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# CHROMATOGRAPHIC DETERMINATION OF AMINES IN BIOLOGICAL FLUIDS WITH SPECIAL REFERENCE TO THE BIOLOGICAL MONITORING OF ISOCYANATES AND AMINES

## III. DETERMINATION OF 4,4′-METHYLENEDIANILINE IN HYDROLYSED HUMAN URINE USING DERIVATIZATION AND CAPILLARY GAS CHROMATOGRAPHY WITH SELECTED ION MONITORING

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SUMMARY

A method is presented for the determination of 4,4′-methylenedianiline in hydrolysed human urine, based on a derivatization procedure with pentafluoropropionic anhydride and capillary gas chromatographic determination with selected ion monitoring. Dideuterated 4,4′-methylenedianiline was used as an internal standard. The ratio of the molecular ion of the 4,4′-methylenedianiline amide derivative (*m/z* 490) and the dideuterated 4,4′-methylenedianiline amide derivative (*m/z* 492) was used for quantitative analysis. The calibration graph was linear in the investigated range (5–30 μg/l in urine) with a correlation coefficient of 0.998. The precision was 3.4% for urine samples containing 26 μg/l and the detection limit was 2 μg/l. The overall recovery for urine samples containing 115 μg/l was 96 ± 5%. Urine samples from eight skin-exposed epoxy resin workers were examined and 4,4′-methylenedianiline was found in the concentration range 2–600 μg/l.

## INTRODUCTION

4,4′-Methylenedianiline (MDA) is a commercially important diamine used as an intermediate in the preparation of epoxy resins, polyurethanes, rubber chemicals and polymers. By far the largest application for MDA is as an intermediate for isocyanates[1,2].

MDA has been reported to be hepatoxic in dogs[3], rats[4,5] and humans[6,7]. It has shown to be mutagenic in the Ames test in the presence of an S9 metabolic activation system[8]. MDA has also been reported to be carcinogenic in rats and mice[9]. MDA is absorbed readily through the skin[10], which causes problems when handling MDA. The determination of MDA in biological fluids from occupationally exposed persons[11,12] is therefore of major importance. The hydrolysis of authentic urine and the possible metabolites N-acetyl-MDA and N,N′-diacetyl-MDA has been describ-

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ed[11,13]. The capillary gas chromatographic (GC) trace determination of MDA has met with considerable difficulties owing to the easy oxidation and adsorbtion of MDA in the analytical system[14], which makes the use of a derivatization procedure necessary. Anhydrides have been reported as derivatization reagents for GC determinations of MDA[11,12,14–17]. Several techniques have been developed for the determination of MDA in matrices such as air sampling solutions[15,16,18], blood[17] and urine[11,12,19,20]. The detection methods used in GC methods are thermionic specific detection (TSD), electron-capture detection (ECD) and selected ion monitoring (SIM). For the determination of MDA in urine, detection limits in the range 5–10 μg/l[11,12,19] have been reported. 4,4′Methylenebis-2-chloroaniline[11] and 4,4′-ethylenedianiline (EDA)[12,17] have been used as internal standards for GC–SIM.

In this paper, a GC–SIM method is described for biological monitoring of MDA, at low μg/l levels, in human urine using dideuterated MDA ([$^2H_2$]MDA) as an internal standard.

## EXPERIMENTAL

### *Equipment*

The analyses were performed on a Shimadzu (Kyoto, Japan) QP1000 EI/CI quadrupole mass spectrometer. The mass spectrometer was connected to a Shimadzu GC-9A gas chromatograph equipped with an SPL-G9 split–splitless injection system and a Shimadzu AOC-9. The carrier gas was helium and the inlet pressure was 0.5 kg/cm$^2$. The injector temperature was 280°C. The split exit valve was closed for 1 min after injection. The oven temperature was isothermal at 100°C for 2 min, then increased at 30°C/min to 300°C, the final temperature being maintained for 2 min. The quadrupole mass-selective detector was operated in the electron-impact (EI) mode at 70 eV using SIM at $m/z$ 490 and 492. The interface temperature was 250°C and the ion source temperature 300°C. The filament of the ion source was on between 7.5 and 10 min after the injection into the GC column. Five measurements were made per second. The mass spectrometer was calibrated with tris(nonafluorobutyl)amine according to the standard procedure.

