Journal of Chromatography, 511 (1990) 185–194
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 419

Determination of 4,4′-methylenedianiline in hydrolysed human urine using liquid chromatography with UV detection and peak identification by absorbance ratio

A. TILJANDER and G. SKARPING*
Department of Occupational and Environmental Medicine, University Hospital, S-221 85 Lund (Sweden)
(First received November 6th, 1989; revised manuscript received February 19th, 1990)

ABSTRACT

A liquid chromatographic method using multi-wavelength UV detection (258 and 285 nm) is presented for the determination of 4,4′-methylenedianiline (MDA) in hydrolysed human urine. The method is based on hydrolysis under strongly acidic conditions followed by derivatization with pentafluoropropionic anhydride. The perfluoro fatty acid amide derivative formed was analysed on a bonded octadecylsilyl column using isocratic elution with acetonitrile–water (67:33, v/v) as mobile phase. The overall recovery for urine samples containing 115 μg/l of MDA was 97 ± 3%. The calibration graph was linear in the investigated range (12–122 μg/l) with a correlation coefficient of 0.998. The precision was 2.3% for urine samples containing 122 μg/l and the detection limit was 8 μg/l. The chromatograms were evaluated using a combination of retention time data and absorbance ratio by the simultaneous monitoring of the wavelengths 285 and 258 nm. The absorbance ratio (285/258 nm) was virtually constant (0.28 ± 0.04) in the range 78–10 000 μg/l. The precision for the absorbance ratio was 6.1% for urine samples containing 124 μg/l and the lowest amount of MDA to give an absorbance ratio was 50 μg/l.

The procedure for the hydrolysis of urine spiked with MDA and N,N′-diacetyl-MDA and urine from skin-exposed workers was studied under strongly acidic, weakly acidic and basic conditions. MDA was found in hydrolysed urines from skin-exposed epoxy resin workers in the concentration range 8–700 μg/l.

INTRODUCTION

4,4′-Methylenedianiline (MDA) is a commercially important aromatic diamine used as an intermediate in the preparation of isocyanates, epoxy resins, polyurethanes and rubber chemicals[1,2]. MDA has been reported to be hepatoxic in dogs[3], rats[4,5] and humans[6,7]. In the Ames test, MDA has been found to be mutagenic[8] and it has been reported to be carcinogenic in rats and mice[9]. MDA has low volatility, but has been found to absorb readily through the skin[10]. The monitoring of MDA in biolog-

0021-9673/90/$03.50

ical fluids from exposed persons[11,12] is therefore of major importance. MDA-related metabolites such as N-acetyl-MDA and N,N'-diacetyl-MDA, have been found in urine[8,11,12]. Several techniques have been developed for the determination of MDA in matrices such as air-sampling solutions[13–16], blood[17,18] and urine[11,12,18–22]. For the determination of MDA in urine gas chromatography–mass spectrometry (GC–MS) has been used with detection limits ranging from 1 to 10 μg/l[11,12,18–21]. The determination of underivatized MDA using high-performance liquid chromatography (HPLC) with UV and electrochemical detection has been reported, with detection limits of total amounts of 32 and 3 ng, respectively[22].

Multi-wavelength detection has been demonstrated to provide specific and reliable evaluation of complicated chromatograms originating from biological samples. The use of absorbance-ratio measurements for peak identification and for peak purity is also feasible with this technique[23–26].

In this paper, a method is described for the determination of MDA in hydrolysed human urine using derivatization with pentafluoropropionic anhydride (PFPA) or heptafluorobutyric anhydride (HFBA) and reversed-phase HPLC with UV detection. The use of absorbance-ratio measurements for low concentrations of MDA in urine was investigated. The procedure for the hydrolysis of urine from skin-exposed workers was studied under strongly acidic and basic conditions.

