Journal of Chromatography, 581 (1992) 57–63
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6470

Diuretic screening in human urine by gas chromatography–mass spectrometry: use of a macroreticular acrylic copolymer for the efficient removal of the coextracted phase-transfer reagent after derivatization by direct extractive alkylation

A. M. Lisi, R. Kazlauskas and G. J. Trout

Australian Government Analytical Laboratory, P.O. Box 385, Pymble, N.S.W. 2073 (Australia)

(First received March 24th, 1992; revised manuscript received May 27th, 1992)

ABSTRACT

A simple and efficient procedure has been developed for the derivatization of diuretic agents in human urine by direct extractive alkylation and their detection by gas chromatography–mass spectrometry. The procedure is an improvement over previous extractive alkylation methods because of the development of a simple clean-up step using a macroreticular acrylic copolymer (SM-7 resin) to remove the coextracted phase-transfer reagent from the organic phase after derivatization. With 1 ml of sample the method gives detection limits in the range 10–50 ng/ml for acetazolamide, probenecid, dichlorphenamide, hydroflumethiazide, furosemide, chlorthalidone, bumetanide, hydrochlorothiazide, quinethazone, bendroflumethiazide, metolazone and cyclopenthiazide.

INTRODUCTION

Extractive alkylation (EA) is a convenient means of derivatizing a wide range of acidic drugs before gas chromatographic (GC) analysis [1–15]. By performing EA directly on biological fluids the extraction and derivatization of these drugs can be carried out quickly in a single step under mild reaction conditions. The procedure utilizes a quaternary ammonium phase-transfer reagent to extract the acid as an anion from an alkaline aqueous phase into an aprotic solvent having poor solvating power for anions. The resultant increase in the reactivity of the anion allows the alkylation reaction to take place rapidly at room temperature.

The phase-transfer reagents most commonly used for EA reactions are tetrabutylammonium (TBA^+), tetrapentylammonium (TPA^+) and tetrahexylammonium (THA^+) salts. Increasing the lipophilicity of the phase-transfer reagent results in increased extraction yields of the ion pairs formed between the quaternary ammonium cations and the anions of acids. The disadvantage of using highly lipophilic phase-transfer reagents in direct EA reactions is the large amount of the reagent that is coextracted into the organic phase because of the formation of extractable ion pairs with some of the naturally occurring anions in the biological fluid. These salts lead to interferences during the GC analysis of the sample. Lindstrom and Molander [7] reported the substitution

Correspondence: A. M. Lisi, Australian Government Analytical Laboratory, P.O. Box 385, Pymble, N.S.W. 2073, Australia.

0378-4347/92/$05.00

of one of the methyl groups of trimethyl furosemide with a hexyl group when the coextracted salts of THA^+ and trimethyl furosemide were injected into a gas chromatograph. Ehrsson [1] reported that the pyrolysis product of the coextracted salt of THA^+ gave an interfering peak during the GC determination of phenobarbital. Graas and Watson [11] reported that residual amounts of the THA^+ salts caused chromatographic interferences due to a broad solvent peak during the determination of benzoylecgonine by direct EA.

In a previous communication [15] we described a gas chromatographic–mass spectrometric (GC–MS) procedure to screen for diuretic agents in human urine using direct EA with THA^+ as the phase-transfer reagent and toluene or dichloromethane as the organic phase. After relatively few injections the pyrolysis product from the residual salts of THA^+ caused a rapid deterioration in the efficiency of the capillary column with analyte retention times slowly increasing and the chromatograms displaying broad tailing fronts with continued column use. To avoid these problems an efficient clean-up procedure is required in order to remove the coextracted salts of THA^+ from the toluene after direct EA. In this communication we address this problem associated with direct EA. We describe a new, efficient and simple clean-up procedure based on solid-phase extraction using a macroreticular acrylic copolymer (SM-7 resin) to remove the coextracted salts of THA^+.

