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Screening for diuretics in human urine by gas chromatography–mass spectrometry with derivatisation by direct extractive alkylation

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ABSTRACT

A rapid, sensitive and reliable gas chromatographic–mass spectrometric (GC–MS) screening procedure for diuretics in human urine has been developed. The procedure uses derivatisation by extractive methylation directly from the urine. The suitability of a number of phase transfer reagents and solvents were studied for the detection of sixteen diuretics. The results obtained indicate that the screening procedure employing tetrahexylammonium hydrogensulphate at pH 12 with methyl iodide in toluene at room temperature was the most effective. This method gives selectivity and sensitivities down to 0.03–0.1 μg/ml and provides a substrate suitable for GC–MS confirmation without further manipulation. The application of the method is demonstrated by the screening of urine for bumetanide, ethacrynic acid, acetazolamide, chlorothiazide and hydrochlorothiazide.

INTRODUCTION

Diuretics and probenecid belong to a class of drugs which have been banned from international sport by the Medical Commission of the International Olympic Committee (IOC). As a result IOC-accredited doping laboratories must be capable of screening human urine for these drugs.

The principal groups of diuretics are the thiazides typified by chlorothiazide (Fig. 1A) and hydrochlorothiazide (Fig. 1B), the carbonic anhydrase inhibitors such as acetazolamide (Fig. 1C) and dichlorphenamide (Fig. 1D), those which have a carboxylic acid group such as bumetanide (Fig. 1E) and furosemide (Fig. 1F) and diuretics such as chlorthalidone (Fig. 1G) and metolazone (Fig. 1H) which do not have a thiadiazine ring, but resemble the thiazides in mechanism and site of action.

The therapeutic value of diuretics lies in their ability to counter the retention of salt and water by the body and as a result are used for the treatment of congestive heart failure, hepatic, renal and pulmonary oedemas. The abuse of diuretics can result in abnormally low concentrations of potassium in the blood which can in turn contribute to variations from the normal rhythm of the heart resulting in

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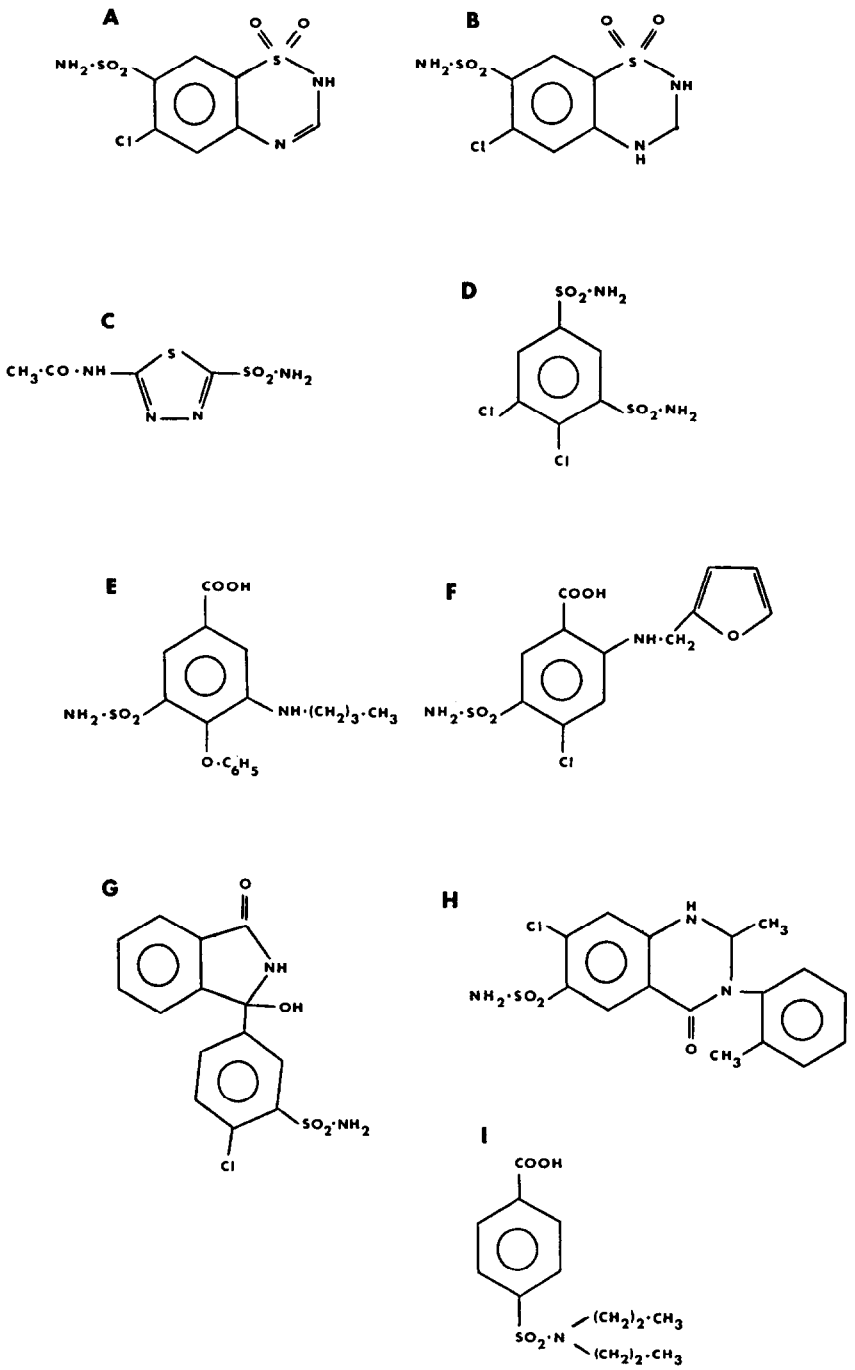

