Gas Chromatography–Negative-ion Chemical Ionization Mass Spectrometry of Hydrolysed Human Urine and Blood Plasma for the Biomonitoring of Occupational Exposure to 4,4′-Methylenebisaniline

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A gas chromatographic–mass spectrometric (GC–MS) method for the biological monitoring of 4,4′-methylenebisaniline (MDA), is presented. MDA was determined in urine and plasma after hydrolysis and analysed as the pentafluoropropionic anhydride derivative. High sensitivity and selectivity were achieved using negative-ion chemical ionization with ammonia as the reagent gas. The hydrolysis procedures for urine and plasma samples were studied under alkaline and acidic conditions. Alkaline conditions gave the highest recovery for both urine and plasma samples. Hydrolyses of urine at 80 °C and of plasma at 100 °C for 16 h were selected owing to the good recovery and precision achieved. Ten analyses of a urine sample containing 11 nmol l^{-1} of MDA gave relative standard deviations (s_r) within and between assays of 2 and 6%, respectively. The determination of MDA in a plasma sample containing 8 nmol l^{-1} of MDA gave s_r = 4 and 6% within and between assays, respectively (n = 5). For the preparation of samples spiked to 10 nmol l^{-1} of MDA, the recovery was 97 ± 3% for urine samples and 96 ± 2% for plasma samples. The detection limit, defined as the blank plus three times the standard deviation of the blank, was 0.2 nmol l^{-1} for aquous solutions containing an internal standard. Determinations of MDA in urine and plasma from exposed workers showed that the method is appropriate for biomonitoring.

Keywords: *Hydrolysis; aromatic amine; gas chromatography; negative-ion chemical ionization mass spectrometry*

Introduction

Exposure to methylenebisaniline (MDA) is a well known occupational hazard. Cases of hepatoxic effects[1] and dermatitis[2] in humans have been reported. Carcinogenicity in mice and rats has been reported[3], implying that exposure of MDA may be a carcinogenic risk to humans. MDA is used in the production of methylene diisocyanate and as an epoxy resin hardener in paints, rubber, composites and glues. A recent report[4] concluded that the occupational exposure is still significant and that skin contact is the dominant exposure route. Dermal uptake is difficult to estimate by monitoring in the work environment. Biomonitoring offers much more powerful methods for assessment of MDA exposure.

MDA and its monoacetyl conjugate have been detected in urine from exposed humans.[5] Therefore, when monitoring MDA in biological samples, effects of differences in human metabolism should be lessened by a hydrolysis procedure that releases the amine from conjugates. Hydrolyses performed in 5 mol l^{-1} NaOH at 80 °C for 2 h[5] and 3 mol l^{-1} HCl at 100 °C for 2 h[6] have been reported to be sufficient to release conjugates from the urine matrix. Determinations of hydrolysed MDA in biological matrices have been based on gas or liquid chromatographic analysis. The derivatization of MDA with perfluoroacid anhydrides was shown to increase the sensitivity for determinations by capillary GC with electron-capture and thermionic detection[7]. Also, gas chromatography–mass spectrometry (GC–MS) with electron impact ionization of MDA as the pentafluoropropionic anhydride (PFPA) derivative gave high sensitivity, demonstrated by a detection limit of 10 nmol l^{-1} of MDA in urine.[8] Negative-ion chemical ionization (NICI) mass spectrometry has been developed in recent years and is now common in biomedical laboratories. The use of electron-capture detection in NICI-MS has been illustrated for fluorinated derivatives of aromatic amines.[9] Studies of the sensitivity of MDA-PFPA by NICI-MS demonstrated detection limits below 10 pmol l^{-1} of MDA in spiked water samples.[10] Most methods for determination of MDA in biological matrices have only been investigated for urine, but MDA has also been detected in rat blood by GC with electron-capture detection[11] and in spiked serum by GC–NICI-MS.[12] The determination of MDA and *N*-acetyl-MDA (monoacetyl-MDA, MAMDA) adducts to hemoglobin, as biomarkers of occupational exposure, has been reported.[13] However, to our knowledge, biomonitoring by analysis of hydrolysed plasma has not been studied. In this paper, a method for the determination of MDA at sub-nanomolar concentrations in hydrolysed human urine and plasma is described.

