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Short Communication

Determination of 4,4′-methylenedianiline in hydrolysed human urine by micro liquid chromatography with ultraviolet detection

Per Brunmark, Pernilla Persson and Gunnar Skarping

Department of Occupational and Environmental Medicine, University Hospital, S-221 85 Lund (Sweden)

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ABSTRACT

4,4′-Methylenedianiline was determined in human urine by micro liquid chromatography with ultraviolet detection. The combination of a thorough work-up and the high mass sensitivity of micro liquid chromatography gave the method very high sensitivity. Derivatization with pentafluoropropionic anhydride enhanced the resolution of the 4,4′-dimethylenedianiline peak. The detection limit, defined as blank plus three times the standard deviation of the blank, was 2 nmol/l of urine, for 10-μl injection volumes. The detection limit, defined as three times the noise, was about ten times better. The within- and between-assay coefficients of variation were 4 and 6%, respectively, for samples containing 40 nmol/l. The method was applied for the monitoring of excreted 4,4′-methylenedianiline in urine, during epicutaneous skin hypersensitivity testing (patch testing).

INTRODUCTION

Methylenedianiline (MDA) is an aromatic amine used in the production of methylenediisocyanate and as an epoxy resin hardener. MDA is hepatoxic [1] and can cause dermatitis [2] in humans. Evidence for its carcinogenicity in mice and rats has been presented [3], indicating that MDA should be treated as if it represents a carcinogenic risk to humans. In a recent report [4], well protected workers were found to have high concentrations of MDA in their urine, owing to contamination of the protection equipment. The use of biological monitoring of human urine was found to be an important tool in the detection of such unexpected exposures. In a study of more than 300 workers with various occupational exposure levels, 80% of the urine samples contained less than 25 nmol/l MDA [5]. The sensitivity needed when screening occupational samples is accordingly very high.

To monitor the total amount of excreted MDA, hydrolysis of the urine is necessary to release the amine from metabolic conjugates. Both alkaline [5] and acidic [6] conditions have been reported to give complete hydrolysis with no losses. The determination of hydrolysed MDA in urine has been based on gas or liquid chromatog-

Correspondence to: Per Brunmark, Department of Occupational and Environmental Medicine, University Hospital, S-221 85 Lund, Sweden.

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raphy (LC). Gas chromatography combined with mass spectrometry (GC–MS) gave a detection limit of 10 nmol/l [7]. In a recently presented LC method using electrochemical detection, a detection limit of 12.5 nmol/l was demonstrated [8].

Microcolumn liquid chromatography (micro-LC) was introduced by the Tsuda and Novotny [9] and Ishii *et al.* [10] in the late 1970s. Use of the technique has expanded only slowly, despite its benefits of high mass sensitivity, high efficiency and low mobile phase consumption, and several favorable detection methods. However, a number of methods using micro-LC equipment have recently been published [11], showing that the technique has the potential for routine use.

This paper describes a method for the determination of hydrolysed MDA at very low concentrations in human urine. A low detection limit is achieved, owing to the high mass sensitivity of micro-LC with UV detection and the injection of 10-μl volumes of samples. The work-up procedure effectively purifies the sample and enriches the concentration of MDA in the sample.

EXPERIMENTAL

Apparatus

The LC equipment consisted of a Shimadzu 9A (Shimadzu, Kyoto, Japan) pump with a Acurate 1/70 split (LC Packings, Amsterdam, Netherlands) positioned before the injector. The Kontron 433 capillary UV detector had a cell volume of less than 90 nl and an optical path length of 22 mm (Kontron, Basel, Switzerland). Fusica microcolumns (150 mm × 0.32 I.D.) were obtained from LC Packings, and packed with 3-μm Hypersil C_{18} particles (Shandon Scientific, Runcorn, UK). Injections were made using a CMA 200 autosampler (CMA/Microdialysis, Stockholm, Sweden) and a Valco C6W loop injector (Houston, TX, USA) with calibrated loop volumes. Chromatograms were evaluated with a Maxima 820 chromatography workstation (Millipore, Milford, MA, USA). The samples were evaporated in a Speed-Vac 290 evaporation centrifuge (Savant, Farmingdale, NY, USA).