Fig. 1. Structures of some diuretics. A = chlorothiazide; B = hydrochlorothiazide; C = acetazolamide; D = dichlorphenamide; E = bumetanide; F = furosemide; G = chlorthalidone; H = metolazone; I = probenecid.

sudden death. In sport they can be abused by athletes in order to achieve rapid weight loss, to mask the presence of other drugs by diluting the urine and to counteract the negative effect of water retention during anabolic steriod abuse. In the case of diuretics which are carbonic anhydrase inhibitors, they can be used to raise the pH of the urine in order to reduce the excretion of some stimulants.

Probenecid (Fig. 1I) increases the urinary excretion of uric acid and hence has therapeutic value for the treament of gout. It also decreases the excretion of some drugs and hence prolongs their plasma concentrations. This aspect of probenecid enables it to be used as a masking agent by athletes in order to reduce the excretion of anabolic steroids.

Numerous methods have been reported for the determination of these drugs in human plasma, blood and urine by high-performance liquid chromatography (HPLC) with spectral detection [1–19].

Cooper *et al.* [20] outlined an HPLC screening procedure with diode array UV detection for the determination of 23 diuretics while Fullinfaw *et al.* [21] outlined a screening procedure for 12 diuretics. The screening of probenecid and diuretics in urine using HPLC with spectral detection suffers from the disadvantage that it is not sufficiently specific and hence is limited in the qualitative information which is required for a positive confirmation. Gas chromatography with mass selective detection (GC–MSD) is a rapid and specific means of screening for the presence of drugs in human urine. The mass spectral fragmentation pattern provides the essential qualitative information for a positive confirmation in doping analysis.

The polar nature of probenecid and most diuretics makes it impossible to determine these drugs by direct GC without derivatisation. Several authors have reported on the determination of some of these drugs in biological fluids using GC after derivatisation by extractive alkylation [22–30]. Methylation of acids by extractive alkylation was first used by Brandstrom and Junggren [31] in preparative organic chemistry. The anion of the acid is extracted as an ion pair from the aqueous phase into an aprotic and polar organic phase which solvates cations strongly and leaves the anions unsolvated and highly reactive especially towards alkylating agents.

This present paper describes the application of extractive alkylation followed by GC–MSD for the rapid screening of diuretics in human urine by performing the derivatisation during the extraction step. Using this technique, recovery problems which characterise liquid extraction techniques prior to derivatisation are minimised and sample preparation time is greatly reduced.

EXPERIMEMTAL

Instrumentation

A Hewlett-Packard Model 5890 Series II gas chromatograph connected to a Model 5970B electron-impact (EI) mass-selective detector via a capillary direct interface was used. All chromatograms were obtained in the selected-ion mode

(SIM) using the screening ions listed in Table III with a 25 m × 0.22 mm I.D. fused-silica cross-linked methyl silicone capillary column coated to a thickness of 0.1 μm (HP Ultra 1). The carrier gas was hydrogen at a flow-rate of 1 ml/min and the split ratio was 10:1. The temperatures were: 250°C for the injector, 280°C for the detector, initial column temperature 130°C and final column temperature 320°C. The column temperature was increased at a rate of 40°C/min. The column remained at its final temperature for 3 min.

Reagents and chemicals

All reagents were of analytical grade. The reference drug samples and tablets were kindly supplied by the manufacturers: chlorthalidone, cyclopenthiazide (Ciba-Geigy, Sydney, Australia); hydroflumethiazide, bendroflumethiazide (E. R. Squibb & Sons, Noble Park, Australia); metolazone, canrenone, spironolactone (Searle, Sydney, Australia); acetazolamide, methazolamide, quinethazone (Lederle, Sydney, Australia); ethacrynic acid, probenecid, dichlorphenamide (Merck Sharp and Dome, Sydney, Australia); furosemide hydrochlorothiazide (Fisons Pharmaceuticals, Sydney, Australia); bumetanide (Astra Pharmaceuticals, Sydney, Australia); chlorothiazide (Fawns & McAllan, Croyden, Australia); mefruside (Bayer, Sydney, Australia). Nanograde toluene and dichloromethane were obtained from Mallinckrodt Australia (Clayton, Australia), silver sulphate from BDH (Melbourne, Australia) and methyl iodide from May and Baker (Melbourne, Australia).

Stock solutions

A 100-ppm stock solution of each drug was prepared by dissolving 5.0 mg of the drug in 50 ml of methanol. The solutions were stored at 4°C.