Experimental

Apparatus

A Carlo Erba Mega gas chromatograph equipped with an A200S autosampler (Fisons Instruments; Carlo Erba, Milan, Italy) connected to a Trio 1000 quadrupole mass spectrometer (Fisons Instruments; VG-Biotech, Altrincham, UK) was used for the analysis of urine and plasma samples. Injection was performed using splitless injection. The injector temperature was 300 °C and the split-exit valve was kept closed for 1 min after injection. For the chromatographic separation, fused-silica columns with a chemically bonded stationary phase, DB-5 (J & W Scientific, Folsom, CA, USA), 25 m × 0.25 mm i.d. with a film thickness of 0.25 μm, were used. The capillary inlet pressure of helium was 80 kPa. The starting temperature of the column oven was 110 °C, kept isothermal for 1 min, then increased at 15 °C min^{-1} to 300 °C, which was maintained for 2 min. The temperature of the ion source was 200 °C and the GC–MS interface temperature was 300 °C. The solvent delay

was to set to 8 min. The instrument was used in the chemical ionization mode with negative-ion monitoring and ammonia as reagent gas. The dwell time for each ion was 0.1 s and the inter-scan delay was 0.01 s. The pressure in the ion source was about 10^{-4} mbar. The emission current was 100 μA and the electron energy was 70 eV. The tuning of the instrument was optimized before every sequence of 50–100 samples, with nonafluorotributylamine as a calibrant.

Chemicals

MDA was obtained from Fluka (Buchs, Switzerland), acetonitrile and toluene from Lab-Scan (Dublin, Ireland), pentafluoropropionic anhydride (PFPA) from Pierce (Rockford, IL, USA), sodium hydroxide, hydrochloric acid and K_2HPO_4 were from Merck (Darmstadt, Germany) and dideuteriated 4,4′-MDA ($^2CH_2[C_6H_4NH_2]_2$) (MDDA) and MDA-PFPA derivative from Synthelec (Lund, Sweden). Monoacetyl-MDA (MAMDA) was synthesized by the reaction of equimolar amounts of MDA and acetic anhydride as described.[13]

Standard Solutions

Stock standard solutions of MDA and MDDA were prepared and further diluted with 0.1 mol l^{-1} HCl. MDA-PFPA stock standard solutions were prepared in acetonitrile, and further diluted with toluene. Stock standard solutions were stored in darkness at room temperature. Diluted solutions were prepared on the day of use.

Sampling and Storage of Samples

Urine samples were collected in polyethylene bottles. Samples that were analysed within 2 d were stored in a refrigerator. Samples analysed more than 2 d after sampling were transferred into polyethylene tubes and kept frozen at −20 °C until analysis. Blood samples were collected in heparinized test tubes (Venoject). The tubes were cooled by storage in a refrigerator for a minimum of 1 h. The plasma fraction was then separated by centrifugation (1500*g*) and transferred into a poly(propylene) tube. Separated plasma samples were kept in a freezer (−20 °C) until analysis.

Hydrolysis

When testing hydrolysis conditions, the internal standard was added in the first work-up step after hydrolysis. Hydrolysis was performed in 5 mol l^{-1} NaOH and 3 mol l^{-1} HCl. Samples hydrolysed in acid were prepared as reported previously.[8] The preparation described under the work-up procedure was used for alkaline hydrolysis. The hydrolysis was studied for test solutions prepared in the biological matrix, urine and plasma.

Urine

Urine matrices were tested under alkaline and acidic conditions with variation of the hydrolysis time. The concentrations of spiked solutions of MDA, MAMDA and pooled urine from subjects exposed to MDA in urine were all approximately 50 nmol l^{-1}. Using the previously reported conditions, the duration of the hydrolysis was varied from 0 to 40 h.

