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High-performance liquid chromatographic determination of 4,4′-methylenedianiline in human urine

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ABSTRACT

A method is described for the determination of urinary 4,4′-methylenedianiline (MDA) by high-performance liquid chromatography (HPLC). MDA was extracted from hydrolyzed urine using C_{18} solid-phase extraction columns. The extract was analyzed by reversed-phase HPLC with electrochemical detection at a cell potential of 0.8 V. The method was very sensitive (detection limit 2.5 μg/l) and quantitation using 4,4′-ethylenedianiline as an internal standard correlated well with results by gas chromatography–mass spectrometry. Run-to-run precision ($n = 25$) averaged 8.9%. In analysis of more than 160 potentially exposed workers, MDA was detected in less than 20% of the urines and concentrations ranged up to 210 μg MDA per g of creatinine.

INTRODUCTION

4,4′-Methylenedianiline (MDA) is used in the production of polyurethane foam and elastomers, Quiana nylon, polyamide–imide resins and epoxy resin composite materials. MDA has long been known to cause human liver damage [1,2] and recent animal studies have shown MDA to be carcinogenic [3,4]. The National Institute of Occupational Safety and Health (NIOSH) recommends that MDA be regarded as a potential carcinogen in the workplace and worker exposure be reduced to the lowest feasible limit [5]. Skin absorption is considered the major route of MDA exposure due to its low volatility and high skin penetration potential. Biological monitoring of urinary MDA would therefore be helpful to assess occupational exposures which air monitoring would underestimate.

Gas chromatographic (GC) methods for the analysis of MDA in biologicals have been described [6–8]; however, due to the polar nature of MDA, derivatization is required to produce a species which can be analyzed reproducibly by GC. Analyses of similar aromatic amines [benzidine and 4,4′-methylene bis(2-chloroaniline)] in urine by high-performance liquid chromatography (HPLC) which avoids the necessity of derivatization have been reported using both UV [9] and electrochemical detection [10]. The method presented here uses HPLC with electrochemical detection for the sensitive and specific analysis of total MDA

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after solid-phase (C_{18}) column extraction of hydrolyzed urine. This is a modification of a preliminary method developed at the National Institute of Safety and Health [11].

EXPERIMENTAL

Materials and reagents

Octadecyl (C_{18}) bonded-silica solid-phase extraction columns were prepared with 500 mg of 40-μm preparative-grade Bondesil obtained from Analytichem International (Harbor City, CA, U.S.A.). Extractions were performed using a 21-position solid-phase extraction manifold (Model SPE-21, J. T. Baker, Phillipsburg, NJ, U.S.A.).

The benzene, methanol and acetonitrile used were glass-distilled, high-purity-grade solvents (Baxter–Burdick and Jackson, Muskegon, MI, U.S.A.) and the sodium hydroxide and sodium acetate were analytical-reagent grade (Mallinckrodt, Paris, KY, U.S.A.).

Standard and controls

Stock standards of MDA (Aldrich, Milwaukee, WI, U.S.A.; 99% purity) and 4,4′-ethylenedianiline (EDA) (Aldrich, 99% purity) were prepared in methanol at a concentration of 1 mg/ml and stored at −15°C. Working standards and spiking solutions were prepared by diluting the stock standards in methanol. Five levels of calibration standards ranging from 5 to 240 μg/l were prepared by spiking pooled urine from unexposed individuals. Urine controls were prepared by spiking pooled urine at two levels, 10 and 100 μg/l, and frozen in 2-ml aliquots for monitoring daily precision of the method. Stock and working standards used for spiking urine controls were prepared separately from those used to prepare calibration standards. The unspiked pooled urine was also aliquoted in 2-ml portions and used as a urine blank with each batch of samples.

Sample preparation procedure

An 80-μl volume of a 5 ng/μl EDA internal standard solution (final urine concentration 200 μg/l) and 2 ml of 10 *M* NaOH were added to 16 × 150 mm screw-cap tubes containing 2.0 ml of urine. This urine mixture was hydrolyzed in a 80°C heating block for 90 min. To each tube 6 ml of reagent-grade water were added and the tubes were centrifuged at 450 *g* for 5 min. The extraction columns were washed with 3 ml of methanol and 5 ml of reagent-grade water. Each sample was transferred to an extraction column and the sample was allowed to pass through the column at a flow-rate between 1 and 2 ml/min. The columns were rinsed with 3 ml of 1 m*M* NaOH and allowed to air-dry for 2–3 min. The remaining water was removed by centrifuging the columns at 450 *g* for 5 min. The columns were returned to the extraction manifold with a 13 × 100 mm tube under each column. The columns were eluted with 3 ml of benzene at a flow-rate

