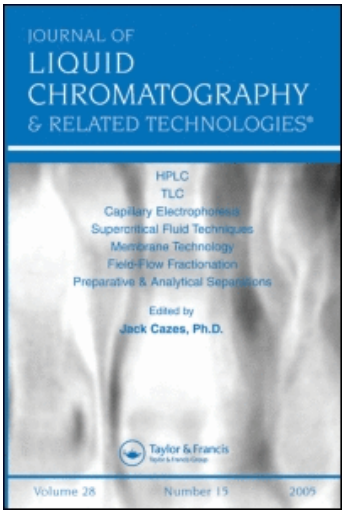

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Tiljander, A. , Skarping, G. and Dalene, M.(1990) 'Determination of 4,4'-Methylene-dianiline in Hydrolysed Human Urine as Chloroformate Derivative Using Column Switching and Liquid Chromatography with UV Detection', Journal of Liquid Chromatography & Related Technologies, 13: 4, 803 — 820

To link to this Article: DOI: 10.1080/01483919008051822

URL: http://dx.doi.org/10.1080/01483919008051822

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(4), 803-820 (1990)

DETERMINATION OF 4,4'-METHYLENE-DIANILINE IN HYDROLYSED HUMAN URINE AS CHLOROFORMATE DERIVATIVE USING COLUMN SWITCHING AND LIQUID CHROMATOGRAPHY WITH UV DETECTION

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ABSTRACT

A liquid chromatographic method with UV-detection (λ=246 nm) is presented for determination of 4,4'-methylenedianiline (MDA, C.A.S No 101-77-9) in hydrolysed human urine. The method is based on a two-phase derivatization procedure with isobutylchloroformate. The formed chloroformate derivative was analysed on a octadecylsilyl column using isocratic elution with acetonitrile-water (75:25 v/v). The overall recovery for urine samples spiked with 32 µg MDA / l was found to be 88 ± 4%. The calibration graph was linear in the investigated range (3-37 µg/l urine) with a correlation coefficient of 0.997. The precision was 3.4% for urine samples spiked with 32 µg MDA / l and the detection limit was 3 µg/l. Methyl-, ethyl-, isobutyl-benzyl-, 2-bromoethyl- and p-nitrobenzylchloroformate as derivatization reagents and clean-up procedures with extraction columns are discussed.

INTRODUCTION

MDA is used as a component in epoxy resins, polyurethanes, rubber chemicals and polymers (1,2). By the fare biggest use for MDA is as an intermediate in the production of 4,4'-methylenediphenyl diisocyanate (MDI). MDI is used in various fields, as for example in fibres and foamed products (1).

MDA are reported to be hepatoxic (3-7), mutagenic (8) and carcinogenic (9). MDA absorbs readily through skin (10), which is of great importance in the industrial set-up (6,11). Metabolites as N-acetyl MDA and N,N'-diacetyl MDA have been found in urine from exposed rats and workers (8,12,13). Several methods have been developed for the determination of MDA in matrices such as air sampling solutions (14-17), blood (18,19) and urine (12,13,19-23). The chromatographic methods for trace analysis of MDA has met considerable difficulties owing to the absorbtion of MDA in the analytical system (24). Perfluorofatty anhydride (22), salicylaldehyddiphenylboron chelate (25), fluoresceamine (26, 27) and aldehydes (27) have been reported as derivatization reagents for liquid chromatographic (LC) determination of MDA. UV (17,22,23,25) electrochemical (26) and fluorescence (26,27) detection have been used.

For urine analysis using GC-MS, detection limits in the range 1-10 µg/l have been found (12,13,19-21). A method for determination of MDA in hydrolysed urine as perfluorofatty amides using LC-UV, with a detection limit of 8 µg/l, has recently been developed at our laboratory (22). Extraction columns have been used for clean-up of serum and urine samples (19).

In this paper a LC method is described for determination of chloroformate derivatives of MDA at the low µg/l level in hydrolysed human urine.