Plasma

When investigating the hydrolysis of plasma, the concentrations of MDA, monoacetyl-MDA and pooled plasma from subjects exposed to MDA were about 4 nmol l^{-1}. The hydrolysis temperature was varied from 60 to 100 °C at a constant hydrolysis time of 16 h. Acidic and alkaline hydrolyses at 100 °C were studied for hydrolysis times in the range 0–40 h.

Work-up Procedure of Urine Samples

A 2 ml volume of a urine sample, 50 μl of internal standard and 2 ml of 10 mol l^{-1} NaOH were added to a 12 ml test-tube. The sample was hydrolysed at 80 °C for 16 h and cooled to room temperature. A 3 ml volume of toluene was added to the screw-capped tube and the solution was shaken for 10 min and centrifuged at 1500*g* for 10 min. A 2 ml volume of the organic phase containing MDA was transferred into a new screw-capped tube. The sample was derivatized by addition of 20 μl of PFPA. The test-tube was immediately shaken for 5 s and the derivatization reaction was completed within 5 min at room temperature. A 2 ml volume of 1.0 mol l^{-1} phosphate buffer (pH 7.5) was added, and the tubes were shaken for 5 s and centrifuged at 1500*g* for 10 min to remove excess of reagent. The organic layer was transferred into vials and analysed by GC–MS.

Work-up Procedure for Plasma Samples

The work-up procedure for plasma samples is similar to that for the urine sample preparation, but the first steps differ and are reported here. To 1 ml of plasma, 50 μl of internal standard and 1 ml of 10 mol l^{-1} NaOH were added. The sample was hydrolysed at 100 °C for 16 h and cooled to room temperature. A 3 ml volume of toluene and 0.5 ml of water were added, after which the tube was shaken for 10 min. The remainder of the work-up procedure was identical with that for the urine preparation.

Quantification

For the determinations of the amine-PFPA derivatives, the ions monitored were *m/z* 470 and 472, corresponding to the $[M - 20]^-$ ions of MDA-PFPA and MDDA-PFPA. The ratio between the peak area of MDA and that of the dideuteriated internal standard was used for quantification. Standard and blank samples were prepared and analysed within every assay.

Results and Discussion

Standards

The isotopic purity of the dideuteriated internal standard, determined by GC–MS, was found to be 97%. A 3% impurity of non-labelled MDA gave an intercept of the calibration graphs of 0.03. For the evaluation of the hydrolysis procedure, we used the crude mixture from the synthesis of acetyl-MDA. By analysis using HPLC with UV detection (254 nm), the mixture was found to contain approximately 7% of MDA, 86% of MAMDA and 7% of diacetyl-MDA. The stock standard solutions of MDA and MDDA in 0.1 mol l^{-1} HCl were stable for at least 2 weeks when stored in darkness at room temperature.

Hydrolysis

The hydrolysis reactions are complicated and dependent on parameters such as temperature, pH and the time of hydrolysis. We investigated the hydrolysis of urine and plasma from humans exposed to MDA. Hydrolysis was also studied in urine and plasma spiked with MDA and MAMDA. The concentrations of MDA and MAMDA in urine and plasma were chosen to represent the concentration of MDA in the pooled urine and plasma solutions. The same solutions in equal volumes were used for acidic and alkaline hydrolysis

studies to achieve comparable results. Within each test 'released MDA' values from the MDA and MAMDA spiked solutions were normalized to arbitrary units with 1.0 as the maximum amount of MDA released. As the 'true' concentration of MDA in the pooled urine and plasma sample is unknown, these 'released MDA' values were normalized separately. The values in the Figs. 2–4 are averages of three measurements. Samples hydrolysed under acidic conditions at 5 °C could not be worked up owing to emulsion formation during extraction.

Urine samples

The release of MDA for different durations was tested in urine at hydrolysis temperatures of 100 °C for acidic conditions and 80 °C for alkaline conditions. For acidic and alkaline hydrolyses no increase in released MDA was obtained on increasing the hydrolysis time above 2 h for urine spiked with MDA and MAMDA. For pooled samples (Fig. 1) the release of MDA continued for the whole period studied of 40 h. However, only a small increase was observed after 16 h. Comparing alkaline and acidic hydrolyses, a higher release of MDA (about 25%) was found under alkaline conditions. From these results, alkaline hydrolysis at 80 °C for 16 h was chosen for the analysis of urine samples.

