ml), the mixture was refluxed for 16 h and vacuum distilled. The product, 4-methyl-Δ^3-tetrahydrophthalic acid monomethyl ester (3 g), was refluxed with acetyl chloride (20 ml) for 23 h followed by vacuum distillation.

$^2H_2$-Labeled 3-M-4-THPA

Dideuterium-labeled maleic anhydride (0.9 g) was dissolved in 7 ml toluene. The mixture was cooled in an ice-bath and *trans*-1,3-pentadiene (1.0 g) was slowly added. The ice-bath was then removed, the mixture refluxed for 23 hours, and thereafter evaporated to dryness. The residue was used without any further clean-up [14,15].

2.5. Storage

The urine was stored without any pre-treatment in 10-ml polyethylene test tubes at −20°C until analysis.

2.6. Preparation of standards

Standard solutions of MTHP acid was prepared from MTHPA. Usually about 50 mg MTHPA was hydrolyzed in 25 ml 0.01 *M* NaOH. Solutions in desired concentrations were prepared from this solution by further dilution in 0.01 *M* NaOH. Urine standards containing MTHP acid were prepared by adding aliquots of these standard solutions to blank urine. For the determination of the amount of each isomer of acid, about 50 mg of the anhydride mixture was dissolved in toluene. This solution was then further diluted in toluene to a concentration of ca. 10 μg/ml and thereafter analyzed by GC–FID. It was assumed that the FID response was equal for all isomers and that no isomerization occurred during the hydrolysis.

In the determination of the recovery, standard solutions of MTHP acid DME were prepared in toluene. About 50 mg of MTHP acid DME was dissolved in 25 ml of toluene. The solution was then further diluted in toluene to desired concentrations. For the determination of the amount of each isomer of the MTHP acid DME, this mixture was dissolved in toluene and analyzed by GC–FID. It was assumed that the FID response was equal for all isomers.

2.7. Work-up procedure

To 2.0 ml of urine in 13 ml test tubes with PTFE screw caps, 100 μl of an internal standard solution containing 100 ng $^2H_2$-labeled 3-M-4-THP acid in 0.01 *M* NaOH were added. The pH of the urine samples was then adjusted with 3.0 ml 0.25 *M* HCl to a pH below 2 prior to the extraction of the urine samples. C_{18} Bond Elut columns were conditioned by passing 5 ml of methanol followed by 10 ml of 0.1 *M* HCl through the columns. The columns were not allowed to dry out between or after these steps. The urine samples were then added to the columns, which resulted in the trapping of the MTHP acid. As a washing step 10 ml of 0.1 *M* HCl were added. The columns were then dried by suction of air for 5 min. Thereafter, the MTHP acid was eluted with 4.0 ml 0.1 *M* Tris buffer (pH 8.8). The eluates were acidified with 4.0 ml of 0.25 *M* HCl to a pH below 2. The same C_{18} columns were reconditioned with 10 ml of methanol and 10 ml of 0.1 *M* HCl and as before the columns were not allowed to dry out between or after these steps. The acidified samples were then added to the columns and retrapped. Again 10 ml of 0.1 *M* HCl were added as a washing step. The columns were then dried for 5 min by suction of air, centrifuged at 1500 *g* for 10 min, and again with air suction for 10 min before the elution with 3.0 ml methanol.

2.8. Derivatization

The eluates were directly derivatized by adding 2.0 ml of 14% BF_3 in methanol. Esterification was performed overnight at 70°C in 13-ml test tubes with PTFE screw caps. The samples were allowed to cool to room temperature and 4.0 ml of saturated sodium hydrogencarbonate solution was slowly added followed by 2.0 ml of toluene. The solutions were shaken for 15 min and then centrifuged for 3 min at 1500 *g* for phase separation. The toluene phase, now containing the MTHP acid DME, was transferred to 2-ml autosampler vials with PTFE screw caps. The vials were stored in a freezer at −20°C until analysis.