MATERIALS AND METHODS

Equipment

The LC system consisted of a Waters (Millipore-Waters, Milford, MA., USA) 600 Multisolvent Delivery System, a Waters 712 WISP autosampler, a Waters 490 Programmable Multiwavelength detector, a three channel SE 130 (ABB Goertz Aktiengesellschaft, Vienna, Austria) and a Shimadzu (Kyoto, Japan) C-R3A integrator. A Waters M6000A LC pump and a Waters Automated Valve Station were used for column switching. The column switching system is described in figure 1. The sample was injected on a clean-up column mounted in a six port valve, off line with the analytical column. After injection the clean-up column was rinsed. The valve was then switched and the clean-up column containing the analyte was on line with the analytical column. Absorption spectrums were recorded on a Shimadzu UV-visible recording spectrophotometer UV-260. For separation of phases a Model 3E-1 centrifuge (Sigma, Harz, F.R.G) was used. For evaporation of the solvent an evaporating unit connected to a Reacti-Therm (Pierce, BA Oud-Beijerland, Netherlands) with nitrogen flow was employed. For liquid-solid sample preparation a Vac-Elut (Analytichem International, Inc., Harbor City, CA, USA) SPS 24 was used. The water was

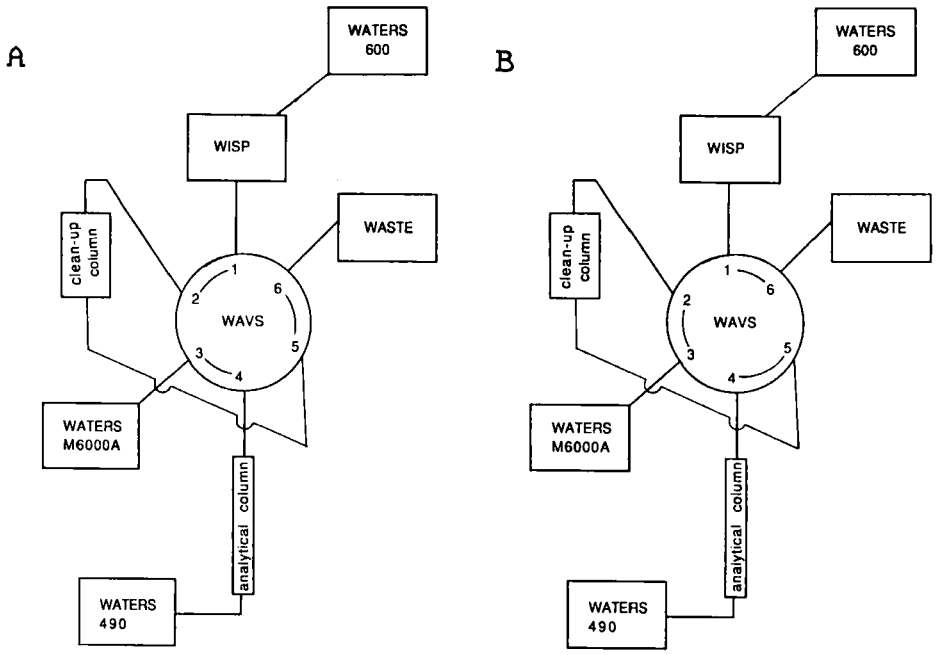

Fig. 1 Configuration of columns switching. A: Injection and rinsing of the worked-up urine samples. B: Eluting the analyte from the clean-up column

produced in a Milli-Q (Millipore, Bedford, MA., USA) apparatus.

Columns

Reversed phase LC columns of stainless steel were used: HICHROM (reading, G.B.) Spherisorb S5ODS1 Excel (20cm x 3.2 mm I.D.), Spherisorb S5ODS2 Excel (20 cm x 3.2 mm I.D.) and Hypersil 5ODS Excel (20 cm x 3.2 mm I.D.); Jones Chromato-

graphy (Hengoed, G.B.) Apex II Octadecyl (25 cm x 4.6 mm I.D.); and Macherey-Nagel (Düren, F.R.G.) Nucleosil $5C_{18}$ (20 cm x 3 mm I.D.). For clean-up of the samples Guard-Pak (Waters) precolumns with Bondapak cartridges and Bond Elut (Analytichem International, Inc.) extraction columns with octadecyl, octyl and cyanopropyl silanes were used. The amount of the sorbentbed was 500 mg and the reservoir volume was 10 ml.

Chemicals

MDA, 2-bromoethyl- and p-nitrobenzylchloroformate were obtained from Aldrich (Beerse, Belgium). N,N-dimethyloctylamine, methyl-, ethyl- and benzylchloroformate were all from Janssen (Beerse, Belgium). Isobutylchloroformate and trimethylamine were from Sigma Company (S:t Louis, USA). LC grades of toluene, acetonitrile and methanol were obtained from Lab-Scan (Dublin, Ireland). Sodium hydroxide, dipotassium carbonate and hydrochloric acid were all from Merck (Darmstadt, FRG). Pyridine was from BDH (Poole, U.K.).