EXPERIMENTAL

Instrumentation

The instrumentation consisted of a Hewlett-Packard gas chromatograph interfaced to an electron-impact mass-selective detector via a capillary direct interface. The details of the instrumentation have been described [15]. The chromatograms presented in this paper were obtained with the mass spectrometer operated in the full-scan mode monitoring the ions in the range 40–500 a.m.u. The carrier gas was helium at a flow-rate of 1 ml/min and the split ratio was 10:1. The GC temperature conditions were: 290°C for the injector and detector, 138°C for the initial column temperature and 300°C for the final column temperature. The column temperature was programmed to increase at 10°C/min. For the chro-

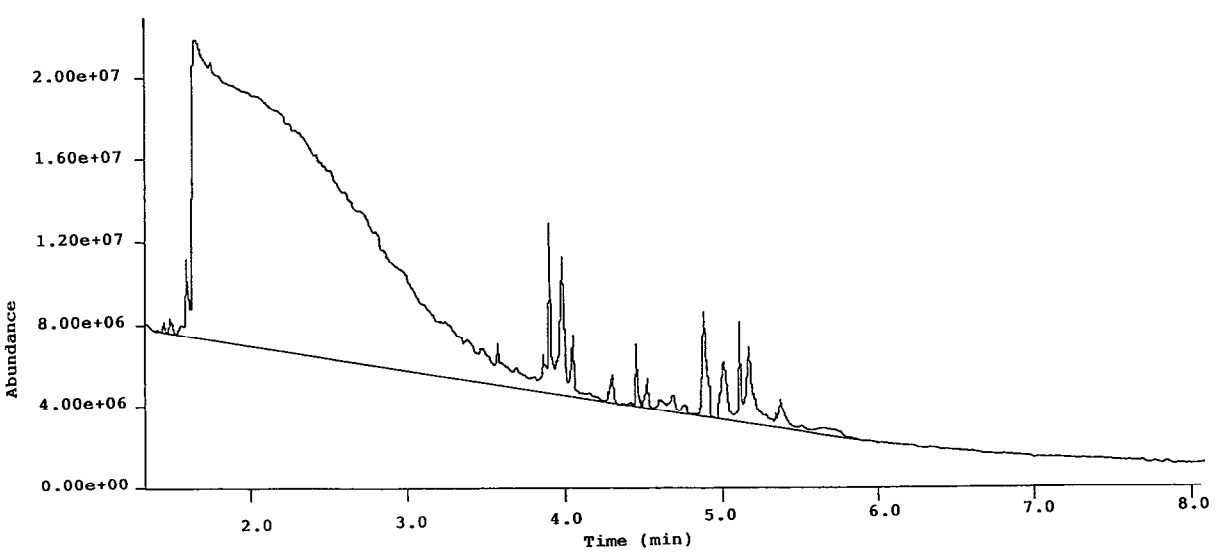

Fig. 1. TIC GC–MS profile obtained after performing direct EA on a urine sample and washing the toluene with a saturated solution of silver sulphate. The trace shows the broad tailing front that results from the overloading of the capillary column with the trihexylamine produced by the pyrolysis of the residual salts of THA^+.

matogram in Fig. 1 the column temperature was increased at a rate of 30°C/min.

Reagents and chemicals

The reference drug samples and tablets, which have been described [15], were kindly supplied by the manufacturers. Nanograde toluene, hexane, cyclohexane, chloroform and liquid chromatography grade methanol, acetonitrile and tetrahydrofuran were obtained from Mallinckrodt Australia (Clayton, Australia); methyl iodide from May and Baker (Melbourne, Australia); tetrahexylammonium hydrogensulphate and trihexylamine from Fluka (Buchs, Switzerland); 200–400 mesh analytical grade SM-7 resin from Bio-Rad Labs. (Sydney, Australia); silanized glass wool from Alltech Australia (Sydney, Australia) and 70–230 mesh silica gel from Merck (Darmstadt, Germany).