2.9. Analysis

Samples were injected with a splitless injection technique. The injector temperature was kept at 250°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 1 min after the injection. The initial column temperature was 110°C for 1 min. The temperature was then increased by 6°C/min to 140°C and kept there for 3 min. Thereafter the temperature was increased again with 40°C/min until 240°C where it was kept for 2 min. The MS interface and the ion source were at 250°C. The carrier gas was helium at a pressure of 85 kPa at 110°C. MS was in the positive chemical ionization (CI) mode with ammonia as reagent gas. The electron energy was 70 eV, the monitoring time for each ion was 0.08 s and the inter scan delay 0.02 s. SIM of MTHP acid DME was performed at *m/z* 213 while *m/z* 215 was chosen for the internal standard. Peak area ratio measurements were used for the determinations.

3. Results and discussion

3.1. Isomeric composition

The studied anhydride was of technical quality. It was made industrially by a Diels–Alder reaction between maleic anhydride and a mixture between isoprene and pentadiene. Thereafter the product was further isomerized via a catalyst. The resulting mixture was a viscous, transparent liquid composed of three main isomers which were identified as 3-M-4-THPA, 4-M-4-THPA and 4-M-3-THPA using ^1H, ^{13}C NMR and GC–MS in electron impact (EI; Fig. 2) mode. The pure isomers of 3-M-4-THPA and 4-M-4-THPA were synthesized. 4-M-3-THPA was also synthesized but we were not able to obtain a pure sample of this isomer.

3.2. Standards

One of the isomers of the commercially used MTHPA mixture was not obtained in a pure

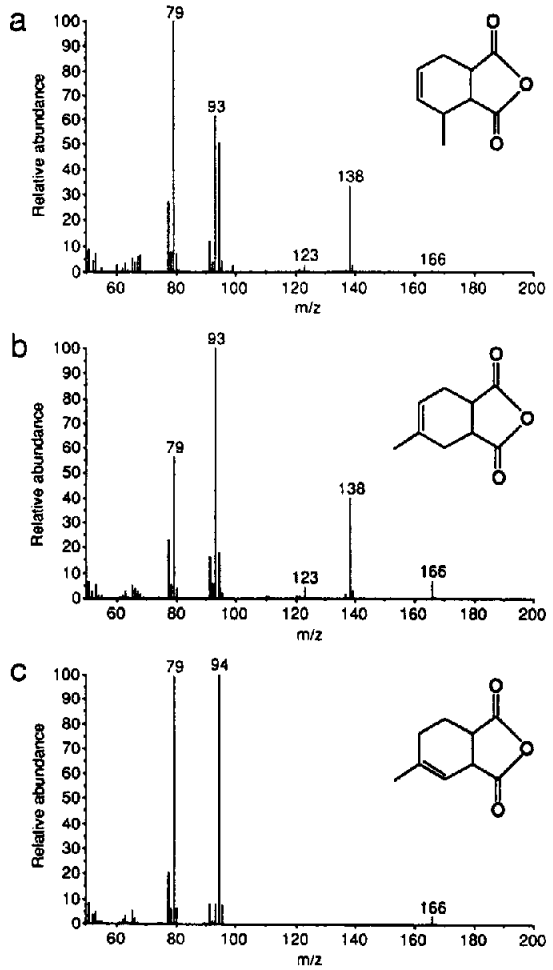

Fig. 2. Electron impact (70 eV) mass spectra of (a) 3-methyl-Δ^4-tetrahydrophthalic anhydride, (b) 4-methyl-Δ^4-tetrahydrophthalic anhydride and (c) 4-methyl-Δ^3-tetrahydrophthalic anhydride.

form and therefore a standard solution containing weighted amounts of this isomer could not be prepared. It was, however, necessary to make individual calibration curves for all isomers since it cannot be assumed that the MS response was equal for all three isomers. This problem was solved by analyzing the commercial MTHPA mixture by GC–FID and assuming an equal response for all isomers with this equipment. There may, of course, be some small error in this procedure but compared to other errors involved in a biological monitoring method, these can be assumed to be minor. This is supported by the fact that weighted amounts of 3-M-4-THPA and 4-M-4-THPA showed equal responses as to those obtained from the commercial mixture using the FID procedure. It seems reasonable to assume that there was no isomerization of the compounds during the hydrolysis in as diluted solutions as 0.01 *M* NaOH. The relative amounts of each isomer in the investigated anhydride mixture were found to be 28, 16 and 56% for 3-M-4-THPA, 4-M-4-THPA and 4-M-3-THPA, respectively.