EXPERIMENTAL

Equipment

The 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, a three-channel SE 130 recorder (ABB Goerz, Vienna, Austria) and a Shimadzu (Kyoto, Japan) C-R3A integrator. The mobile phase was acetonitrile–water (67:33, v/v) and the injection volume was 50 μl. Absorption spectra were recorded on a Shimadzu UV-260 UV–visible recording spectrophotometer. 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 aspiration pump was employed. The vacuum desiccator was equipped with an electrically heated oven. The water was produced in Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.).

Columns

Reversed-phase HPLC columns of stainless steel were used: Spherisorb S5 ODS-1 Excel (20 cm × 3.2 mm I.D.), Spherisorb S5 ODS-2 Excel (20 cm × 3.2 mm I.D.) and Hypersil 5-ODS Excel (20 cm × 3.2 mm I.D.) from Hichrom (Reading, U.K.), Apex II octadecyl (25 cm × 4.6 mm I.D.) from Jones Chromatography (Hengoed, U.K.); and Nucleosil 5 C_{18} (20 cm × 3 mm I.D.) from Macherey-Nagel (Düren, F.R.G.).

Chemicals

MDA and acetic anhydride were purchased from Aldrich (Beerse, Belgium), PFPA and HFBA from Pierce (Rockford, IL, U.S.A.), sodium hydroxide and hydrochloric acid from Merck (Darmstadt, F.R.G.), acetonitrile, methanol and toluene of

HPLC grade from Lab-Scan (Dublin, Ireland) and ethanol from Kemetyl (Stockholm, Sweden).

Synthesis

N,N'-Diacetyl-MDA. A 150-ml volume of an aqueous solution containing 7.5 ml of acetonitrile and 1.6 g of sodium hydroxide was added to a solution containing 10 g of MDA and 10 ml of methanol. Acetic anhydride (50 ml) was added dropwise. After 15 min the solution was heated in a water-bath at 80°C for 15 min, then cooled to room temperature. The crystals formed were filtered and washed with water and then with methanol. The crystals were dried in an incubator at 40°C.

MDA–perfluoro fatty acid 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 heated slowly 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 MDA–HFBA derivative was synthesized in an analogous way.

Preparation of standard solutions

Stock solutions of 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 N,N'-diacetyl-MDA was prepared in methanol and diluted with 0.1 *M* HCl to the desired concentrations. Stock solutions of MDA–perfluoro fatty amide derivatives were prepared in ethanol at the 1 g/l level and further dilutions were made with acetonitrile–water (67:33, v/v). The stock 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 1.2-ml volume of 6 *M* HCl was added to a 0.8-ml urine sample in a 10-ml test-tube, equipped with a PTFE cap. The test-tube was heated at 98°C for 2 h and then cooled to room temperature. A 3-ml volume of toluene and 4 ml of saturated NaOH solution were added to the test-tube. The mixture was shaken for 10 min and then centrifuged at 1500 *g* for 10 min. A 2-ml aliquot of the organic phase was separated and transferred to a new test-tube, 20 μl of PFPA or HFBA were added to the organic phase and the solution was shaken for 10 min. A 1-ml volume of the organic phase was transferred to a new test-tube and the toluene and the excess of derivatization reagent were removed by evaporation in a vacuum desiccator at 30°C. The residue was dissolved in 0.7 ml of the mobile phase.

RESULTS AND DISCUSSION

Standards

The identities of the MDA–perfluoro fatty acid amide derivatives and N,N'-

diacetyl-MDA were confirmed by GC–MS. The purities were determined using HPLC with UV detection and capillary GC with thermionic specific detection and found to be better than 99%. The purities were further confirmed by elemental analysis; the experimental values for carbon, hydrogen, nitrogen and oxygen did not differ by more than 0.2% from the calculated values.

Work-up procedure

Hydrolysis. The urine samples were acidified according to the sampling procedure.