Preparation of the SM-7 sorbent and the columns

The fines were removed by suspending the commercially available SM-7 sorbent in methanol and decanting the supernatant. This procedure was repeated until the supernatant was clear. The sorbent was then stored as a slurry under methanol until use.

To prepare the columns, disposable glass pipettes (6 mm I.D.) were fitted with small plugs of silanized glass wool to act as bed supports and with a second pipette the resin slurry was added until columns of length 2.5–3.0 cm were obtained. Before use the columns were conditioned with 2 ml of toluene.

Synthesis of milligram amounts of the methyl derivatives of probenecid, dichlorphenamide, furosemide and hydrochlorothiazide

An 80-mg amount of the diuretic was dissolved in 10–15 ml of acetonitrile before the addition of 300–400 mg of potassium carbonate and 1 ml of methyl iodide. The mixture was refluxed at 60°C for 5 h before evaporating the acetonitrile, extracting the residue with toluene and washing the toluene with two 5-ml aliquots of 0.5 *M* NaOH. The diuretics that gave more than one reaction product, *i.e.* dichlorphenamide, furosemide, and hydrochlorothiazide, were purified on a 10 × 1 cm silica gel 40 column using hexane or toluene modified with tetrahydrofuran as the eluent to isolate the desired product. The methyl ester of probenecid was recrystallized from hexane, trimethylfurosemide from cyclohexane, tetramethyldichlorphenamide from toluene–hexane and tetramethylhydrochlorothiazide from chloroform–hexane. The purity of the derivatives was checked using thin-layer chromatography and GC–MS.

Direct extractive alkylation

The details of the method have been described [15]. The following modifications were employed. Urine samples (1 ml) were made alkaline with 25 μl of 6 *M* NaOH before the addition of 100 μl of 10 μg/ml mefruside (internal standard), 150 μl of 0.2 *M* tetrahexylammonium hydrogensulphate (prepared by dissolving 4.5 g of the salt in 50 ml of 0.5 *M* NaOH) and 5 ml of 0.5 *M* methyl iodide in toluene. The urine and toluene phases were mixed at room temperature for 20 min on a Clements laboratory suspension mixer (Phoenix Scientific, Sydney, Australia) and then centrifuged at 1500 *g* for 5 min. The toluene phases were passed through the pre-prepared columns of 200–400 mesh macroreticular acrylic copolymer sorbent (SM-7 resin), collected in Quickfit B14 test-tubes (Mowbray Glass, Gosford, Australia) and evaporated to dryness under a stream of nitrogen. The residues were reconstituted in 100 μl of toluene before injection of 2-μl aliquots into the GC–MS system.

RESULTS AND DISCUSSION

Interference from the THA^+ counter ion

During direct EA reactions the THA^+ counter ion can coextract into the organic phase as either the iodide salt, generated during the methylation reaction, or the chloride and uric acid salts, generated from ion pair formation with the Cl^- ions and uric acid present in the urine. Inside the heated GC injection port quaternary ammonium salts undergo pyrolysis to their corresponding tertiary amines [16,17]. The retention time and mass spec-

trum of the pyrolysis product of the coextracted THA^+ salts were found to correlate with that of authentic trihexylamine, the expected product from the thermal degradation of these salts.

Removal of the interference from the THA^+ counter ion

The coextraction of large amounts of THA^+ salts during direct EA and their pyrolysis in the GC injection port means that an efficient procedure for their removal is required if chromatographic interferences and column deterioration are to be avoided during analysis. This is particularly important if capillary columns are used as they have low capacities and are easily overloaded.