The MTHP acid DME, used in the recovery study, was synthesized in a commercial laboratory from the commercially used anhydride and methanol with acid as catalyst. The mixture was thereafter distilled. This resulted in a different concentration ratio between the isomers as compared to the original MTHPA. Hence, the same method as described above for the MTHP acid solutions was also used for the calculations of the amount of each isomer of MTHP acid DME.

3.3. Storage

Urine samples from a worker with MTHP acid in the range 0–250 ng/ml were analyzed before and after 8 months storage at −20°C. No degradation of the MTHP acid was found.

3.4. Conjugation of MTHP acid

Some carboxylic acids are known to be excreted in urine as conjugates with glucuronides [18]. These conjugates can often be hydrolyzed in as mild conditions as 0.2 *M* HCl. To examine if there was such a conjugation, a hydrolysis test was performed. Both urine from a MTHPA exposed worker and blank urine spiked with MTHP acid were treated with 1 *M* HCl and 100°C for between 30 min and 45 h. No elevation of the MTHP acid concentration was observed for the MTHPA exposed worker. However, in both the urine from the worker and in the spiked urine, some isomerizations and looses of the MTHP acid could be seen even at these rather mild conditions. At 6 *M* HCl the losses and isomerizations were more severe. Still, the result indicates that there was no conjugation of the

MTHP acid with glucuronides. However, there is still a possibility that the MTHP acid my be conjugated with other substances. For example, conjugates between MTHPA and lysine seem not to be successfully hydrolyzed with even 6 *M* HCl for 100°C in 24 h (data not shown).

3.5. Work-up procedure

Numerous papers have been published on work-up methods for dicarboxylic acids in biological samples. Liquid–liquid extraction techniques have been frequently used [9,12,13]. However, they often give insufficient clean-up and are generally laborious when many samples need to be treated. Recently the use of liquid–solid extraction techniques have proved to be successful in the work-up of biological samples with complicated analytical matrixes. A technique based on the combination of C_{18} and trimethylaminopropylsilyl (SAX) liquid–solid extraction columns for determination of HHP acid has been described [10]. This technique was tried for the work-up of MTHP acid with a clean matrix as a result but with a decreased recovery of the MTHP acid. The main losses were found during the washing step using the C_{18} columns and the method was therefore optimized by testing different ,washing solutions for the these columns. HCl solutions (0.1 *M*) with different concentrations of methanol in the range 0–30% were tried but an increasing loss was found for all concentrations of methanol added compared to pure 0.1 *M* HCl. Different buffer solutions were also tried such as phosphoric buffer and Tris buffer with different pH and additions of methanol. However, all buffers gave losses of more than 40% and it was concluded that the best result was obtained with 0.1 *M* HCl without any addition of methanol. Since this also gave a sufficiently clean matrix, this solution was used for the washing. The step with the SAX columns was more laborious than with the C_{18} columns. The possibility to use a double extraction procedure with C_{18} columns in stead was therefore investigated. This was found to still give sufficiently low detection limits and was therefore chosen. The use of a single C_{18} extraction procedure was also investigated but resulted in a very impure matrix. It was concluded that the elution step with the Tris buffer was necessary for obtaining a good purification. The C_{18} columns were reused up to 8 times without any noticeable change in performance.

3.6. Mass spectrometry

The mass spectra of 3-M-4-THPA, 4-M-4-THPA and 4-M-3-THPA using EI are shown in Fig. 2. 3-M-4-THPA and 4-M-4-THPA showed rather similar spectra with abundant M − 28 (M − CO), M − 72 (M − C_2O_3), M − 73 (M − C_2O_3H; base peak in 4-M-4-THPA), and M − 87 (phenonium ion; base peak in 3-M-4-THPA) fragments. Both had also a small fragment at M − 43 (M − CO − CH_3) but 4-M-4-THPA had a more abundant molecular ion (M) at $m/z = 166$ as compared to 3-M-4-THPA. 4-M-3-THPA had small M and M − 73 (M − C_2O_3H), abundant M − 72 (M − C_2O_3) and M − 87 (phenonium ion) fragments, but showed none of the other characteristic fragments.