Plasma samples

The release of MDA after hydrolysis of plasma samples was studied for different hydrolysis temperature and time (Figs. 2 and 3). Hydrolysis for 16 h at 5–100 °C is shown in Fig. 2. For acidic conditions the releases of MDA from the spiked solutions were similar for 60 and 80 °C but lower at 100 °C, indicating a loss of MDA [Fig. 2(*a*)]. Alkaline hydrolysis showed about the same release in the temperature range 60–100 °C. On comparing alkaline and acidic hydrolyses of pooled plasma from exposed persons, a higher release of MDA was seen for the alkaline hydrolysis and the release increased with increase in temperature. The alkaline release of MDA from MAMDA-spiked and pooled plasma was low at 5 °C, showing the necessity for hydrolysis. On altering the duration of the hydrolysis only a small difference was found between long and short hydrolysis times for acidic and alkaline hydrolyses of the spiked samples. The acidic and alkaline hydrolysis of the pooled plasma showed a slightly increasing release of MDA with time (Fig. 3), with a small difference between 16 and 40 h. About a 10% higher release of MDA was seen with alkaline hydrolysis. We adopted alkaline hydrolysis at 100 °C for 16 h when analysing plasma samples from exposed subjects.

Interestingly, there were differences in the release patterns of pooled samples compared with samples spiked with MDA and MAMDA (Fig. 2). This may indicate a binding to, *e.g.*, proteins or additional metabolite(s) to those investigated.

Except for free MDA, only one metabolite, monoacetyl-MDA, has been found in a human biological matrix. Several other metabolites are likely as they arise from structurally related compounds. The formation of oxidized metabolites of MDA has been shown in metabolism studies by a rabbit microsome model.[14] Oxidized metabolites may react and form covalent bindings to proteins. Covalent bindings to hemoglobin have been found to release under weak hydrolysis conditions.[13] However, bindings to other biomolecules may not necessarily release MDA during hydrolysis. The hydrolysis procedure should therefore never be expected to release the total amount of 'MDA' in the sample. What can be measured are the free MDA and the fraction of hydrolysable conjugates. For this reason, it is obviously important to study

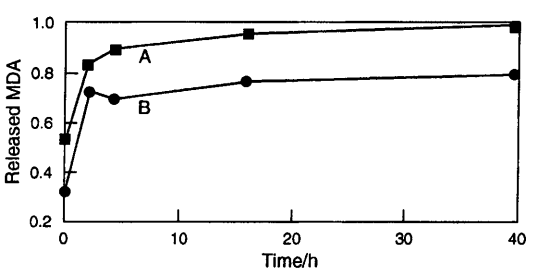

Fig. 1 Release of MDA from pooled urine during acidic and alkaline hydrolysis for 0–40 h. The pooled urine was obtained from exposed volunteers and hydrolysed under acidic conditions at 100 °C and alkaline conditions at 80 °C. A, Pooled urine during alkaline hydrolysis at 80 °C and B, pooled urine during acidic hydrolysis at 100 °C.