To date users of EA have taken one of two approaches in attempts to separate the coextracted THA^+ salts from the analyte before GC analysis. Firstly the solvent used for the EA reaction is evaporated to dryness and the methyl derivatives are extracted from the residue with a non-polar solvent in which the salts of THA^+ have low solubility [6–10]. This approach suffers from the disadvantage that some of the analyte may be lost due to entrapment in the crystalline matrix of the residue giving poor recovery and loss of sensitivity. Graas and Watson [11] found that the resulting physical entrapment of the analyte gave highly variable recoveries for the determination of benzoylecgonine. The second technique that has been widely used has involved washing the organic phase with a saturated solution of silver sulphate [12–15]. This approach gives incomplete removal of the coextracted salts of THA^+ so that when the toluene extract is concentrated and analysed the trihexylamine produced by the pyrolysis of the residual THA^+ salts results in the contamination of the injection port liner and overloading of the capillary column causing a rapid deterioration in column efficiency. As a result the routine application of direct EA becomes difficult without frequent column and injection port maintenance. Fig. 1 gives an example of the total ion current (TIC) GC–MS chromatograms that can be expected when urine samples are routinely subjected to direct EA and the toluene is washed with a saturated solution of silver sulphate. The broad tailing front in the chromatogram is due to the overloading of the coloumn with trihexylamine. The high background signal that is produced results in poor signal-to-noise ratios at low drug concentrations.

The new clean-up procedure described in this paper overcomes the above problems by utilizing a short column of a moderately polar macroreticular acrylic copolymer (SM-7 resin) to adsorb the polar salts of THA^+ from the toluene, while allowing the less polar methyl derivatives of the diuretics to pass through the column unretained. The efficiency of the SM-7 resin in separating the coextracted salts of THA^+ from the methyl derivatives of the diuretics is demonstrated in Fig. 2. Chromatogram 1 was obtained after a blank urine sample was subjected to direct EA and the toluene passed through a column of the resin. The absence of a peak for trihexylamine at 4.06 min demonstrates that the coextracted salts of THA^+ have been adsorbed by the SM-7 resin. The region of interest for diuretic analysis (>6 min) has very few coextracted endogenous urinary compounds capable of interfering with the analysis. Chromatogram 2 was obtained after direct EA on a composite urine sample consisting of urines taken from volunteers who ingested a single oral dose of acetazolamide (250 mg), probenecid (500 mg), dichlorphenamide (50 mg), furosemide (50 mg) and hydrochlorothiazide (50 mg). The methyl derivatives of the diuretics have eluted with the toluene and the THA^+ salts have been extracted by the SM-7 resin.

The pre-prepared SM-7 resin columns may be regenerated and reused by eluting the adsorbed salts of THA^+ with 2 ml of methanol and conditioning the columns with 2 ml of toluene before the addition of the next sample. With this regeneration procedure we did not observe any carry over of the methyl derivatives from one sample to the next. Other solid phases that were investigated with little success included silica, XAD-2, octadecyl silica, florisil and a macroporous cation exchanger.

Dichloromethane is the most commonly used solvent in EA reactions. The polarity of this sol-