Fig. 3 shows the mass spectra of 3-M-4-THP acid DME and $^2H_2$-labeled 3-M-4-THP acid DME using CI with ammonia as reagent gas. This gave M + 1 as the base peak at m/z 213 for the MTHP acid DME and m/z 215 for the internal standard. The pressure of ammonia in the ion source was adjusted to give a relative abundance of the M + NH_3 fragment of ca. 25% as compared to the base peaks. The two other isomers of MTHP acid DME showed similar fragmentations. The technique using CI with ammonia as reagent gas was chosen since EI strongly fragmented the molecule. The base peak in the EI spectrum of MTHP acid DME was m/z 93 and the matrix in urine at that fragment was very impure. However, the matrix at m/z 213 and 215 was fairly clean. It was also possible to use the less abundant fragments at m/z 230 and 232 for the monitoring but this gave a less clean matrix. On the other hand, the similar results when using both the M + 1 and the M + NH_3 fragments for the monitoring presented an evidence that the compound analysed in the urine in fact is MTHP acid.

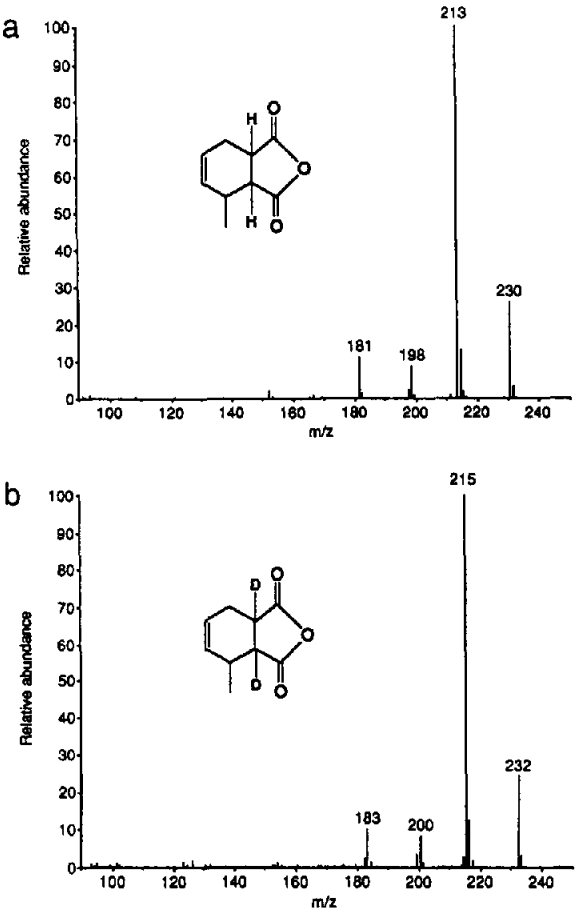

Fig. 3. Mass spectra of (a) 3-methyl-Δ^4-tetrahydrophthalic acid dimethylester and (b) $^2H_2$-labeled 3-methyl-Δ^4-tetrahydrophthalic acid dimethylester using positive chemical ionization with ammonia as reagent gas.

3.7. Chromatography

Analysis of urine samples using GC–FID showed very complex chromatograms and attempts to separate MTHP acid DME from the matrix were not successful. MS gave a much cleaner matrix and was therefore chosen. Chromatograms of a urine sample from a MTHPA-exposed worker at a total MTHP acid concentration of 23 ng/ml and a blank urine sample are shown in Fig. 4. The chromatographic behavior of the dimethyl esters were good. A non-polar column with a thin film thickness was used for the analysis because of the lower elution temperature and lower column bleeding achieved. Thousands of injections have been made without any observed column aberrations. A slow temperature program had to be chosen to separate the MTHP acid DME isomers from some interfering peaks.

3.8. Quantitative analysis

Calibration graph

Data on calibration graphs of 3-M-4-THP acid, 4-M-4-THP acid and 4-M-3-THP acid, made from spiked and worked-up blank urine, are shown in Table 1.

Detection limit

Urine samples were collected from ten volunteers who were presumed to be unexposed to MTHPA or MTHP acid. Internal standard was added and the samples were worked up and analyzed according to the method described above. The detection limit was calculated as reported by Miller and Miller [19] as the concentration corresponding to the peak height ratios with the same retention time as MTHP acid DME plus three times the standard deviation of these. The detection limit was <2 ng/ml for each of the three isomers giving a total detection limit of <6 ng MTHP acid/ml urine.