The hydrolysis procedure was studied for urine samples from unexposed persons with a spiked concentration of 130 μg/l of MDA. The hydrolysis were performed under strongly acidic, weakly acidic and basic conditions. For strongly acidic conditions 0.8 ml of urine sample was mixed with 1.2 ml of 6 *M* HCl. For weakly acidic conditions 2 ml of urine sample alone were used. For basic conditions 1 ml of 10 *M* NaOH was added to 1 ml of urine sample. The urine samples were hydrolysed at 98°C and the hydrolysis time was varied between 15 min and 7 days. The work-up procedure was then performed and one determination with duplicate injections was made for each time and condition.

No losses were found under strongly acidic and basic conditions (Fig. 1A) for a hydrolysis time of 0.25–16 h. However, with hydrolysis under basic conditions for 2 days or more, more than 20% was lost. With hydrolysis under weakly acidic conditions the losses were unacceptable (> 50%).

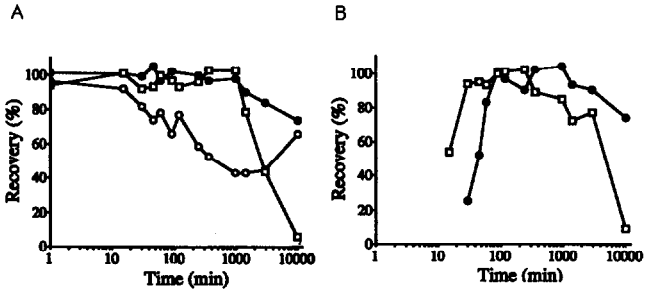

Fig. 1. Recovery of MDA–PFPA derivative in worked-up urine *versus* time. Urine sample spiked with (A) 130 μg/l of MDA and (B) 130 μg/l of N,N′-diacetyl-MDA. (●) Strongly acidic conditions; (○) weakly acidic condition; (□) basic conditions.

The hydrolysis procedure was also studied for urine samples with a spiked concentration of 130 μg/l of N,N′-diacetyl-MDA using the same procedure as for MDA-spiked urine samples. The recovery of MDA in N,N′-diacetyl-MDA-spiked urine under strongly acidic and basic conditions (Fig. 1B) showed a plateau, representing *ca.* 100% recovery, between 1 and 16 h and between 0.5 and 8 h, respectively. With basic conditions a prolonged hydrolysis time resulted in a decreased recovery.

The hydrolysis procedure was also studied for urine samples originating from three skin-exposed workers, using the same procedure as for MDA-spiked urine samples. The largest amount of MDA found for each urine was assumed to represent a 100% recovery. For each time and condition an average was calculated from the

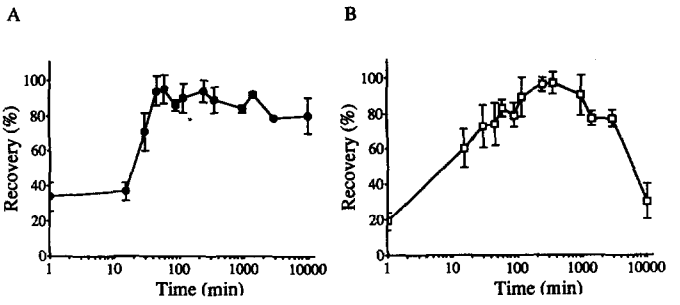

Fig. 2. Recovery of MDA from urine from three exposed workers (251, 545 and 645 μg/l) *versus* hydrolysis time under (A) strongly acidic and (B) basic conditions. The average recovery and the range are plotted.

three different urine samples (Fig. 2). Under strongly acidic conditions the amount of MDA found increased to a plateau, which virtually reached a maximum after 45 min. Within experimental error it was not possible to determine whether the recovery decreased if the hydrolysis time was prolonged. With hydrolysis under basic conditions the amount of MDA found reached a maximum much later than under strongly acidic conditions. It was also demonstrated that the amount of MDA found decreased if the hydrolysis time was prolonged to more than 16 h.