Internal standard solution

A 100-ppm mefruside solution was prepared by dissolving 10.0 mg of the drug in 100 ml of 0.1 *M* sodium hydroxide.

Phase transfer reagents

Solutions of 0.2 *M* tetrabutylammonium hydrogensulphate (TBA^+), 0.2 *M* tetrapentylammonium hydroxide (TPA^+) and 0.2 *M* tetrahexylammonium hydrogensulphate (THA^+) were prepared by dissolving 1.4, 1.3 and 1.8 g, respectively, of the salts in 20 ml of 1.0 *M* sodium hydroxide.

Methyl iodide solution

A 0.5 *M* solution of methyl iodide was prepared by dissolving 7 g of the liquid in 100 ml of dichloromethane or toluene.

Derivatization

To six 10-ml Quickfit B14 test tubes (Mowbray Glass, Gosford, Australia) were added 25 μl of 100 μg/ml stock solution of furosemide, mefruside (internal standard) and probenecid along with 50 μl of 100 μg/ml stock solutions of acetazolamide, bumetanide, bendroflumethiazide, canrenone, cyclopenthiazide, chlorthalidone, chlorothiazide, dichlorphenamide, ethacrynic acid, hydrochlorothiazide, hydroflumethiazide, metolazone and quinethazone. The methanol was evaporated to dryness under a nitrogen purge and the residue dissolved in 1 ml of 0.5 *M* sodium hydroxide to give a final concentration of 2.5–5.0 μg/ml for the individual drugs. To each of the test tubes were added 100 μl of 0.2 *M* phase transfer reagent and 5 ml of 0.5 *M* methyl iodide solution as outlined in Table I. Each test tube was stoppered and agitated on a Clements Laboratory Suspension mixer (Phoenix Scientific, Sydney, Australia) for 20 min at room temperature, after which they were centrifuged for 10 min at 1500 *g*. A reaction time of at least 20 min was found to be necessary in order to achieve maximum recovery of diuretics. The organic fractions containing the methyl derivatives were transferred to a second series of six 10-ml test tubes. For the trials in which dichloromethane was used the solvent was evaporated to dryness under a stream of nitrogen and the residue was reconstituted in 5 ml of toluene. The six toluene fractions were washed with 3 ml of saturated silver sulphate solution and centrifuged for 10 min at 1500 *g* after which the organic fractions were transferred to six 10-ml glass centrifuge tubes and evaporated to dryness under a stream of nitrogen.

The residues were redissolved in 100 μl of toluene before 1 μl aliquots were injected into the GC–MSD system. The mass/charge (*m/z*) ions that were used in SIM mode in order to detect the methyl derivatives of the diuretics are outlined in Table III.

Urinary excretion studies

In order to demonstrate the screening procedure, single doses of bumetanide, ethacrynic acid, hydrochlorothiazide, chlorothiazide and acetazolamide were administered to healthy volunteers whose urine samples were collected at regular intervals. Direct extractive alkylation was carried out on the urine samples by the addition of 5 ml of 0.5 *M* methyl iodide solution in toluene to 5-ml aliquots of urine that were made alkaline with 100 μl of 6 *M* sodium hydroxide and to which were added 150 μl of 0.2 *M* THA^+ and 25 μl of 100 μg/ml mefruside (internal standard). Fleuren *et al.* [27] reported that after administration of mefruside to humans less than 1% of the dose was found in the urine as unchanged drug. As a result this diuretic was chosen as an internal standard for this work. If an athlete had taken mefruside then this would become apparent by the presence of the metabolite 5-oxomefruside in the urine.

Detection limits

Representative detection limits were established with chlorothiazide, acetazol-

amide, bumetanide, ethacrynic acid and hydrochlorothiazide by spiking 5 ml of drug-free urine to a concentration of 0.2 μg/ml using 10 μl of 100 μg/ml stock solutions and subjecting to direct extractive alkylation as outlined above.

RESULTS AND DISCUSSION

Derivatisation

Extractive alkylation was carried out on sixteen diuretics using six different combinations of organic phases and phase transfer reagents (Table I). Figs. 2 and 3 show the chromatograms obtained for the six trials.

In this system trimethylchlorothiazide coeluted with tetramethylhydroflumethiazide, tetramethylhydrochlorothiazide coeluted with tetramethylquinethazone and although methazolamide was not included in this study it is known that dimethylmethazolamide coelutes with trimethylacetazolamide. As the coeluting diuretics can be detected by their differing mass spectra, their identification presents little difficulty.