Synthesis of MDA derivatives

A 0.5-g amount of pyridine and 100 ml of 5 M NaOH were added to a solution containing 2 g of MDA and 50 ml of toluene. The chloroformate reagent (5 g) was dropwise added during stirring. After 10 minutes the organic phase, containing the precipitate of the formed derivative, was

separated. The toluene mixture was then shaken with 0.1M HCl. The mixture was filtrated and the precipitate was washed with toluene. The precipitate was dried in an incubator at 80 °C and thereafter kept in a vacuum exicator. The derivatives of methyl-, ethyl- and isobutylchloroformate were synthesised.

Preparation of standard solutions

Stock solutions of MDA were prepared in 0.1 M HCl at the 1 g/l level and diluted with 0.1M HCl to the appropriate concentrations. Stock solutions of the chloroformate derivatives of MDA were prepared in acetonitrile at the 1 g/l level and further diluted in an acetonitrile-water solution, 3:1. 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 6M HCl per 100 ml of urine was added to the urine samples. The acidified urine samples were stored in a refrigerator until analysis.

Procedure

Two-phase derivatization. A 0.8 ml urine sample was added to a 10 ml test-tube fitted with a PTFE cap containing 1.2 ml of 6M HCl. The test-tube was heated at 95-100 °C for 2h

(hydrolysis) and then cooled to room temperature. A 3-ml volume of toluene and 4 ml of a saturated aqueous NaOH solution were added to the test-tube. The mixture was shaken for 10 minutes and thereafter centrifugated at 1,500 g for 10 minutes. A 2-ml volume of the organic phase was separated and transferred to a new test-tube. Pyridine (5 µl), 3 ml of an aqueous 2M carbonate buffer (pH=9.5) and 10 µl of the isobutylchloroformate reagent were added to the organic phase. The mixture was shaken for 10 minutes. A 1-ml aliquote of the organic phase was transferred to a new test-tube and the sample was evaporated into dryness by a gentle stream of nitrogen.

Clean-up with column switching. The dry residue was dissolved in 0.25 ml of an acetonitrile-water solution (3:1). The clean-up column was conditioned with 3 ml methanol followed by 6 ml of water. A 50-µl volume of the sample was injected on the clean-up column. The clean-up column was then rinsed with 5 ml of an acetonitrile-water solution (1:1) at a flow of 1 ml/min. After 5 minutes the valve was switched and the clean-up column was on-line with the analytical column. The sample retained on the clean-up column was eluted with the mobile phase. After elution the clean-up column was washed with 2 ml of a solution containing 6 ml triethylamin in acetonitrile-water (4:1). The pH was adjusted with phosphoric acid to 3.

Clean-up with extraction column. The two-phase derivatization procedure was in this case performed with methylchloroformate. The dry residue was dissolved in 1.5 ml of an acetonitrile-water solution (3:1). The extraction column was conditioned with 5 ml of methanol followed by 5 ml of water

(1-2 ml/min). A 1-ml aliquote of the sample and 4 ml of water was applied on the cartridge which was thereafter rinsed with 5 ml of water. The retained sample was eluted with 2 ml of 0.02 M N,N-dimethyloctylamine in acetonitrile. The extraction column was carefully sucked dry. The eluat was evaporated into dryness with nitrogen. The dry residue was dissolved in 0.5 ml of an acetonitrile-water solution (3:1). The solution (50 µl) was injected into the chromatographic system.

RESULTS AND DISCUSSION

Standards

The identities of the chlorofomate derivatives of MDA were confirmed by mass spectrometry. The purities of the chlorofomate derivatives of MDA were determinated using LC-UV, λ=246 nm and by GC with thermionic specific detection, and were found to be better than 99%. The purities were further examined by elemental analysis and the experimental values for carbon, hydrogen, nitrogen and oxygen were found to differ less than 0.2% from the calculated values.

Procedure

Choise of derivatization reagent. Methyl-, ethyl- and isobutylchloroformate derivatization reagents were investigated. No difference was found in the recovery of the different derivatives in the two-phase derivatization procedure.

Different aqueous phases consisting of 1M phosphate (pH=7.4), 2M carbonate (pH=9.5) and 2M carbonate (pH=10.5) buffer solutions and 5 M NaOH were tested. The 2M carbonate buffer solution (pH=9.5) was found to give the best recovery, 98±5% with 5 µl pyridine used as catalyst. Variation of the pyridine concentrations in the range 2-50 µl did not influence the recovery. However chromatographic artifacts increased with the amount of pyridine added. Without pyridine the recovery was lowered to 73±3%. Ammonia, triethylamine and picolin were also tested as catalysts but gave less recovery.