between 1 and 2 ml/min. The elution was repeated with a second 3-ml volume of benzene and the extracts were combined. The benzene was evaporated with a stream of nitrogen at 35°C and the residue was reconstituted with 200 μl of methanol. After vortex-mixing the tubes briefly, the extracts were transferred to 2-ml screw-cap vials with 100-μl glass inserts and 10 μl were injected by the autosampler.

Urine samples were extracted for gas chromatographic–mass spectrometric (GC–MS) confirmatory analysis according to the method described by Cocker *et al.* [6]. The same volume of urine (2 ml) was hydrolyzed in the manner described in the above HPLC method and extracted with 10 ml of diethyl ether. The extract was derivatized with 1% heptafluorobutyryl chloride, concentrated to 100 μl and injected (1.0 μl) into the GC–MS apparatus.

Instrumentation and chromatographic conditions

The Shimadzu HPLC system included a Model LC-6A pump, a Model SCL-6A system controller, a Model C-R5A chromatography integrator, a Model SIL-6A autosampler set for 10-μl injections and a Model LC-ECD-6A electrochemical detector with a glassy carbon electrode set at + 0.8 V. The HPLC system was equipped with a 25 cm × 6.4 mm I.D. (5 μm) APEX ODS Symmetry column (Jones Chromatography, Littleton, CO, U.S.A.). The mobile phase was acetonitrile–methanol–0.01 *M* sodium acetate (27:11:62, v/v) with a flow-rate of 1 ml/min at ambient temperature.

The Hewlett Packard Model 5988A GC–MS system was operated in the negative-ion chemical ionization (NCI) mode using methane as the reagent gas. A 30 m × 0.25 mm I.D. DB-5 fused-silica column (J & W Scientific, Folsom, CA, U.S.A.) was programmed from 100 to 240°C at 30°C/min, then from 240 to 300°C at 5°C/min. Two ions were monitored each for MDA (570 *m/z* and 571 *m/z*) and for the internal standard, D_8-benzidine (564 *m/z* and 565 *m/z*).

RESULTS AND DISCUSSION

Hydrolysis of urine

Cocker *et al.* [6] performed several hydrolysis experiments to determine the effect of heat, the addition of strong base and the length of time required to completely hydrolyze the N-acetyl and the di-N-acetyl metabolites and conjugates of the parent compound MDA. They determined the time required for complete hydrolysis at 80°C with 10 *M* NaOH to be 90 min. These conditions were duplicated to facilitate a comparison of the HPLC and GC–MS methods.

Chromatographic analysis of MDA

Typical HPLC profiles of an MDA standard, urine blank and a positive urine from an MDA-exposed worker are shown in Fig. 1A, B and C, respectively. The internal standard was ethylene dianiline (EDA). Chromatogram A shows the

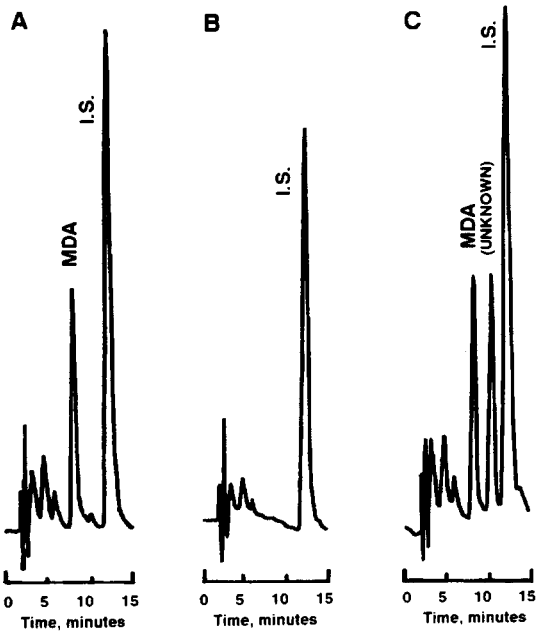

Fig. 1. Typical HPLC profiles of (A) an MDA-spiked urine standard, (B) a urine from an unexposed individual and (C) a urine from a worker occupationally exposed to MDA. The internal standard is EDA, spiked into urine at 200 μg/l. The MDA standard concentration was 60 μg/l. The quantitated concentration of MDA in the worker was 65 μg/l. The unknown peak in (C) was present in all positive specimens. Instrumental conditions and parameters are given in the Experimental section.