Extraction of MDA. The recovery for the extraction of MDA into the organic phase was *ca.* 100%, irrespective of whether 5 *M* or saturated sodium hydroxide solution was added to the hydrolysed urine sample. The foaming tendency was found to be less pronounced when saturated sodium hydroxide solution was used. For separations of phases it was necessary to centrifuge the urine samples.

Evaporation. Evaporation to dryness was performed in order to enrich the sample, to remove the excess of derivatization reagent and the acid formed and to choose a suitable solvent for the subsequent HPLC analysis. No losses were found in the evaporation step.

Chromatography

Choice of derivatization reagents. Initial attempts to determine underivatized MDA by HPLC with UV detection gave a bad resolution relative the urine matrices. Considerable efforts were made to clean up the sample without sample losses. However, in analyses for MDA at the μg/l level, these attempts were unsuccesful. The chromatograms obtained using either PFPA or HFBA as derivatization reagents were very similar. For the problems related to the matrix no differences were found. One of the advantages with MDA–perfluoro fatty amide derivatives is that the mobile phase did not need to be buffered to minimize absorption, which eliminated the problems due to salt precipitation in the HPLC system.

Choice of chromatographic conditions. The capacity factors (k') for the MDA–PFPA and MDA–HFBA derivatives were recorded for aqueous acetonitrile (Table I) to determine the optimum chromatographic conditions. Aqueous acetonitrile was found to be better than aqueous methanol owing to the k' values and the better resolution between the biological matrix and the MDA–PFPA and MDA–HFBA derivatives. Aqueous acetonitrile as the mobile phase also required a lower pressure and gave a better resolution.

TABLE I

RETENTION TIMES, t_R (min), AND CAPACITY FACTORS (k') FOR INVESTIGATED SUBSTANCES ON A 5-μm NUCLEOSIL C_{18} PACKING

Conditions: column, Nucleosil 5 C_{18} (20 cm × 3 mm I.D.); flow-rate, 1.0 ml/min; mobile phase, acetonitrile–water with various volume ratios.

Derivative	*Acetonitrile–water (v/v)*									
	85:15		*80:20*		*75:25*		*70:30*		*65:35*	
	t_R	k'	t_R	k'	t_R	k'	t_R	k'	t_R	k'
MDA–PFPA	4.6	1.8	5.5	2.4	6.5	3.2	8.8	4.8	13.5	7.2
MDA–HFBA	5.4	2.3	7.0	3.3	9.1	4.9	13.5	7.8	22.7	12.9

For low water contents in the mobile phase the MDA–PFPA and MDA–HFBA derivatives eluted fairly rapidly. The k' values increased with increasing water content, which reflects the dominant influence of the non-polar parts of the MDA–perfluoro fatty acid amide derivatives on retention. As expected, an increased chain length of the alkyl group on the derivatization reagent gave an increased retention (Table I). No significant differences in the chromatographic matrices could be seen between strongly acidic and basic conditions at their respective plateaux.

Choice of columns. Columns packed with 5-μm octadecylsilyl particles supplied by different manufacturers were tested. Significantly different k' values and resolutions relative to the matrices were obtained. The Apex II octadecyl column gave the best resolution relative to the matrices (Fig. 3). No endogenous peaks from the urine extract interfered in the analysis.

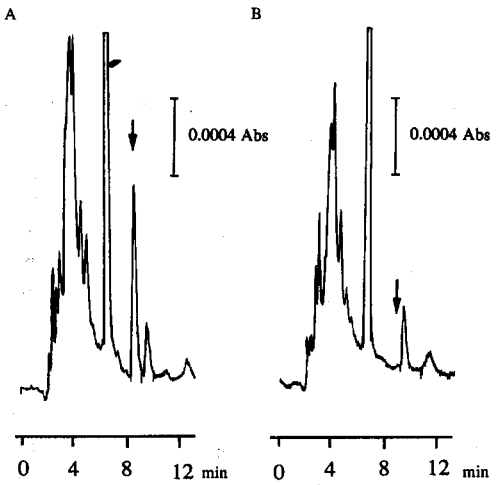

Fig. 3. Chromatograms of urine samples. (A) MDA–PFPA derivative from a urine sample from an exposed worker. The peak corresponds to a concentration of 220 μg/l. (B) Chromatogram from an unexposed worker. Conditions: Column, Apex II octadecyl; eluent, acetonitrile–water (67:33, v/v); flow-rate, 1 ml/min; injection volume, 50 μl; UV detection (258 nm); time constant, 1.0 s.