The high-performance liquid chromatography (HPLC) system consisted of a Waters 600 multi solvent delivery system (Millipore-Waters, Milford, MA, U.S.A.), a Waters 712 WISP with variable injection volume, a Waters 490 programmable multi-wavelength detector and a Shimadzu C-R3A integrator.

A Varian (Walnut Creek, CA, U.S.A.) 3500 gas chromatograph equipped with a Varian thermionic specific detector and a Varian 8035 automatic on-column injector was employed. For separation of phases, a Model 3E-1 centrifuge (Sigma, Harz, F.R.G.) was used. For enrichment and evaporation, a vacuum desiccator connected to an aspirating pump was employed. The vacuum desiccator was equipped with an electrically heated oven, designed and manufactured in our laboratory.

### *Columns*

Fused-silica capillary columns, with chemically bonded apolar stationary phases, were tested: Chrompack (Middelburg, The Netherlands) CP-Sil 8 CB (25 m × 0.32 mm I.D.) with film thicknesses of 1.2, 0.50 and 0.25 μm and CP-Sil 8 CB (25 m × 0.25 mm I.D.) with film thickness of 0.12 μm; Nordion (Helsinki, Finland) NB-54

(25 m × 0.32 mm I.D.) with film thicknesses of 1.0, 0.50 and 0.25 μm; and J & W Scientific (Folsom, CA, U.S.A.) DB-5 (30 m × 0.25 mm I.D.) with a film thickness of 0.25 μm.

The HPLC column was made of stainless steel (25 cm × 3 mm I.D.) and was packed with Nucleosil $C_{18}$ particles (5 μm) (Marcherey-Nagel, Düren, F.R.G.).

*Chemicals*

MDA, EDA and 2,4-toluenediamine (2,4-TDA) were obtained from Aldrich (Beerse, Belgium). The [$^2H_2$]MDA [$C^2H_2(C_6H_4NH_2)_2$ hydrochloride] was synthesized at Synthelec (Lund, Sweden). Trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA) and heptafluorobutyric anhydride (HFBA) were obtained from Pierce (Rockford, IL, U.S.A.), toluene and acetonitrile of HPLC grade from Lab-Scan (Dublin, Ireland), tris(nonafluorobutyl)amine, sodium hydroxide, hydrochloric acid, potassium dihydrogenphosphate and dipotassium hydrogenphosphate (anhydrous) from Merck (Darmstadt, F.R.G.) and ethanol from Kemetyl (Stockholm, Sweden).

*Synthesis of MDA amide derivatives*

A 17.5-g amount of PFPA was added to a solution containing 5.0 g of MDA and 90 ml of toluene. The mixture was slowly heated to 70°C, then cooled and evaporated to dryness on a rotating evaporator. The residue was recrystallized from ethanol–water (7:1) and the crystals were filtered and washed with the same mixture. The crystals obtained were dried in a vacuum desiccator. The HFBA derivative of MDA was synthesized in a similar manner.

*Preparation of standard solutions*

Stock solutions of MDA and [$^2H_2$]MDA were prepared in 0.1 *M* HCl at the 1 g/l level and diluted with 0.1 *M* HCl to the appropriate concentrations. A stock solution of MDA-PFPA derivative was prepared in ethanol at the 1 g/l level and was diluted with toluene to the desired concentrations. The solutions were stable for more than 10 weeks without any noticeable degradation when stored in a refrigerator.

*Sampling*

A 2-ml volume of 6 *M* HCl per 100 ml of urine was added to the urine samples. The acidified urine samples were stored in a refrigerator until analysis.

*Work-up procedure*

A 0.8-ml urine sample was placed in a 10-ml test-tube fitted with a PTFE cap and 1.2 ml of 6 *M* HCl and 0.1 ml of 0.5 mg/l [$^2H_2$]MDA solution were added. The test-tube was heated at 95–100°C for 2 h and then cooled to room temperature. Toluene (3 ml) and 4 ml of saturated aqueous NaOH solution were added and the mixture was shaken for 10 min and then centrifuged at 1500 *g* for 10 min. A 2-ml volume of the organic phase was separated and transferred to a new test-tube, 20 μl of PFPA were added and the solution was shaken for 5 min. Then, 2 ml of 1 *M* aqueous phosphate buffer (pH 7.5) were added and the mixture was shaken for 10 min. A 1-ml volume of the organic phase was transferred to a new test-tube and evaporated to dryness in a vacuum desiccator at 30°C. The dry residue was dissolved in 0.1 ml of toluene and 1 μl of this solution was injected into the GC–mass spectrometric (GC–MS) system.