In the trials that involved the use of methyl iodide dissolved in toluene as the organic phase (Fig. 2), the number of diuretics that were successfully methylated increased with decreasing hydrophilic character of the phase transfer reagent (Table II). In the trials where methyl iodide dissolved in dichloromethane was used as the organic phase (Fig. 3), all the diuretics with the exception of chlorothiazide were readily methylated irrespective of which ion-pair reagent was used. Fagerlund *et al.* [29] attempted to methylate chlorothiazide using methyl iodide and dichloromethane as the organic phase and found that several unidentified peaks were produced. We found that chlorothiazide formed the expected trimethyl derivative only when the methylation reaction was carried out using THA^+ phase transfer reagent with methyl iodide and toluene as the organic

TABLE I

SOLVENT–PHASE TRANSFER REAGENT COMBINATIONS USED FOR THE METHYLATION REACTION

Trial	Solvent	Phase transfer reagent[a]
1	0.5 *M* CH_3I in toluene	0.2 *M* TBA^+
2	0.5 *M* CH_3I in dichloromethane	0.2 *M* TBA^+
3	0.5 *M* CH_3I in toluene	0.2 *M* TPA^+
4	0.5 *M* CH_3I in dichloromethane	0.2 *M* TPA^+
5	0.5 *M* CH_3I in toluene	0.2 *M* THA^+
6	0.5 *M* CH_3I in dichloromethane	0.2 *M* THA^+

[a] TBA^+ = Tetrabutylammonium ion; TPA^+ = Tetrapentylammonium ion; THA^+ = Tetrahexylammonium ion.

phase at room temperature. The full-scan EI mass spectrum of the trimethyl derivative of chlorothiazide is presented in Fig. 4.

For acetazolamide two derivatives were observed. Fagerlund *et al.* [29] also reported two derivatives and identified them as dimethyl- and trimethylacetazolamide. They also presented the mass spectral data for the trimethyl derivative but not for the dimethyl derivative. We were able to observe the reported trimethyl derivative but not the dimethyl derivative. In theory acetazolamide may be mono-, di- or trimethylated. The uncertainty relating to the degree of methylation of acetazolamide was resolved by obtaining the positive-ion chemical ionisation mass spectra of the two derivatives using a Finnigan 4500 mass spectrometer and

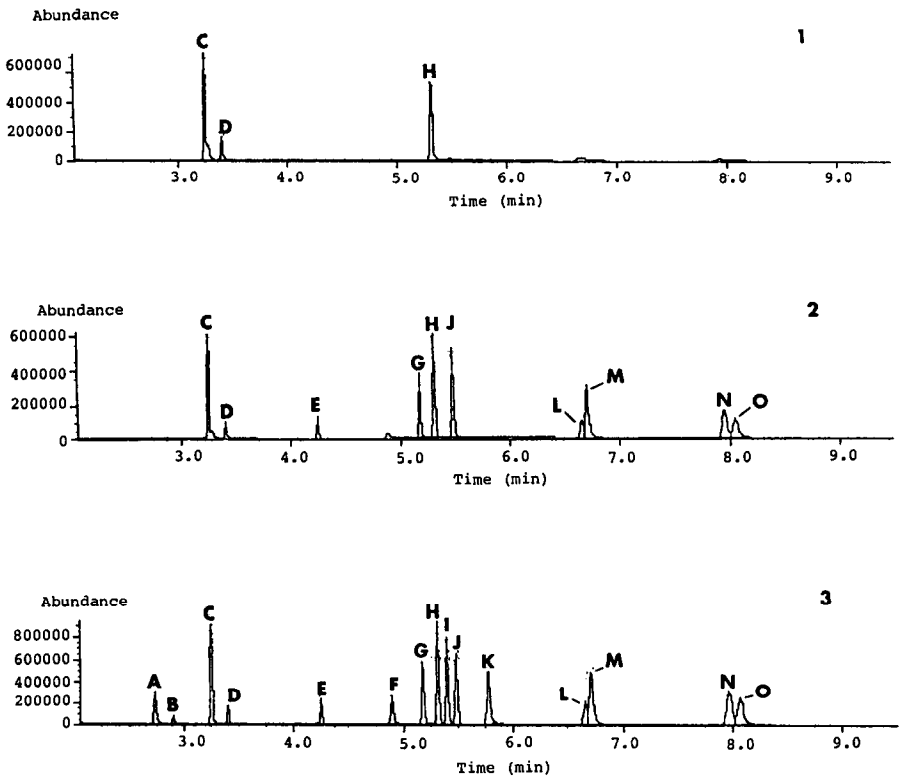

Fig. 2. SIM GC–MS profiles obtained for the methylation reaction carried out with methyl iodide dissolved in toluene as the organic phase. (1) Tetrabutylammonium counter ion; (2) tetrapentylammonium counter ion; (3) tetrahexylammonium counter ion. Peaks: A = monomethylated acetazolamide; B = trimethylated acetazolamide; C = monomethylated probenecid; D = monomethylated ethacrynic acid; E = tetramethylated dichlorphenamide; F = trimethylated chlorothiazide and tetramethylated hydroflumethiazide; G = trimethylated furosemide; H = dimethylated mefruside (internal standard); I = tetramethylated chlorthalidone; J = trimethylated bumetanide; K = tetramethylated hydrochlorothiazide and tetramethylated quinethazone; L = canrenone; M = tetramethylated bendroflumethiazide; N = trimethylated metolazone; O = tetramethylated cyclopenthiazide.