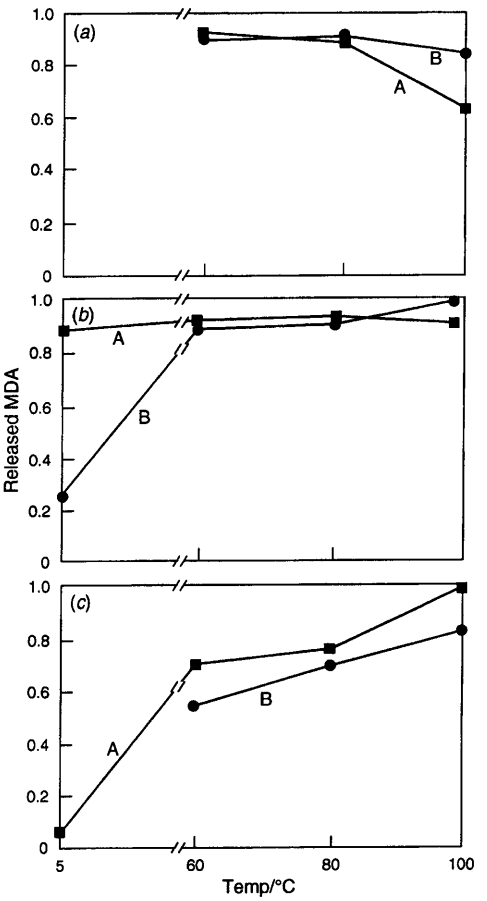

Fig. 2 Release of MDA with acidic and alkaline hydrolysis for 16 h at different temperatures. Plasma solutions of MDA and monoacetyl-MDA under (*a*) acidic conditions: A, MDA and B, MAMDA; and (*b*) alkaline conditions: A, MDA and B, MAMDA. Diagram (*c*) shows the hydrolysis of pooled plasma samples from exposed volunteers under A, acidic and B, alkaline conditions.

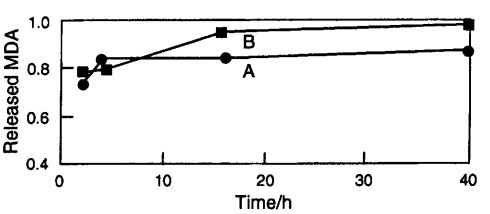

Fig. 3 Release of MDA from pooled plasma during A, acidic and B, alkaline hydrolysis at 100 °C for 2–40 h. The pooled plasma was obtained from exposed volunteers and hydrolysed under acidic and alkaline conditions at 10 °C.

biological samples from exposed humans when selecting optimum hydrolysis conditions. Further studies on these aspects are in progress.

Work-up Procedure

The relatively simple work-up procedure gave a high throughput and a short analysis time. The available volume of a plasma sample is often limited and therefore only 1 ml samples are used. Addition of water to the plasma samples was useful to avoid foam formation during the extraction. The work-up may also be performed by liquid–solid extraction of MDA in hydrolysed biological matrices. However, the high concentration of sodium hydroxide demands either neutralization or dilution of the sample.

Gas Chromatography–Mass Spectrometry

Spectra from the PFPA derivatives of MDA and the internal standard MDDA gave very simple spectra. The fragment corresponding to $[M - HF]^-$ dominates the spectrum but also the $[M - 2HF]^-$ fragment was apparent, giving ions of *m/z* 470 and 450, respectively, for MDA. The relative abundance of ions varies with the ion source temperature and the ammonia pressure. Chromatograms of the $[M - HF]^-$ ions obtained from urine and plasma samples showed good separation of the MDA-PFPA derivative and the internal standard from the matrix (Fig. 4). Several background peaks were found in plasma samples but they did not interfere with the MDA peak. The urine samples were usually very clean from peaks not originating from MDA. Initial attempts to use methane and isobutane as reagent gases gave about the same NICI mass spectra as ammonia. However, using methane and isobutane contamination of the ion source occurred, and the source had to be rinsed after 1–2 d. The use of ammonia as the ionization gas gave very stable conditions for several weeks of operation.