## RESULTS AND DISCUSSION

*Standard*

The identity of the MDA-PFPA derivative was confirmed by GC–MS and the purity was determined using both HPLC–UV detection ($\lambda = 245$ nm) and capillary GC–TSD. The purity was found to be better than 99%. The purity was further examined by elemental analysis and the experimental carbon, hydrogen and nitrogen percentages were found to differ by less than 0.2% from the calculated values.

*Isotopic purity*

The ratio of the mass fragment of *m/z* 492 to *m/z* 490 of the MDA-PFPA derivative was 3%, as expected from the naturally occurring isotopes in the MDA-PFPA derivative. The ratio of the mass fragment of *m/z* 490 to *m/z* 492 of the [$^2H_2$]MDA-PFPA derivative was 6% as a result of the remaining parent material.

*Work-up procedure*

*Hydrolysis.* Urine samples from workers exposed to MDA were hydrolysed under weakly acidic, strongly acidic and strongly basic conditions. The optimum conditions were 2 h at 95–100°C under strongly acidic conditions[13].

*Extraction of MDA.* The recovery was almost completely independent of whether 5 *M* NaOH or saturated NaOH was added to the hydrolysed urine sample. The foaming tendency was found to be less pronounced when saturated NaOH was used.

*Derivatization.* Three derivatization reagents, TFAA, PFPA and HFBA, were tested. The recoveries for the derivatization reactions were *ca.* 100%. Excess reagent and liberated acid were removed by extraction with 1 *M* phosphate buffer (pH 7.5) without any measurable losses of the MDA amide derivative.

*Enrichment.* The components of interest were enriched 10-fold. The removal of the remaining excess of reagent and the volatile impurities present in the sample increased the lifetime of the capillary column. The opportunities to enrich the sample further are obvious and the possibility of choosing an optimum solvent for the subsequent GC analysis is also feasible.

*Chromatography*

Capillary columns with an apolar stationary phase with a film thickness in the range 0.12–1.2 $\mu$m were tested. Of the three derivatives tested, the MDA-PFPA derivative showed the best column performance. The elution temperature was 300°C. No endogenous peaks from the urine extracts interfered in the analysis. The necessity to achieve an appropriate vacuum in the MS system defined the chromatographic system. A relative long capillary column, (20–30 m) of I.D. 0.25 mm was used to avoid a sub-atmospheric inlet pressure. The pressure in the ion source was *ca.* $2 \cdot 10^{-5}$ Torr. The column oven was maximally temperature programmed at 30°C/min owing to the better signal-to-noise ratio obtained. More than 1000 chromatographic analysis were made without any noticeable column degradation.

*Internal standard*

EDA, 2,4-TDA and [$^2H_2$]MDA were tested. EDA and 2,4-TDA gave unsatis-

factory precision. [$^2H_2$]MDA was found to be the best choice on the basis of the similar fragmentation pattern and chemical performance, and it is not expected to appear in a workplace environment.

*Mass spectra*

For the purpose of identifying and selecting suitable fragment ions for quantitative analysis, mass spectra of the PFPA derivatives of MDA and [$^2H_2$]MDA were obtained (Fig. 1). The fragmentation patterns for the two isotopes are very similar. Typical fragmentations were found[16,17] and the most abundant peaks were the molecular ions.

*Detection*

*Thermionic specific detection.* The molar sensitivity using GC–TSD is similar for the three derivatives of MDA investigated. The detection limit was *ca.* 40 fmole of MDA-PFPA derivative injected. Interfering peaks occur in the chromatograms from the urine samples, giving a practical detection limit of *ca.* 600 μg/l. The efficient use of a GC–TSD method for urine samples demands a more complete sample clean-up or a more selective derivatization procedure.