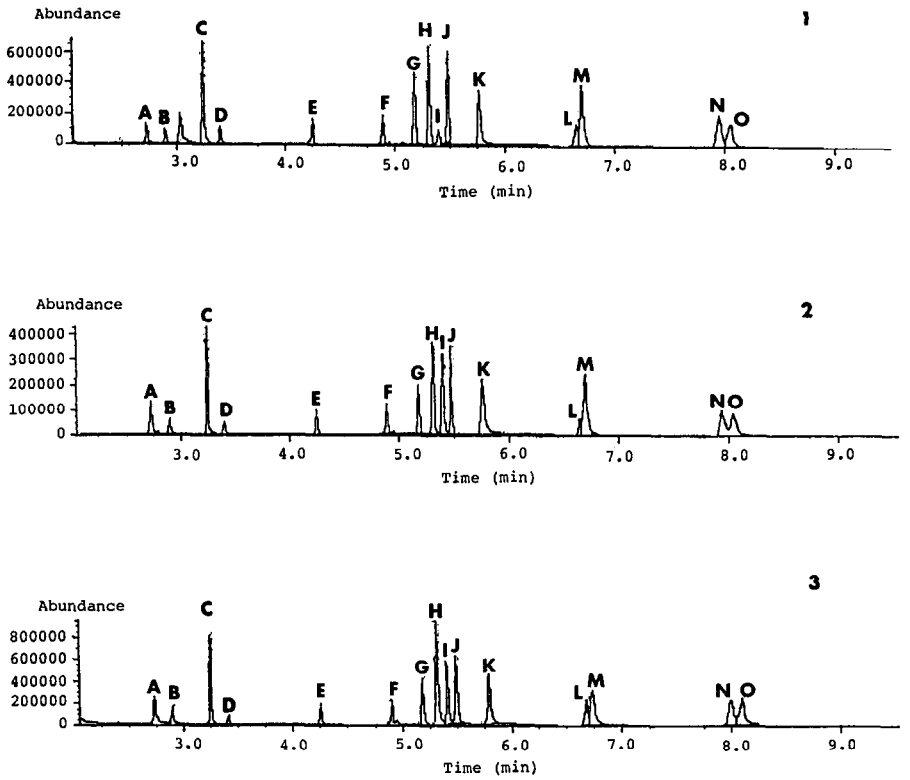

Fig. 3. SIM GC–MS profiles obtained for the methylation reaction carried out with methyl iodide dissolved in dichloromethane as the organic phase. (1) Tetrabutylammonium counter ion; (2) tetrapentylammonium counter ion; (3) tetrahexylammonium counter ion. Peaks: A = monomethylated acetazolamide; B = trimethylated acetazolamide; C = monomethylated probenecid; D = monomethylated ethacrynic acid; E = tetramethylated dichlorphenamide; F = tetramethylated hydroflumethiazide; G = trimethylated furosemide; H = dimethylated mefruside (internal standard); I = tetramethylated chlorthalidone; J = trimethylated bumetanide; K = tetramethylated hydrochlorothiazide and tetramethylated quinethazone; L = canrenone; M = tetramethylated bendroflumethiazide; N = trimethylated metolazone; O = tetramethylated cyclopenthiazide.

methane reagent gas. Since the ion molecule reactions used in chemical ionisation techniques are low in energy compared to the EI process, abundant quasi-molecular ions are often observed. From the observed quasi-molecular ions (M + 1) we were able to establish that the derivatives were mono- and trimethylacetazolamide. The full-scan EI mass spectra of the two derivatives are presented in Fig. 5.

Table III shows the retention time (t_R), relative retention time (RRT) and the ions used for screening and confirming in SIM mode along with their relative abundance (RA) for eighteen diuretics.

TABLE II

METHYLATION OF DIURETICS IN TOLUENE USING TBA^+, TPA^+ AND THA^+ PHASE TRANSFER REAGENTS

TBA^+	TPA^+	THA^+
Ethacrynic acid	Bumetanide	Acetazolamide
Mefruside	Bendroflumethiazide	Bumetanide
Probenecid	Canrenone	Bendroflumethiazide
	Cyclopenthiazide	Canrenone
	Dichlorphenamide	Cyclopenthiazide
	Ethacrynic acid	Chlorthalidone
	Furosemide	Chlorothiazide
	Mefruside	Dichlorphenamide
	Metolazone	Ethacrynic acid
	Probenecid	Furosemide
		Hydrochlorothiazide
		Hydroflumethiazide
		Mefruside
		Metolazone
		Probenecid
		Quinethazone

Urinary excretion studies

For routine screening, derivatisation by direct methylation of the diuretics in urine using THA^+ ion and methyl iodide dissolved in toluene as the organic phase was found to be the most advantageous.

Firstly the largest number of diuretics could be methylated using this combination and secondly the use of toluene instead of dichloromethane as the solvent enabled the elimination of one evaporation step resulting in reduced sample preparation time.