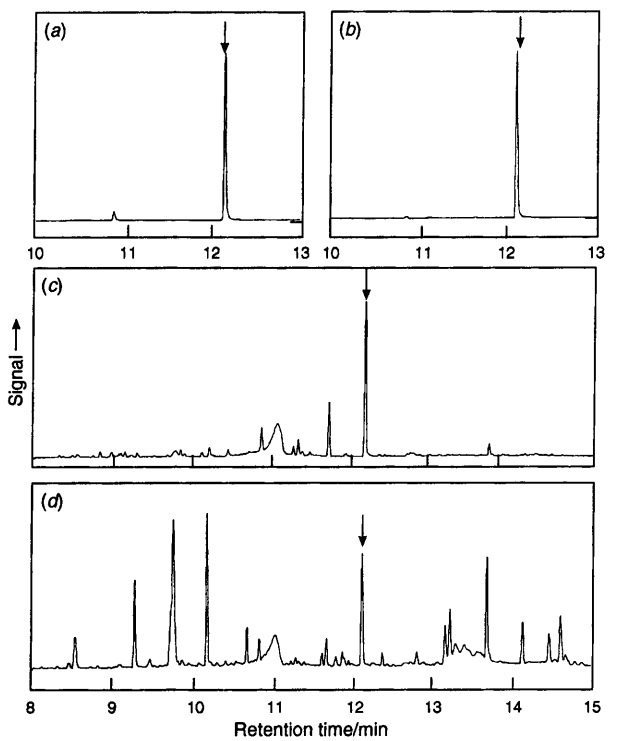

Fig. 4 Selected ion monitoring of (*a*) and (*b*) a urine sample and (*c*) and (*d*) a plasma sample from a subject, dermally exposed to 1.5 μmol of MDA. The urine sample was collected in the interval 6.25–8.25 h after exposure and the blood sample 8.25 h after exposure. The urine and plasma samples contain 19 and 4 nmol of MDA per litre of matrix, respectively. For chromatographic conditions, see Experimental. The determined MDA (*m/z* 470) and the internal standard MDDA (*m/z* 472) are plotted separately.

Recovery

The over-all recovery was studied by performing the work-up procedure for 10 urine samples and 10 plasma samples spiked with 10 nmol of MDA per litre sample. Comparisons were made with MDA-PFPA standards diluted to the same concentration. The over-all recovery for urine samples was found to be 97 ± 3% ($P = 0.05$). For plasma samples the over-all recovery was 96 ± 2% ($P = 0.05$).

Calibration Graphs and Linearity

Peak area and peak height measurements were both useful for the quantification. The calibration graph was linear up to an MDA : MDDA ratio of about five. For higher relative amounts of MDA the calibration graph bent towards a lower relative response. For this reason, the concentration of the internal standard corresponding to a concentration of one quarter of the highest MDA concentration in the calibration was selected. Calibration graphs in the ranges 0–60 nmol l^{-1} for urine and 0–8 nmol l^{-1} for plasma were prepared using urine and plasma from unexposed humans. A calibration graph with six concentrations in the range 0–60 nmol of MDA per litre of urine ($n = 12$) gave a correlation coefficient of 0.999. For the concentration range 0–8 nmol of MDA per litre of plasma ($n = 12$), the calibration graph gave a correlation coefficient of 0.998.

Repeatability

Samples originating from an exposed volunteer were studied. For five analyses during 1 d of a urine sample containing 11 nmol l^{-1} of MDA, the relative standard deviation (s_r) was 2%. Five analyses during 1 d of a plasma sample containing 8 nmol l^{-1} of MDA gave an s_r value of 4%.

Reproducibility

For peak area measurements during a period of 4 weeks, the s_r value was 6% for five preparations of a urine sample containing 12 nmol l^{-1} of MDA. For peak area measurements during a period of 4 weeks the s_r value was 6% for five preparations of a plasma sample containing 8 nmol l^{-1} of MDA. The urine and plasma samples were taken from an exposed volunteer.

Detection Limit

The instrumental detection limit, defined as three times the noise, was 50 pmol l^{-1} of MDA for a 1 μl injection volume. The detection limit, defined as the blank plus three times the standard deviation of the blank, was 0.2 nmol l^{-1} for aqueous solutions containing 4 nmol l^{-1} of internal standard.[15]

Biological Monitoring of MDA

The proposed method makes possible sensitive and precise determinations of MDA in hydrolysed human urine and plasma. The simple work-up procedure makes a high sample throughput possible and the method has been used in our laboratory for hundreds of samples. This method is suitable for biological monitoring of occupational exposure. For the complete interpretation of the analytical results, with regard

to the exposure of MDA, further studies are necessary. A study of MDA-exposed workers and volunteers is in progress. MDA will be determined in urine and plasma and the concentrations will be associated with exposure.

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