sensitivity of the electrochemical detector when a 2-ml aliquot of urine fortified with MDA at a concentration of 60 μg/l is extracted. The internal standard (EDA) at a urine concentration of 200 μg/l is well separated from MDA. None of the following common aromatic amines interfere chromatographically with either MDA or the internal standard: benzidine, 3,3′-dichlorobenzidine, methylene-bis(2-chloroaniline) (MBOCA), *m*-phenylenediamine (MPDA), *o*-tolidine, *o*-toluidine, 3,3′-dimethoxybenzidine and 4-methoxy-1,3-phenylenediamine. Only one aromatic amine tested, N-phenyl-1,4-phenylenediamine, potentially interferes with the internal standard at the instrumental conditions described. Such an interference would artificially reduce the calculated MDA concentration.

The urine blank (Fig. 1B) shows the interference-free background in urine from unexposed individuals obtained with the electrochemical detector. The detection limit using 2 ml of urine was determined to be 2.5 μg/l. This compares favorably to detection limits of 1 and 10 μg/l in biologicals obtained by GC–MS [7] and GC–electron-capture detection [8], respectively. Our earlier attempts to utilize a UV detector for MDA analysis were unsuccessful due to the appearance of numerous, random background interferences. Also, the large urine sample

volumes (>20 ml) required to achieve low μg/l detection limits led to HPLC column fouling and degradation of chromatographic efficiency.

The chromatogram of a positive urine specimen (Fig. 1C) contains a third, unidentified peak. This peak has been present in all positive urine specimens analyzed in this laboratory. We are continuing attempts to identify this peak to determine whether it is an unhydrolyzable metabolite of MDA or another substance to which the workers are concurrently exposed. Fortuitously, it is well separated from both the MDA and internal standard peaks and therefore does not interfere with the MDA quantitation.

The linearity of the method was studied by spiking blank urine at six levels and analyzing them in the same manner as urine specimens. The method was linear over a range between 5 and 240 μg/l. The equation of the calibration curve was $y = 1.4x + 0.029$ ($r^2 = 0.998$), where y is the detector response relative to the internal standard and x is the ratio of the MDA concentration to the internal standard concentration (200 μg/l).

Recovery and precision studies

The absolute recovery (total amount recovered *versus* the theoretical maximum yield) using this extraction procedure was 68% for MDA and 53% for the internal standard EDA. To determine if the extraction of MDA and EDA varied with urine from different individuals, urines from twelve unexposed individuals spiked at 50 μg/l were analyzed. The measured ratio of MDA to EDA for the twelve spiked urines had a precision (relative standard deviation) of 3.5%, thus indicating that the extraction efficiencies for both MDA and EDA are unaffected by the variability of the urine matrix. Calibration of the instrument was therefore performed using spiked urine standards which were carried through the entire sample preparation scheme. This calibration method effectively corrects for the incomplete recovery of the analyte.

As a part of the laboratory's quality assurance program, spiked urine controls at two concentrations, 10 and 100 μg/l, were analyzed with each batch of specimens. Precision data collected on a run-to-run basis were used to evaluate method performance. Run-to-run precision ($N = 25$) for the two control concentrations over a six-month period, expressed as relative standard deviation, was 9.8% and 8.0% for the low (10 μg/l) and high (100 μg/l) control levels, respectively.

Sample stability

A study of the stability of MDA in human urine stored at 2–4°C was performed with urine specimens collected from exposed workers. The levels of total MDA in urine remained unchanged for at least sixteen days. The average loss of MDA in three specimens (concentration range 18.9–33.9 μg/l) at the end of 46 days storage was 21%. After six months storage of a fourth urine specimen at 2–4°C the concentration decreased 88% from the initial measurement. Aliquots of urine spiked at 10 and 100 μg/l (urine controls) stored at −20°C were stable for at least