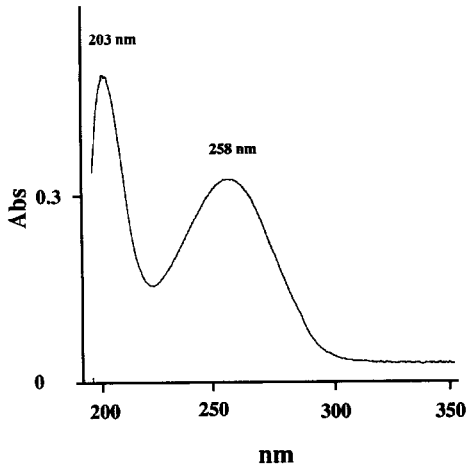

Fig. 4. Absorbance spectra of MDA–PFPA derivative, diluted with acetonitrile, at a concentration of 2.12 mg/l. Conditions: slit, 0,5 nm; speed, 95 nm/min; path length, 1.0 cm.

Detection

Absorbance maxima of the MDA–PFPA and MDA–HFBA derivatives were found at 203 and 258 nm (Fig. 4). The signal-to-noise ratio for the MDA–PFPA and MDA–HFBA derivatives were optimum and the same at 258 nm.

Absorbance ratio

The low concentrations of MDA in complex matrices such as urine samples from exposed persons necessitate sensitive detection and appropriate work-up procedures. More than ten urine samples were worked up as described under Experimental and no interfering peaks were found, although the matrix varied greatly from sample to sample. However, it could not be disregarded that the urine samples could possibly contain co-eluting UV-absorbing compounds originating from the diet, medication, etc. The use of a more selective determination was therefore examined to ensure peak identity and peak purity. Detection wavelengths of 258 and at 285 nm were applied simultaneously and the absorbance ratios between these wavelengths were monitored. The absorbance ratio was 0.28 ± 0.04 (95% confidence, n = 7) when the MDA concentration was varied between 78 and 10 000 μg/l (Fig. 5). On injecting

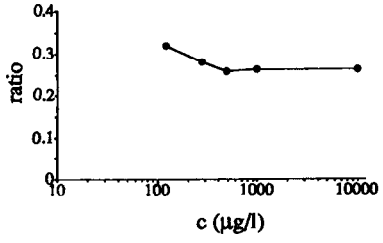

Fig. 5. Absorbance ratio (285/258 nm) *versus* concentration (c) of MDA. Each point on the graph represents the average of six measurements of the absorbance ratio.