Recovery

The recovery of the overall method was investigated by working up ten urine samples spiked with a mixture of the MTHP acid isomers but with no addition of $^2H_2$-labeled 3-M-4-THP acid. However, prior to the analysis, a toluene solution containing the internal standard, $^2H_2$-labeled MTHP acid DME, was added. This internal standard was prepared by working up water solutions of hydrolyzed $^2H_2$-labeled 3-M-4-THPA according to the method described above for urine. Comparisons were made with standard solutions of MTHP acid DME in toluene with the same concentration of internal standard added. The recoveries of the method are shown in Table 2.

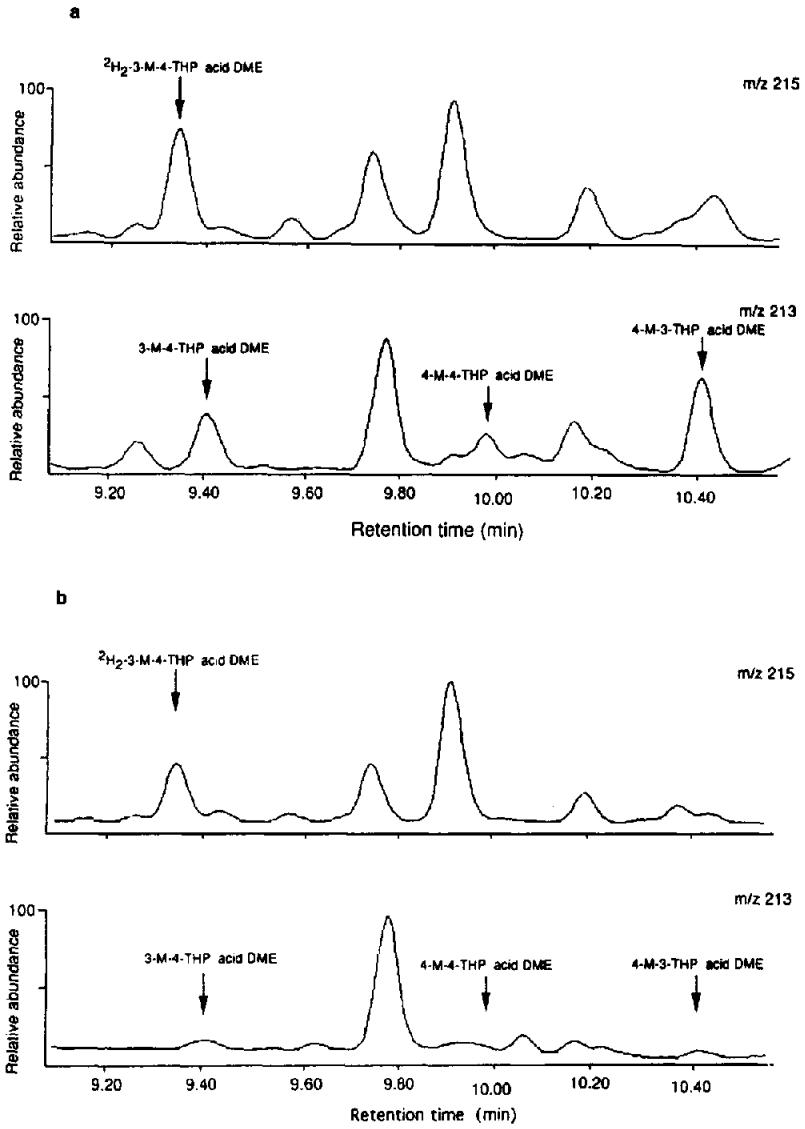

Fig. 4. (a) Chromatograms of a urine sample from a worker exposed to MTHPA. The concentration of MTHP acid was 6, 3 and 14 ng/ml, respectively for the three isomers. (b) Chromatograms of a urine sample from a non-exposed volunteer. For analytical conditions see text.

Precision

The repeatability (precision within a run of ca. 3 h) of the method was determined by analysis of ten different urine samples spiked with MTHP acid. The coefficients of variation for the ratio between the area of MTHP acid DME and the internal standard for all three isomers are shown in Table 3. In addition, the reproducibility between different assays during a period of three days was determined. The results are shown in Table 3.