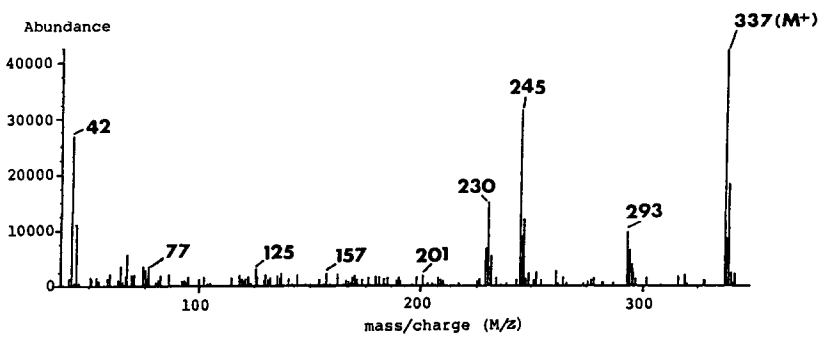

Fig. 4. Electron-impact (70 eV) mass spectrum of trimethylated chlorothiazide.

TABLE III

RETENTION TIMES (t_R), RELATIVE RETENTION TIMES (RRT), PARENT (M^+), SCREENING AND CONFIRMING IONS WITH RELATIVE ABUNDANCE (RA) FOR THE METHYL DERIVATIVES OF EIGHTEEN DIURETICS

Methyl derivative	t_R (min)	RRT	M^+ (RA)	Screening ions (RA)	Confirming ions (RA)
Monomethylacetazolamide	2.69	0.57	236(20)	70(100),44(74),236(20)	70(100),44(74),129(31),236(20),237(4)
Trimethylacetazolamide	2.90	0.61	264(31)	249(100),264(31),108(20)	249(100),264(31),43(28),83(20),108(20),265(4)
Dimethylmethazolamide	2.90	0.61	264(30)	249(100),264(30),108(20)	249(100),264(30),43(20),108(15),83(13),265(4)
Monomethylprobenecid	3.23	0.68	299(3)	270(100),199(30),135(25)	270(100),199(30),135(25),228(4),299(3)
Monomethylethacrynic acid	3.33	0.70	316(13)	261(100),263(64),243(47), 316(13)	261(100),263(64),243(47),45(45),245(30),316(13), 318(9),317(4),315(2),319(2)
Tetramethyldichlorphenamide	3.94	0.83	360(8)	44(100),253(35),255(24), 360(8)	44(100),253(35),255(24),108(16),360(8),362(7), 316(3),361(1),363(−[a])
Trimethylchlorothiazide	4.34	0.92	337(100)	337(100),245(75),339(44), 230(36)	337(100),245(75),42(64),339(44),230(36),293(23), 338(19),340(7)
Tetramethylhydroflumethiazide	4.38	0.92	387(100)	387(100),344(50),252(32)	387(100),344(50),322(38),236(36),252(32),172(25), 388(18)
Trimethylfurosemide	4.65	0.98	372(20)	81(100),372(20),374(9)	81(100),372(20),96(13),374(9),339(7),373(6), 375(2)
Dimethylmefruside	4.74	1.00	410(−[a])	85(100)	85(100)
Tetramethylchlorthalidone	4.81	1.02	394(−[a])	363(100),287(59),365(41)	363(100),287(59),365(41),176(29),364(23),289(21), 220(9),366(9),394(−[a]),396(−[a])
Trimethylbumetanide	4.90	1.03	406(82)	254(100),406(82),363(78)	254(100),406(82),363(78),318(49),77(49),196(29), 407(18)
Tetramethylhydrochlorothiazide	4.99	1.05	353(100)	353(100),310(93),355(43), 218(41)	353(100),310(93),288(46),355(43),312(43),218(41), 202(41),354(20),352(17),290(17),356(7)
Tetramethylquinethazone	5.05	1.06	345(4)	316(100),208(48),318(39)	316(100),208(48),318(39),42(33),173(22),317(17), 345(4),346(−[a]),347(−[a])
Dimethyl-5-oxomefruside	5.14	1.08	381(−[a])	99(100),325(17)	99(100),325(17),327(8),218(8),282(7),326(2), 328(−[a]),381(−[a]),382(−[a]),383(−[a])
Canrenone	5.57	1.18		340(100),267(93),107(32)	340(100),267(93),107(32),91(28),341(22),325(20)
Tetramethylbendroflumethiazide	5.62	1.19	477(−[a])	386(100),278(25),42(10)	386(100),278(25),42(10),91(4),145(3),458(−[a]), 477(−[a]),478(−[a])
Trimethylmetolazone	6.23	1.32	407(5)	392(100),394(38),284(14)	392(100),394(38),393(21),284(14),118(9),395(9), 407(5),409(4),408(3)
Tetramethylcyclopenthiazide	6.33	1.34	435(1)	352(100),354(44),244(10)	352(100),354(44),244(10),42(6),145(3),435(1), 436(−[a]),437(−[a])

[a] Relative abundance less than 1%.