*Selected-ion monitoring.* The molecular ion was chosen owing to the sensitivity and the more significant information provided. The sensitivities of the PFPA and

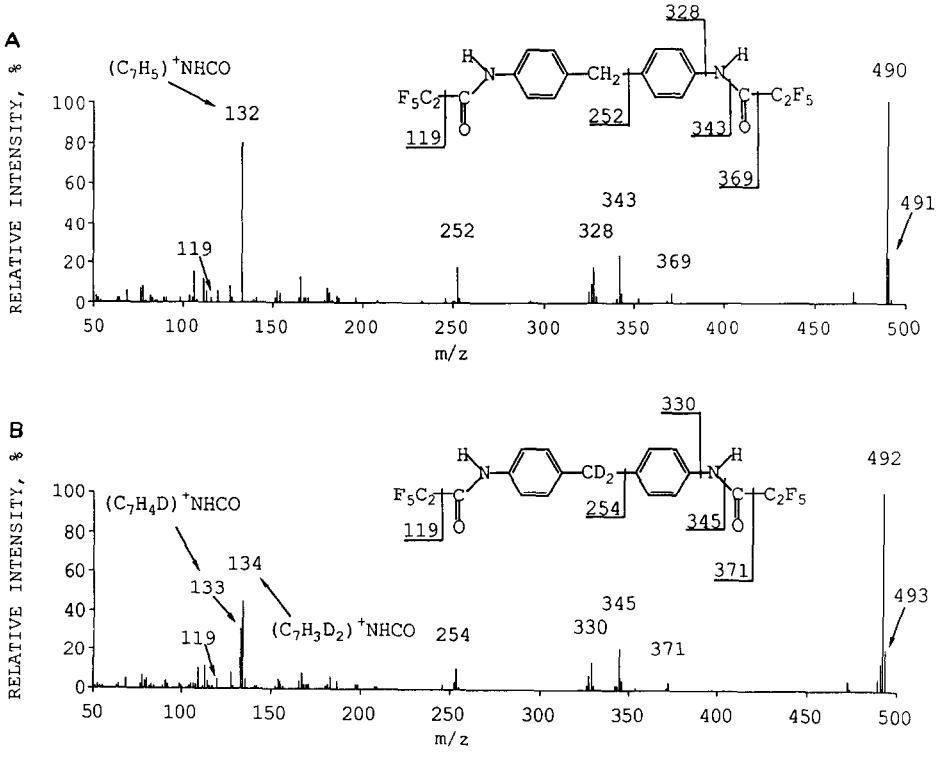


Fig. 1. Mass spectra obtained in the EI mode at an ionization potential of 70 eV: (A) MDA-PFPA derivative; (B) [$^2H_2$]MDA-PFPA derivative.

HFBA derivatives of MDA were about the same. However, the MDA-TFAA derivative was 10–20 times less sensitive. In this study, the detection limit was set by the instrument as no interfering peaks were found. Obviously for urine samples a lower detection limit can be obtained with further enrichment. However, the concentration of the internal standard influences the detection limit as [$^2H_2$]MDA unfortunately contains 6% of MDA.

*Quantitative analysis*

*Recovery.* The overall recovery was studied for seven urine samples containing 115 μg/l of MDA using an HPLC–UV system. The recovery was 96 ± 5% (95% confidence and $n=7$).

*Calibration graph.* Seven different concentrations of MDA in urine were prepared, including a blank, according to the work-up procedure. For each concentration three determinations with duplicate injections were made by GC–MS. The calibration graph (Fig. 2) for the investigated concentration range of 5–30 μg/l of MDA in urine was linear and gave a correlation coefficient of 0.998 ($n=7$; $y = 0.0158x + 0.0108$). Correction for the contribution of MDA from the [$^2H_2$]MDA internal standard was made by subtraction of a value of 0.06. Each point on the graph represented the corrected average value of the ratio of the peak area of the MDA-PFPA derivative to that of the [$^2H_2$]MDA-PFPA derivative.

*Detection limit.* The detection limit, defined as the urine sample concentration giving a signal equal to the blank signal plus three standard deviations[21], was 2 μg/l of MDA. No interfering peaks appeared when urine samples from ten persons were examined.