Chemicals

MDA was obtained from Fluka (Buchs, Switzerland), and acetonitrile and toluene from Lab-Scan (Dublin, Eire). Pentafluoropropionic anhydride (PFPA) was from Aldrich (Milwaukee, WI, USA) and triethylamine from Janssen (Geel, Belgium). Sodium hydroxide, hydrochloric acid and phosphoric acid were from Merck (Darmstadt, Germany) and the MDA-PFPA derivative from Synthelec (Lund, Sweden). For epicutaneous patch testing a preparation of MDA in petrolatum at 0.5% (w/w) from Chemotechnique (Malmö, Sweden) was used.

Standard solutions

Standard solutions of MDA were prepared in 0.1 *M* hydrochloric acid. MDA-PFPA standard stock solutions were prepared in acetonitrile. Further dilution was made in 40% acetonitrile–60% aqueous solution containing 1.5% triethylamine and adjusted to pH 3.0 with phosphoric acid. The stock solutions were stored in darkness at room temperature. The diluted solutions were prepared daily before use.

Work-up procedure

A 4-ml volume of 10 *M* sodium hydroxide was added to a 12-ml screw-cap tube containing 4 ml of urine. The sample was hydrolysed at 80°C in a heating oven for 2 h and cooled to room temperature. A 3-ml aliquot of toluene was added to the screw-cap tube; the solution was shaken for 10 min and centrifuged at 1500 *g* for 10 min. Then 2 ml of the organic phase, containing the MDA, were transferred to a new screw-cap tube, followed by 2 ml of toluene and 2 ml of 0.5 *M* hydrochloric acid. MDA was extracted to the acidic aqueous phase by shaking for 10 min and the toluene phase was discarded. The acidic aqueous phase was washed by shaking for 5 s with 2 ml of toluene, and again the toluene phase was discarded. The addition of 1 ml of 1.5 *M* sodium hydroxide and 2 ml of toluene and shaking for 10 min extracted MDA to the organic phase. The toluene phase was then transferred to a further screw-cap tube. Another 2-ml volume of toluene was added to the screw-cap tube containing the aque-

ous phase, the tube was shaken, and the organic extracts were combined. A 50-μl volume of PFPA was then added, and the screw-cap tube was shaken for 5 s. The derivatization reaction was complete within 5 min at room temperature. Subsequently the toluene phase was evaporated to dryness in a Speed-Vac system. The evaporation residue was dissolved in 250 μl of 40% acetonitrile–60% aqueous solution containing 1.5% triethylamine and adjusted to pH 3.0 with phosphoric acid. Before the chromatographic analysis the sample was treated for 5 min in an ultrasonic bath and transferred to a 250-μl micro-vial.

Chromatography

Samples prepared according to the work-up procedure were analysed by micro-LC the same day. The mobile phase was 70% acetonitrile–30% aqueous solution containing 1.5% triethylamine and adjusted to pH 3.0 with phosphoric acid, at a flow-rate of typically 4 μl/min. The injection volume was 9.6 μl and detection was performed at 258 nm. Every sample was taken through work-up and injection in duplicate. Calibration graph was obtained from the determination of urine standards spiked to appropriate concentrations. The mean value of peak heights was used for the quantitative analyses.

Sampling and storage of samples

Urine samples were collected in polyethene bottles. Samples analysed within two days were stored in a refrigerator. Samples analysed more than two days after sampling were transferred to polyethene tubes and kept at −20°C until analysis.

RESULTS AND DISCUSSION

Storage

The stock solutions of MDA in 0.1 *M* hydrochloric acid were stable for at least two weeks when stored in darkness at room temperature.