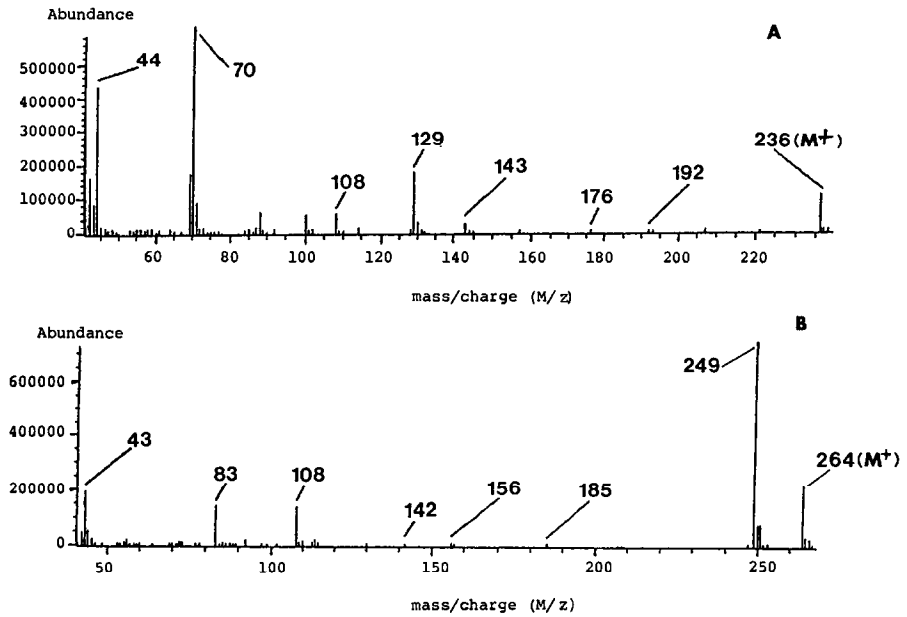

Fig. 5. Electron-impact (70 eV) mass spectrum of (A) monomethylated and (B) trimethylated acetazolamide.

Unidentified urinary endogenous material was found to interfere by inhibiting the methylation reaction in urine. The inhibition is thought to occur by the endogenous material binding the phase transfer reagent and making it unavailable to the analyte for the formation of ion pairs. In order to overcome the observed interference and to ensure the methylation of chlorthalidone, bendroflumethiazide and quinethazone, 150 μl of 0.2 *M* THA^+ were added to each urine sample.

The screening procedure was applied to urine samples collected from subjects that ingested either 1 mg of bumetanide, 50 mg of ethacrynic acid, 50 mg of hydrochlorothiazide, 500 mg of chlorothiazide or 250 mg of acetazolamide. For urine samples containing bumetanide and ethacrynic acid, 5-ml aliquots were used for screening and for samples containing hydrochlorothiazide, chlorothiazide and acetazolamide, 1 ml aliquots diluted to 5 ml with blank urine were used. Figs. 6–10 illustrate typical chromatograms that were obtained in the SIM mode using the screening ions listed in Table III after direct extractive methylation from urine showing the presence of bumetanide, ethacrynic acid, hydrochlorothiazide, chlorothiazide and acetazolamide.

All the diuretics are readily identifiable and free from any interferences from urinary endogenous material.

Although the results for five diuretics were presented in this paper, the same method has been successfully used to screen for probenecid and other diuretics such as dichlorphenamide, hydroflumethiazide, furosemide, 5-oxomefruside (me-

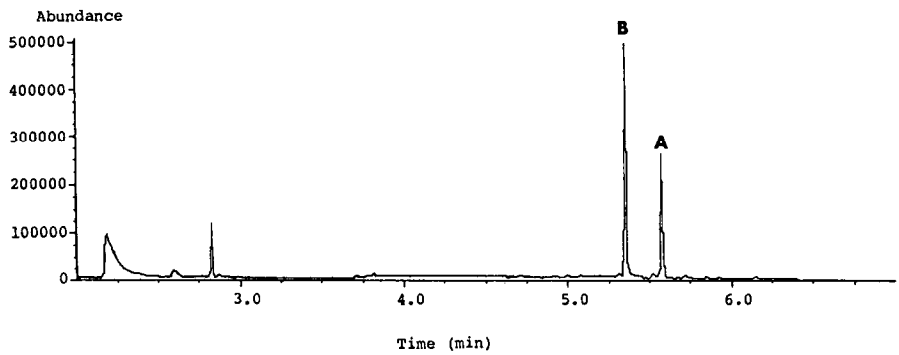

Fig. 6. SIM GC–MS profile obtained from human urine 4.2 h after the oral administration of a 1-mg dose of bumetanide. Peaks: A = trimethylated bumetanide; B = dimethylated mefruside (internal standard).

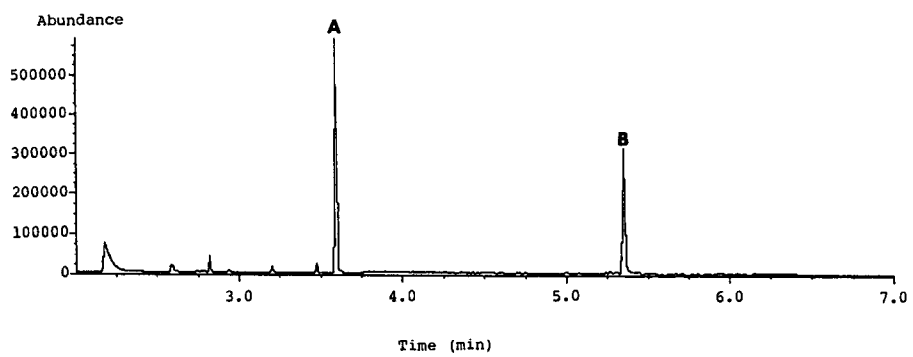

Fig. 7. SIM GC–MS profile obtained from human urine 1.6 h after the oral administration of a 50-mg dose of ethacrynic acid. Peaks: A = monomethylated ethacrynic acid; B = dimethylated mefruside (internal standard).