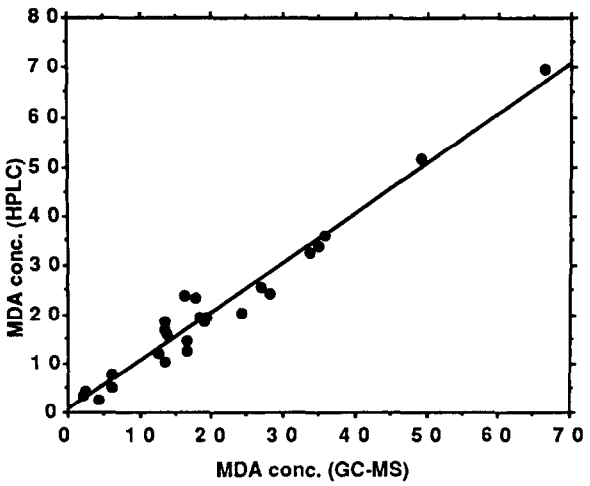

Fig. 2. Correlation between quantitative MDA levels from occupationally exposed worker specimens when measured by both HPLC with electrochemical detection and GC–MS.

six months as indicated by the results of daily quality control analyses described above.

Comparison to quantitation by GC–MS

A comparison of results from positive urine samples analyzed by HPLC and GC–MS methods is shown in Fig. 2. The samples were extracted by two different methods (see Experimental section). Quantitation was based on separately pre-

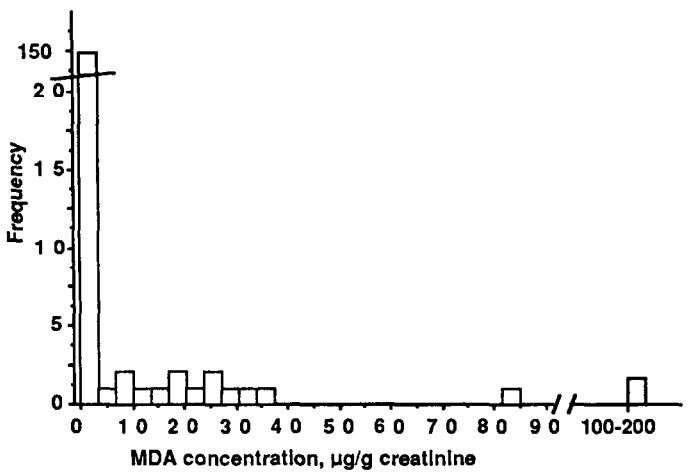

Fig. 3. Distribution of urinary MDA levels of potentially exposed workers from ten different industrial sites.

pared calibration standards. Detection limits for both methods were 2.5 μg/l. An excellent correlation between the two methods is evident ($r^2 = 0.958$) indicating an apparent lack of interference using this HPLC method with electrochemical detection. In addition, of over 300 urine samples analyzed by the two methods, only five specimens (1.6%) produced false positives (positive by HPLC and negative by GC–MS). All the false positives had apparent concentrations less than 6 μg/l. There were no false negatives (negative by HPLC and positive by GC–MS).

Analysis of workers' specimens

The HPLC analysis of over 160 urine specimens from different individuals working at ten industrial sites resulted in the concentration distribution presented in Fig. 3. MDA concentrations of positives ranged from 2.9 to 210 μg/g of creatinine while over 80% of the samples were below the detection limits. This distribution is quite similar to that obtained by Cocker *et al.* [6] for 323 MDA workers at five sites; however, one should expect this distribution to vary from site to site according to the work practices maintained at each facility and this similarity may only be coincidental.

CONCLUSIONS

Due to the relative non-volatility of MDA, dermal exposure is considered the major route of workplace exposures. Therefore, analysis of urinary MDA is an important industrial hygiene tool to assess the effectiveness of workplace safety practices. Biological monitoring can only be used if a valid method of analysis is available. The method presented here, based on the solid-phase extraction of hydrolyzed urine and analysis by HPLC with electrochemical detection, is specific, sensitive and reproducible. Recovery of MDA spikes is unaffected by the variable matrix from different urines and positive results from the analysis of workers' urines compare favorably to those determined by GC–MS.

While benzene was found to be the solvent which produced the highest recoveries without background interferences, another solvent with perhaps slightly lower recoveries would be preferable in light of benzene's carcinogenic properties. Further experiments are needed to identify such a solvent and evaluate its performance with workers' specimens.

The sample preparation described here results in the determination of the total MDA (free, acetylated and conjugated) concentration. However, the determination of just free MDA is possible if the base hydrolysis step is omitted. Other related aromatic amines may also be analyzed by this method with slight chromatographic modifications; however, further method validation would be required.

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