*Accuracy and precision.* Eight urine samples containing 26 μg/l of MDA were analysed. The relative standard deviation of the MDA peak area was 35%. The relative standard deviation of the peak area ratio of MDA to [$^2H_2$]MDA was 3.4%.

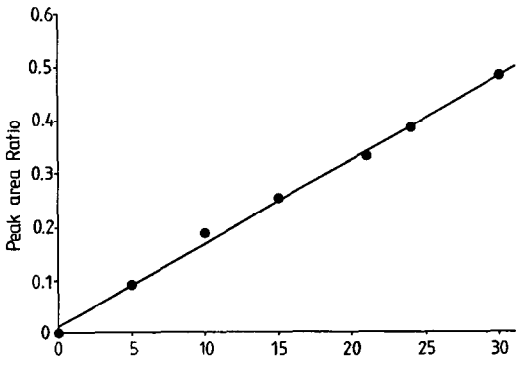


Fig. 2. Calibration graph for MDA in urine: ratio between the peak areas of the MDA-PFPA derivative and the [$^2H_2$]MDA-PFPA derivative from the worked-up urine *versus* the concentration of MDA in the original urine sample. Column, CP-Sil 8 CB (25 m × 0.25 mm I.D.) with a film thickness of 0.25 μm; inlet pressure of carrier gas (helium), 0.5 kg/cm$^2$; 1-μl splitless injection; temperature programme, isothermal at 100°C for 2 min, increased at 30°C/min to a final temperature of 300°C, then isothermal for 2 min.

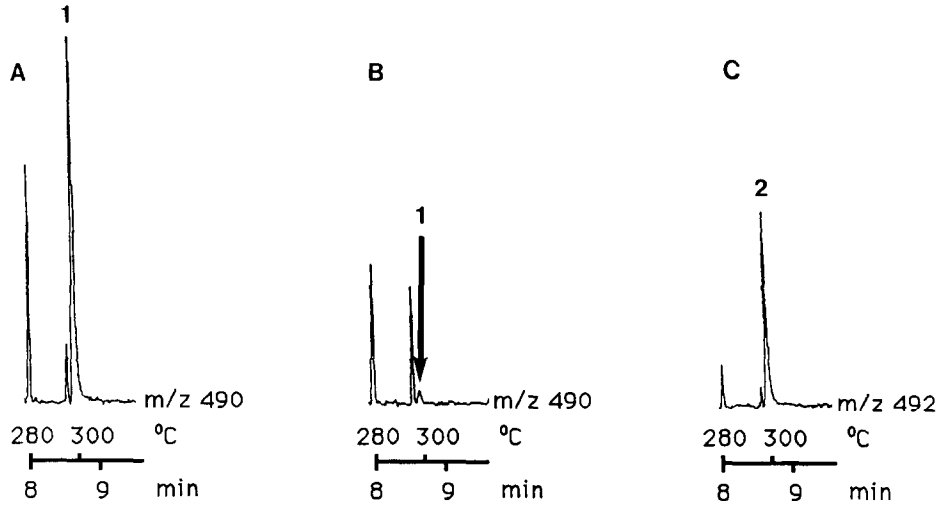


Fig. 3. Selected-ion chromatogram of urine samples using the EI mode. (A) Molecular ion of MDA-PFPA derivative from urine sample from an exposed worker. The peak corresponds to a concentration of 31 μg/l. (B) Molecular ion of MDA-PFPA derivative from urine sample from an unexposed worker. (C) Molecular ion of $[^2H_2]$MDA-PFPA derivative. Chromatographic conditions as in Fig. 2. Peak 1 = MDA-PFPA derivative; peak 2 = $[^2H_2]$MDA-PFPA derivative.

*Application*

An important application of the method is the biological monitoring of workers who are skin-exposed to MDA. Typical SIM of urine samples from occupationally exposed and non-exposed workers are shown in Fig. 3. MDA was found in the concentration range 2–600 μg/l in the hydrolysed urine samples from skin-exposed epoxy workers.

## CONCLUSIONS

The method developed for assessing occupational exposure to MDA provides the selective and sensitive determination of MDA at low μg/l levels using GC–SIM. The use of $[^2H_2]$MDA as internal standard results in accurate and precise determinations.

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