Work-up procedure

The use of micro-LC for the determination of MDA in a complex matrix such as urine demands a proper clean-up procedure. Compared with other reported procedures for the determination of MDA in urine the method presented here includes a thorough clean-up of the sample. In the first alkaline extraction step of the hydrolysed urine, amines and lipids are transferred to the toluene phase. After derivatization and phase exchange, this toluene extract can be analysed by high-performance liquid chromatography even if it contains interfering compounds. Consequently, the detection limit will be high with the non-selective UV detector. An additional clean-up by extraction of MDA into 0.1 *M* hydrochloric acid and washing with toluene markedly purifies the sample. MDA is back-extracted into toluene by alkalization, and the derivatization is performed by the addition of PFPA. The work-up procedure gives a ten-fold enrichment of the sample.

The stability of the sample solution resulting from the work-up procedure was studied by preparing urine samples originating from an exposed worker containing 40 nmol/l MDA. The solution was then injected into the chromatographic system during three consecutive days. When the peak heights were compared with a freshly diluted MDA-PFPA standard no degradation, within experimental error, was observed. When the evaporation residue was dissolved in acetonitrile–water, with no triethylamine or acetic acid added, more than 15% was lost in 24 h.

Chromatography

Micro-LC with UV detection was chosen because of its high mass sensitivity compared with conventional LC. This is achieved by the injection of several microlitres of a solution of the sample dissolved in a liquid phase which gives the solutes a very high capacity factor. During the injection, the MDA-PFPA is focused at the top of the column and starts to elute when the plug of injection solution is replaced by the mobile phase. Recently we described this technique for the determination of toluene diisocyanate in air [12]. No noticeable difference in band broadening was seen when the injection volume was varied from 1 to 200 μl, but the analysis time increased for larger injection volumes. The injection of

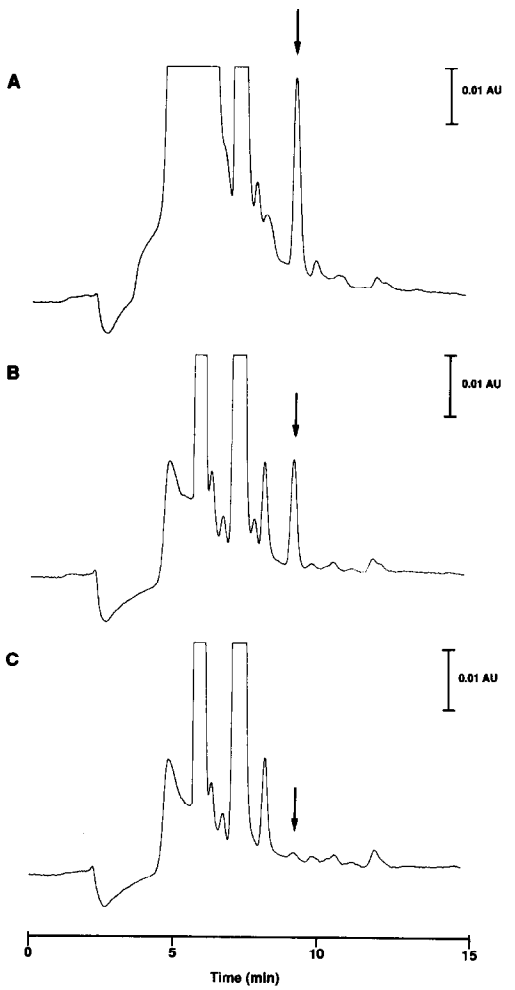

Fig. 1. Micro-LC chromatograms of urine samples. (A) Urine sample containing 40 nmol/l MDA, from an exposed worker; (B) urine spiked to 20 nmol/l MDA; (C) blank urine sample. Column, Fusica 150 mm × 0.32 mm I.D. fused-silica column packed with 3-μm C_{18} Hypersil; mobile phase, 70% acetonitrile–30% aqueous solution containing 1.5% triethylamine and adjusted to pH 3.0 with phosphoric acid; injection, 9.6-μl sample dissolved in 40% acetonitrile–60% aqueous solution containing 1.5% triethylamine and adjusted to pH 3.0 with phosphoric acid. UV detection at 258 nm, with 5 s response time.

10-μl sample volumes was chosen as a compromise between the time per analysis and high sensitivity.