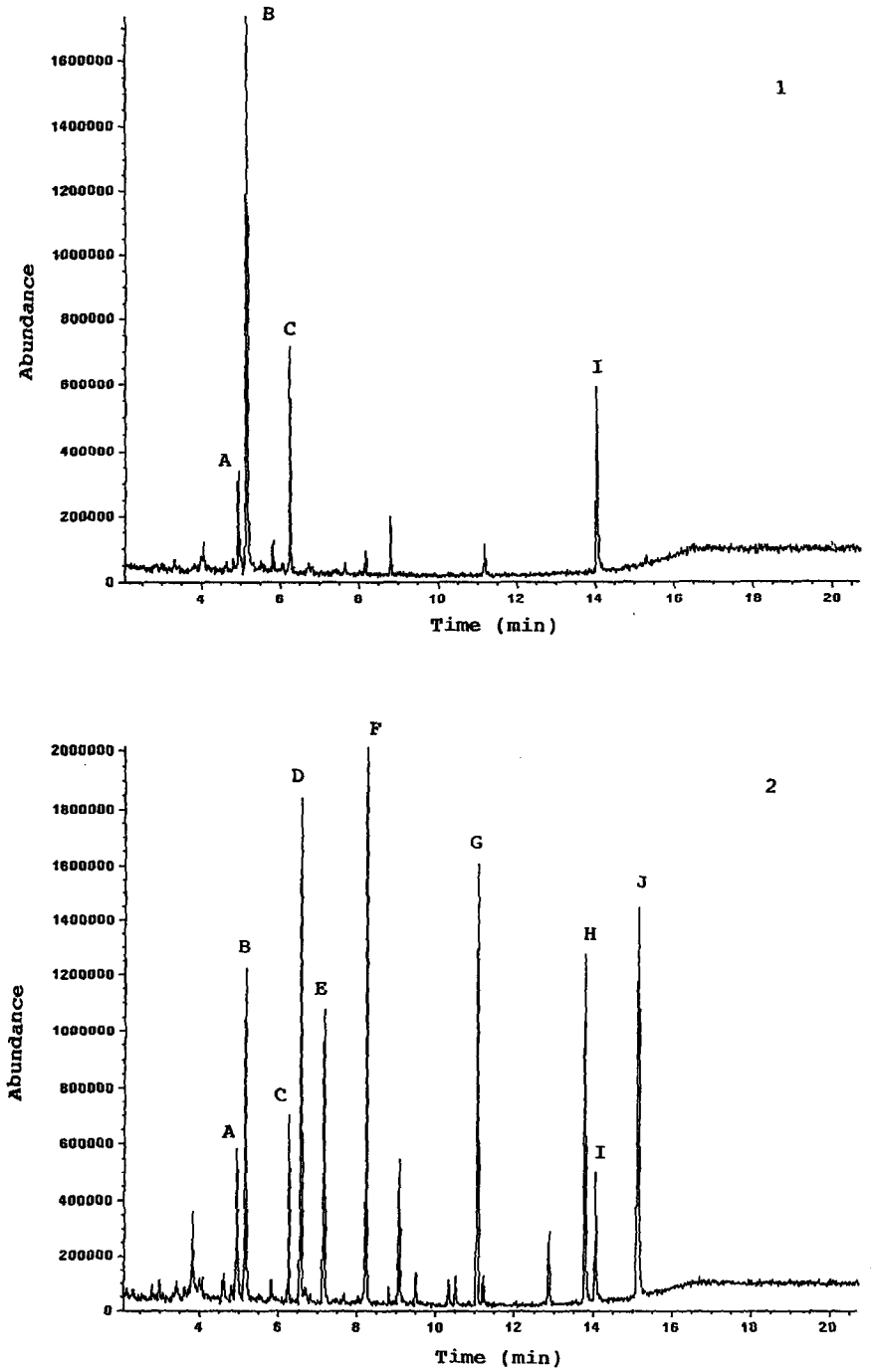

Fig. 2. TIC GC–MS profiles obtained after performing direct EA on two urine samples and extracting the THA^+ salts from the toluene with a 2.5–3.0 cm column of SM-7 resin. (1) Blank urine sample; (2) composite urine sample from subjects who ingested a single oral dose of acetazolamide, probenecid, dichlorphenamide, furosemide and hydrochlorothiazide. Peaks: A = methyl ester of indole-3-acetic acid; B = caffeine; C = unknown; D = monomethylated acetazolamide; E = trimethylated acetazolamide; F = methyl ester of probenecid; G = tetramethylated dichlorphenamide; H = trimethylated furosemide; I = dimethylated mefruside (internal standard); J = tetramethylated hydrochlorothiazide. Under these experimental conditions any trihexylamine from the pyrolysis of THA^+ salts would have eluted at 4.06 min.

vent was found to be too high to effect a separation of the methyl derivatives of the diuretics from the coextracted salts of THA^+ on SM-7 resin.

Detection limit

The detection limit was determined with the mass-selective detector operated in the selected-ion mode, and was defined as the concentration of analyte that gave a signal-to-noise ratio of 3. The ions that were monitored have been listed [15]. The limits of detection for twelve diuretics are given in Table I.