Application

Urine was sampled from a worker exposed to MTHPA at an 8-h time-weighted average of 11 $\mu g/m^3$ (total amount). All urine was collected

Table 1
Calibration graph for three isomers of MTHP acid in urine

Isomer	Concentration range (ng/ml)	Slope (ml/ng)	Intercept	Correlation coefficient
3-M-4-THP acid	2–200	0.0070	0.0455	0.9996
4-M-4-THP acid	2–110	0.0047	0.0386	0.9937
4-M-3-THP acid	4–420	0.0050	0.0492	0.9983

Table 2
Recovery of MTHP acid DME from ten urine samples spiked with different amounts of three isomers of MTHP acid

Isomer	Concentration (ng/ml)	Recovery (%)	Precision[a] in recovery (%)
3-M-4-THP acid	5	61	16
3-M-4-THP acid	50	84	7
4-M-4-THP acid	3	73	11
4-M-4-THP acid	30	102	4
4-M-3-THP acid	11	117	10
4-M-3-THP acid	110	102	4
Total[b]	19	94	9
Total[b]	190	97	3

[a]Given as coefficients of variation.
[b]Sum of the three isomers.

during the 24 h in 4-h samples during the daytime and 7-h samples during the night. Assuming an inhaled volume of 10 m^3 during a working day about 70% of the inhaled dose was excreted in the urine as MTHP acid. In Fig. 5 the ratio between MTHP acid and creatinine in urine from the exposed worker is plotted versus the time. The half-times in the body were 3, 3 and 6 h for 3-M-4-THP acid, 4-M-4-THP acid and 4-M-3-THP acid, respectively.

4. Conclusions

The method was designed to monitor concentrations of MTHP acid in urine from workers

Table 3
Precision in the GC–MS analysis of ten urine samples spiked with different amounts of three isomers of MTHP acid

Isomer	Concentration (ng/ml)	Repeatability[a] (%)	Reproducibility[a] (%)
3-M-4-THP acid	5	7	
3-M-4-THP acid	50	4	4
4-M-4-THP acid	3	8	
4-M-4-THP acid	30	5	6
4-M-3-THP acid	11	5	
4-M-3-THP acid	110	4	7

[a]Given as coefficients of variation.

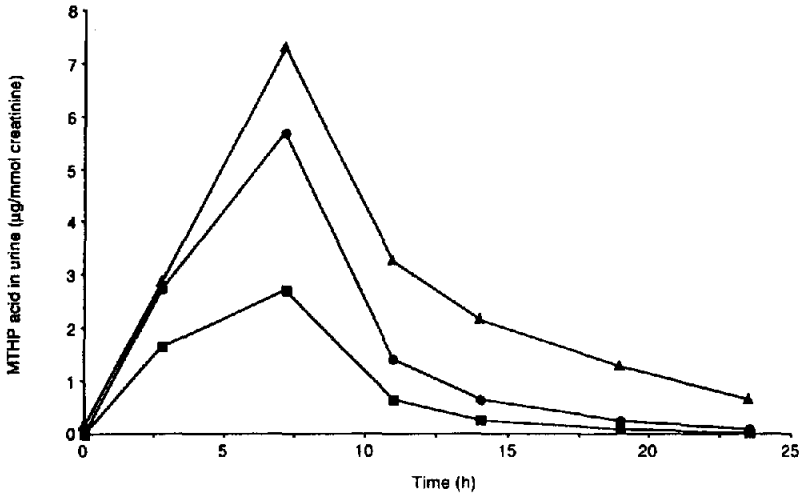

Fig. 5. Amount of MTHP acid in urine from a worker exposed to 11 μg MTHPA/m^3 plotted versus time. (●) 3-Methyl-Δ^4-tetrahydrophthalic acid; (■) 4-methyl-Δ^4-tetrahydrophthalic acid; (▲) 4-methyl-Δ^3-tetrahydrophthalic acid. The worker was unexposed to MTHPA on the day before the sampling.

exposed to MTHPA. The work-up procedure is relatively simple and fast. The method has a high precision and an equally good recovery. The detection limit is sufficient to monitor low exposure levels of MTHPA, thus making it suitable for biological monitoring of MTHPA.

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