Chromatograms obtained from a urine sample from an unexposed person, the same urine sample spiked with 20 nmol/l MDA and a urine sample from an MDA-exposed worker are shown in Fig. 1. All samples were prepared according to the work-up procedure discussed above. The chromatograms show that the MDA derivative peak is well resolved from interfering peaks in less than 15 min. Compared with free MDA, the MDA-PFPA derivative has a much higher capacity factor. This enhances the resolution from matrix peaks in the chromatogram and makes it possible to trap the substance during the injection, as discussed above. The stability and repeatability of the system are excellent if normal micro-LC rules, such as careful filtering of the mobile phase, are followed.

Recovery

The overall recovery was studied by performing the work-up procedure for ten urine samples spiked to 47 nmol/l MDA. Comparisons were made with an MDA-PFPA standard diluted to the same concentration. The overall recovery was found to be 89%, with a coefficient of variation (C.V.) of 4%.

Linearity and concentration range

A typical calibration graph in the concentration range 0–50 nmol/l MDA (n = 12) gave an intercept of $2.0 \cdot 10^{-4}$ A.U. and a slope of $8.4 \cdot 10^{-4}$ A.U./(nmol/l^{1}). The correlation coefficient was 0.9999. A lower relative response was observed when samples containing more than 50 nmol/l were analysed; these samples were reanalysed after dilution.

Repeatability

The repeatability for the chromatographic system was studied by performing ten repeated injections of a solution corresponding to a urine sample spiked to 40 nmol/l MDA. The C.V. for the retention time was 0.3% and for peak-height and peak-area measurements it was 0.9 and 0.8%, respectively. For ten preparations during one day of a urine sample containing 40 nmol/l MDA, the C.V. was 4%.

Reproducibility

The C.V. for peak-height measurements during a period of four weeks was 6% for five prep-

arations of a urine sample containing 40 nmol/l MDA. The sample was obtained from an exposed worker.

Detection limit

The detection limit for the method, defined as the blank signal plus three times the standard deviation of the blank, is 2 nmol/l [13]. Urine samples from ten persons, who were presumed unexposed, were used to test the detection limit. A slight unpredictable exposure may therefore have given a false higher detection limit than the true one. The detection limit, defined as three times the noise, was *ca.* ten times better, corresponding to a urine sample containing 0.2 nmol/l MDA for a 10-μl injection volume. When the injection volume was increased the detection limit was further improved. The lowest detection limits described previously were obtained using GC–MS: 10 nmol/l MDA [7]. Modern MS instrumentation is expected to result in much lower detection limits. Work on this is in progress at our laboratory.

Application

The method was applied to the monitoring of excreted MDA in urine, during epicutaneous skin hypersensitivity testing (patch testing). The Finn Chamber technique with 33 mg of the test preparation of MDA in petrolatum at 0.5% was applied in the alumium chamber. The chamber was attached with adhesive to the back skin of a volunteer and removed after 48 h. Urine samples were collected every hour during the first day and at longer intervals the following two days, then hydrolysed and analysed by the presented method. The measured concentration of MDA related to the creatinine concentration of the samples was plotted *versus* mid-time (Fig. 2). The total amount of MDA excreted was *ca.* 13% of that applied.

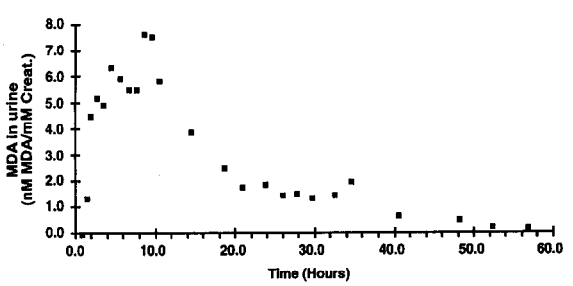

Fig. 2. Excretion of MDA in hydrolysed urine. MDA in petrolatum was applied to the skin of a volunteer in an epicutaneous patch test. The MDA concentration related to creatinine concentration is plotted against the mid-time of each observation period.

CONCLUSIONS

This method is demonstrated to give highly sensitive and precise determinations for the assessment of occupational exposure to MDA. The sensitivity is achieved by the high level of enrichment in the work-up procedure and by the use of micro-LC with large injection volumes.

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