Recovery

To demonstrate the method recovery, eight 1-ml aliquots of urine were spiked to 2.5 μg/ml with probenecid, dichlorphenamide, furosemide and hydrochlorothiazide and taken through the direct EA procedure. The yields for the four diuretics were compared with pure solutions of tetramethyldichlorphenamide, trimethylfurosemide, tetramethylhydrochlorothiazide and the methyl ester of probenecid. The recoveries and standard deviations for the four diuretics are shown in Table II.

TABLE I

DETECTION LIMITS FOR TWELVE DIURETICS

Diuretic	Detection limit (ng/ml)
Acetazolamide	50
Probenecid	10
Dichlorphenamide	25
Hydroflumethiazide	10
Furosemide	10
Chlorthalidone	10
Bumetanide	10
Hydrochlorothiazide	50
Quinethazone	10
Bendroflumethiazide	10
Metolazone	10
Cyclopenthiazide	10

TABLE II

RECOVERIES OF FOUR DIURETICS

Samples were 1 ml of urine, spiked with 2.5 μg/ml; $n = 8$.

Diuretic	Recovery (mean ± S.D.) (%)
Probenecid	93 ± 4
Dichlorphenamide	93 ± 5
Furosemide	80 ± 7
Hydrochlorothiazide	79 ± 11

CONCLUSIONS

We have introduced a new more efficient procedure for the removal of the salts of THA^+ from the toluene phase after performing EA directly on urine samples. This simple clean-up procedure overcomes the chromatographic problems caused by the coextraction of these salts resulting in improved signal-to-noise ratios and enabling EA to be more easily applied to the routine GC analysis of acidic drugs in biological fluids.

ACKNOWLEDGEMENTS

We thank Professor Graham Johnston, Department of Pharmacology, Sydney University, for his valuable discussion of the manuscript, and the Australian Sports Drug Agency for their continuing financial support of the AGAL sports drug testing division.

REFERENCES

1 H. Ehrsson, *Anal. Chem.*, 46 (1974) 922.
2 F. C. Churchill and D. N. Ku, *J. Chromatogr.*, 189 (1980) 375.
3 S. Floberg, K. Landbeck and B. Lindstrom, *J. Chromatogr.*, 182 (1980) 63.
4 D. J. Hoffman, *J. Pharm. Sci.*, 68 (1979) 445.
5 N. A. Adinoife and M. K. L. Bicking, *J. Chromatogr. Sci.*, 23 (1985) 348.
6 M. Ervik and K. Gustavii, Anal. Chem., 46 (1974) 39.
7 B. Lindstrom and M. Molander, *J. Chromatogr.*, 101 (1974) 219.

8 B. Beermann, M. Groschinsky-Grind and B. Lindstrom, *Eur. J. Clin. Pharmacol.*, 10 (1976) 293.
9 W. Schneider and P. H. Degen, *J. Chromatogr.*, 217 (1981) 263.
10 B. Lindstrom, M. Molander and M. Groschinsky, *J. Chromatogr.*, 114 (1975) 459.
11 J. E. Graas and E. Watson, *J. Anal. Toxicol.*, 2 (1978) 80.
12 P. Hartvig and C. Fagerlund, *J. Chromatogr.*, 140 (1977) 170.
13 P. Stenberg, T. E. Jonsson, B. Nilsson and F. Wollheim, *J. Chromatogr.*, 177 (1979) 145.
14 P. Hartvig, C. Fagerlund and B. M. Emanuelsson, *J. Chromatogr.*, 228 (1982) 340.
15 A. M. Lisi, G. J. Trout and R. Kazlauskas, *J. Chromatogr.*, 563 (1991) 257.
16 D. R. Matthews, W. D. Shults and J. A. Dean, *Anal. Lett.*, 6 (1973) 513.
17 J. MacGee and K. G. Allen, *Anal. Chem.*, 42 (1970) 1672.