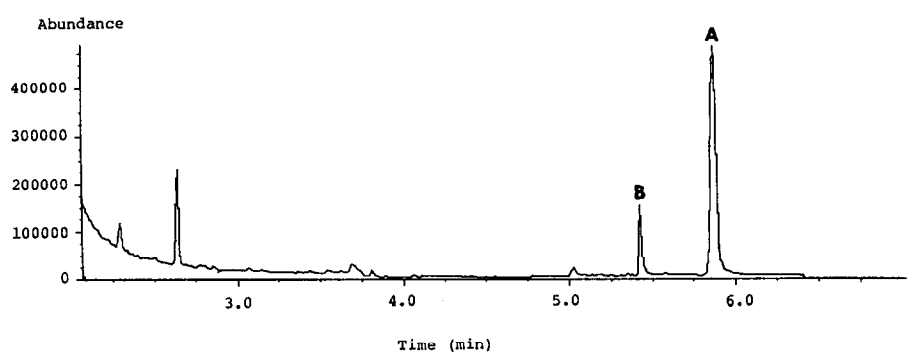

Fig. 8. SIM GC–MS profile obtained from human urine 3.2 h after the oral administration of a 50-mg dose of hydrochlorothiazide. Peaks: A = tetramethylated hydrochlorothiazide; B = dimethylated mefruside (internal standard).

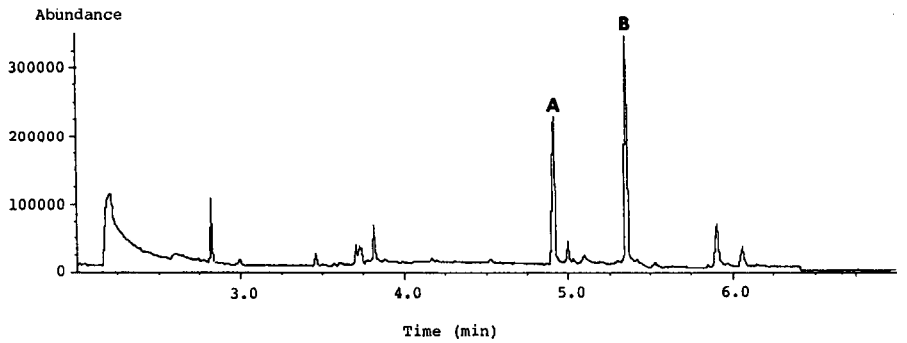

Fig. 9. SIM GC–MS profile obtained from human urine 4.5 h after the oral administration of a 500-mg dose of chlorothiazide. Peaks: A = trimethylated chlorothiazide; B = dimethylated mefruside (internal standard).

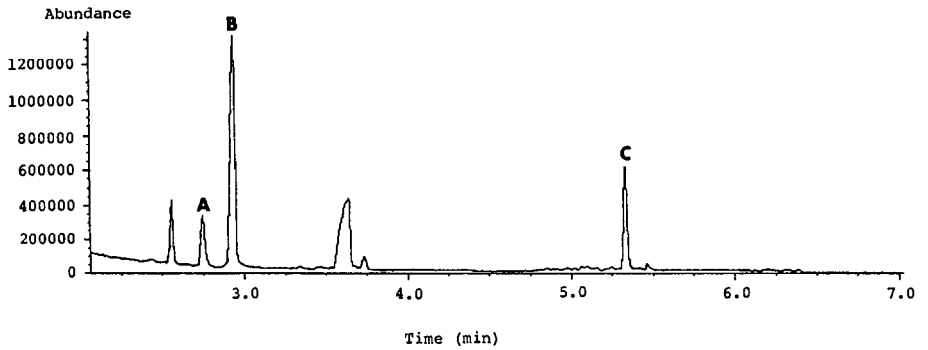

Fig. 10. SIM GC–MS profile obtained from human urine 4.0 h after the oral administration of a 250-mg dose of acetazolamide. Peaks: A = monomethylated acetazolamide; B = trimethylated acetazolamide; C = dimethylated mefruside (internal standard).

tabolite of mefruside), chlorthalidone, quinethazone, canrenone (metabolite of spironolactone), bendroflumethiazide, metolazone, methazolamide, cyclopenthiazide, clopamide, ozolinone (metabolite of etozolin) and piretanide.

Detection limit

The detection limit was defined as the concentration of diuretic that gave a signal-to-noise ratio of 3. The limits of detection for the five diuretics that were examined ranged from 0.03 to 0.10 μg/ml.

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