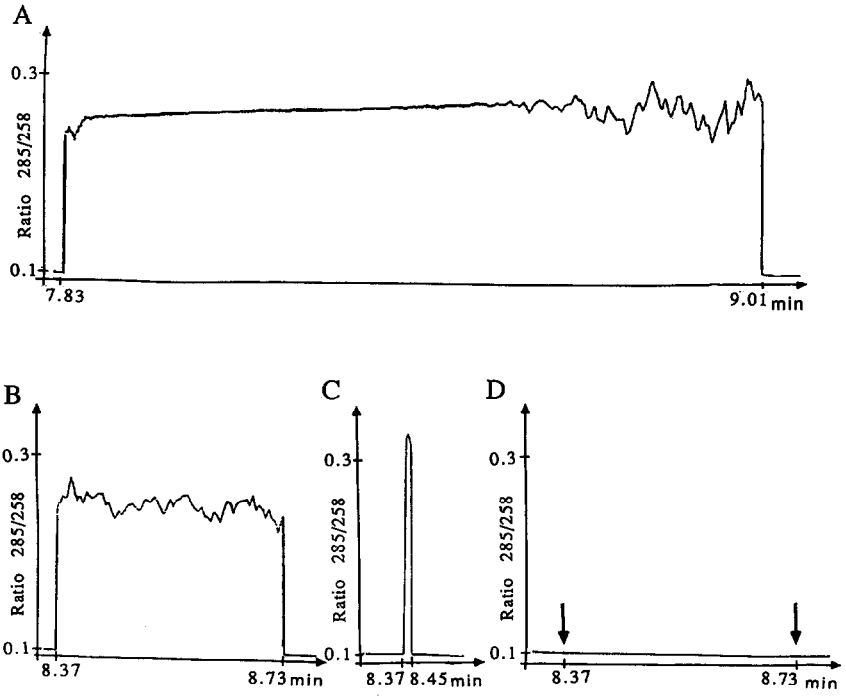

Fig. 6. Ratio chromatograms obtained monitoring of the absorbance ratio (285/258 nm). (A) Standard solution of MDA–PFPA at a concentration of 10 mg/l; (B) urine sample originating from an exposed worker at a concentration of 220 μg/l; (C) MDA-spiked urine sample at a concentration of 75 μg/l; (D) urine sample from an unexposed person. Chromatographic conditions as in Fig. 3 and ratio min, 0.1; absorbance threshold, 0.0001.

urine samples containing 124 μg/l of MDA the precision for the absorbance ratio was 6.1% (n = 14). The shape of the ratio chromatogram for low concentrations was noisy, especially at the peak start and the peak end. The ratio measurements were, of course, impossible when the absorbance approached the detection limit. An absorbance threshold of 0.0001 was therefore used for the two wavelengths in the ratio determination. The limit for ratio monitoring was 50 μg/l of MDA and was set by the absorbance at the least sensitive wavelength, 285 nm. The absorbance ratios were the same for diluted MDA–PFPA derivative, spiked urine and urine from exposed workers (Fig. 6). This confirms the identification and the peak purity. The absorbance ratio was therefore used as an extra quality measurement.

Quantitative analysis

Recovery. The overall recovery was studied for eight urine samples containing 115 μg/l of MDA. The peak areas obtained were compared with a standard. The recovery was 97 ± 3% (95% confidence, n = 14).

Calibration graph. Eight different concentrations of MDA in urine and urine blanks were prepared according to the work-up procedure. For each concentration, two determinations with duplicate injections were made. The calibration graph for

the investigated range of 12–122 μg/l of MDA was linear with a correlation coefficient of 0.998 ($n = 8$; $y = 120x - 344$).

Detection limit. The detection limit, defined as the concentration in a urine sample giving a signal equal to the blank signal plus three standard deviations[27], was 8 μg/l of MDA. No interfering peaks appeared when urine samples from ten persons were examined.

Precision. Eight urine samples containing 122 μg/l of MDA were analysed. The relative standard deviation of the MDA peak area was 2.3%.

CONCLUSIONS

The method developed for assessing occupational exposure to MDA provides the selective and sensitive determination of MDA at the 8–10 000 μg/l level using HPLC with UV detection. Multi-wavelength detection and ratio chromatograms were demonstrated to give the selective determination of MDA in human urine. The use of a derivatization procedure with perfluoro-fatty acid anhydrides produced stable derivatives resolved from the urine matrices. The low detection limit achieved together with the opportunity to use ratio detection gave safer quantification and identification.

ACKNOWLEDGEMENTS

The authors are indebted to Professor Staffan Skerfving, Head of the Department of Occupational and Environmental Medicine, for his interest in this work. They also gratefully acknowledge the Swedish Work Environment Fund (AMFO 88-0161) for financial support.

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