The recovery in the derivatization step was virtually not influenced by the different amount of chloroformate (2-100 µl) added. The addition of > 200 µl chloroformate created gas evolution and for > 1000 µl chloroformate the solution became hazy.

Evaporation. The evaporation of the organic phase to dryness made it possible to enrich and choose optimal solvents for the column switching or the extraction column procedure. No losses were found in the evaporation step.

Column switching. The isobutylchloroformate derivative of MDA was retained on the clean-up column. However when testing the methyl- and ethylchloroformate derivative of MDA considerable losses were found due to the lower k'-value. The washing of the clean-up column with an acetonitrile:water (80:20) solution containing triethylamine. This step also increased the column lifetime.

Extraction column. The clean-up procedure for the sample solution containing formed MDA derivative with the use of extraction columns were studied. The recovery of the derivatives were tested for different sorbentbeds, Table I. The

TABLE I

MDA recovery using different extraction columns and 0.02M N,N-dimethyloctylamine in acetonitrile. Three urine samples spiked with 148 µg/l MDA were determinated with triple injection.

sorbentbed	methyl	ethyl	isobutyl
C18	96±2%	92±2%	87±3%
C8	90±6%	95±4%	84±4%
CN	90±4%	94±2%	79±3%

TABLE II

MDA recovery using C_{18}-extraction columns with different elution solvents. Three urine samples spiked with 148 µg/l MDA were determinated with triple injections.

elution solvent	methyl	ethyl	isobutyl
Acetonitrile	94±2%	91±4%	81±3%
0.01M Trimethylamine and acetonitrile	-	84±8%	86±12%
0.02M N,N'-dimethyloctylamine and acetonitrile	96±2%	92±2%	87±3%

methylchloroformate reagent and C_{18} extraction column was preferred due to better recovery. The choice of elution solvent were also tested, Table II, and 0.02 M N,N'-dimethyloctylamine in acetonitrile was optimal. High recovery were obtained and interfering compounds in the chromatograms were eliminated.

Chromatography

Choice of separation conditions. The k'-values for MDA derivatives of methyl-, ethyl-, isobutyl-, benzyl-, 2-bromoethyl- and p-nitrobenzylchloroformate were investigated for

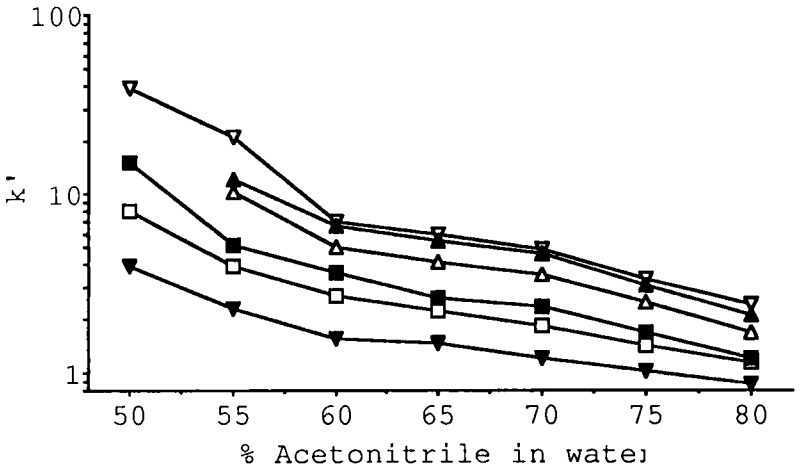

Fig. 2 Relationship between capacity factor (k'), for chloroformate derivatives of MDA of methyl- (▼), ethyl- (□), isobutyl- (▽), benzyl- (▲), 2-bromoethyl- (■) and p-nitrobenzyl-chloroformate (△) and the composition of the mobile phase. Conditions: Eluent: acetonitrile-water; Flow rate: 1 ml/min; Column: Nucleosil $5C_{18}$; Injection volume: 50 µl; UV detection: 246 nm.

aqueous acetonitrile, Fig 2. Aqueous acetonitrile compared with aqueous methanol was preferred due to better resolution relative the matrix. Aqueous acetonitrile as mobile phase gave also low pressure.

Low water content in the mobile phase make the chloroformate derivatives of MDA elute fairly rapidly from the column. The k'-values increased with increasing water content. This reflects the dominant influence of the non-polar parts of the chloroformate derivatives of MDA on retention. As

expected, increased chain length of the alkyl group on the derivatization reagent increased retention.

The methyl-, ethyl- and isobutylchloroformate derivatives of MDA showed the best resolution relative the biological matrix. These derivatives were therefore studied in more detail.

Detection

Absorbance maxima of the standards of the methyl-, ethyl- and isobutylchloroformate derivative of MDA were found at 202 and 246 nm. The signal/noise level were optimal at 246 nm. The detection of coeluting compounds in the matrix were also minimized at this wavelength. The derivative is eluted on the tailing matrix, Fig. 3.

Absorbance ratios (230-260 nm / 246 nm) chromatograms were studied. Compounds in the matrix coeluting with the MDA derivative showed some absorption in the range 230-260 nm. Ratio chromatograms was therefore not possible to use in the studied concentration range.

Quantitative analysis

Recoveries. The overall recovery was studied for eight urine samples spiked with MDA. Triple injections of each sample were made. Comparison were made by analysing standards of the chloroformate derivative of MDA diluted to actual concentrations. The overall recovery was found to be 88 ± 4%

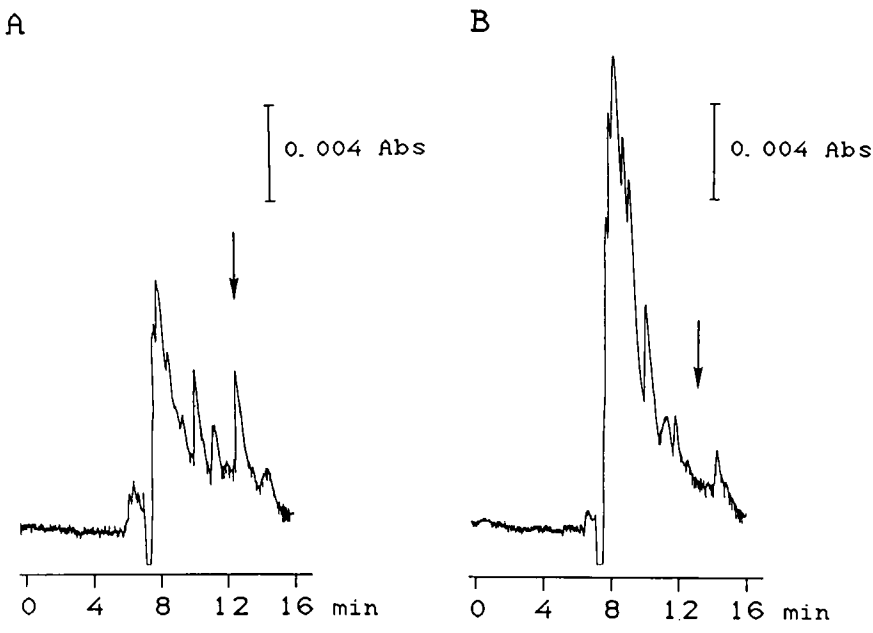

Fig. 3 Chromatograms of urine samples. A: isobutylchloroformate derivative of MDA from a urine sample from an exposed worker. The peak corresponds to a concentration of 36 µg/l of urine. B: Chromatogram from an unexposed workers. Conditions: Column: Apex II C_{18}; Eluent: acetonitrile-water 3:1; others conditions as in Fig. 2.

(95% confidence and n=8) for isobutylchloroformate reagent and column switching. It was 96 ± 2% (95% confidence and n=8) for methylchloroformate reagent and using extraction columns. The MDA concentration was 32 µg/l and 148 µg/l of urine respectively.

Calibration graphs. Different concentrations of MDA in urine and blanks were prepared according to procedure. For

each concentration two determinations were made with triple injections. The calibration graph using the column switching technique was linear and gave a correlation coefficient of 0.997 (n=8, y=545x-340), for the concentration range 3-37 µg/l of MDA in urine. For the extraction column the calibration graph was linear and gave a correlation coefficient of 0.999 (n=6, y=236x-868), for the concentration range 48-150 µg/l of MDA in urine.

Detection limits. The detection limit, defined as the urine sample concentration giving a signal equal to the blank signal plus three standard deviations (28), was 3 µg/l urine using the column switching technique and 13 µg/l urine with extraction columns. No interfering peaks appeared when urine from ten persons were examined using column switching technique respectively extraction columns.

Accuracy and precisions. Nine urine samples were analysed. The relative standard deviation of the MDA peak areas were 3.4% using the column switching technique and 3.4% using extraction columns, at concentrations of 32 µg/l and 148 µg/l respectively.

CONCLUSIONS

The described LC method are developed for determinations of MDA in hydrolysed human urine. Selective and sensitive determinations in biological matrices, at the low µg/l level, are feasible using clean-up procedures with column switching or extraction columns.

ACKNOWLEDGEMENTS

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

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