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Comparison of derivatization procedures for the determination of diuretics in urine by gas chromatography–mass spectrometry

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Abstract

Three different GC–MS screening procedures, which use different ways of derivatization (methylation), are compared. In the first one, derivatization with iodomethane in acetone and a previous solid–liquid extraction is used; the second one is based on an extractive alkylation method using iodomethane in toluene (liquid–liquid extraction); and the last one is flash methylation by pyrolysis of tetraalkylammonium salts in the injector of the gas chromatograph using trimethylanilinium as the derivatization agent. The speed of the extraction, reproducibility and accuracy have been compared for 20 diuretics including the ones most often used in sports, such as bumetanide, ethacrynic acid, acetazolamide, dichlorphenamide, furosemide, hydroflumethiazide, hydrochlorothiazide and chlorthalidone; they have also been applied to the two uricosuric agents probenecid and benzbromarone.

1. Introduction

Diuretics are therapeutic agents used in the treatment of edema and hypertension resulting from cardiac or renal failure [1].

Uricosuric agents increase urinary excretion of uric acid, and hence they are effective compounds for the treatment of gout.

Diuretics and uricosuric agents are on the doping list of pharmaceutical forbidden substances indicated by the Medical Commission of the International Olympics Committee (IOC) [2].

As diuretics are drugs used to increase the volume of urine excreted by the kidneys, they are employed as doping substances first to reduce the body weight in sports with weight categories, and secondly so as not to detect other doping agents properly by reducing their concentrations in urine.

Probenecid, an uricosuric agent, is what athletes use as masking substance to reduce urinary excretion of anabolic steroids.

Diuretics are usually classified according to their pharmacological properties into four different groups: carbonic anhydrase inhibitors (such as acetazolamide and dichlorphenamide), thiazide (i.e. bendroflumethiazide, chlorothiazide and hydrochlorothiazide) and thiazide type (e.g. clopamide and chlorthalidone), loop (such as bumetanide, ethacrynic acid and furosemide), and potassium-sparing diuretics (i.e. triamterene) [3].

The detection and determination of these drugs in biological fluids is quite complex specially due to their variety of chemical structures (Fig. 1).

There are several methods available for the

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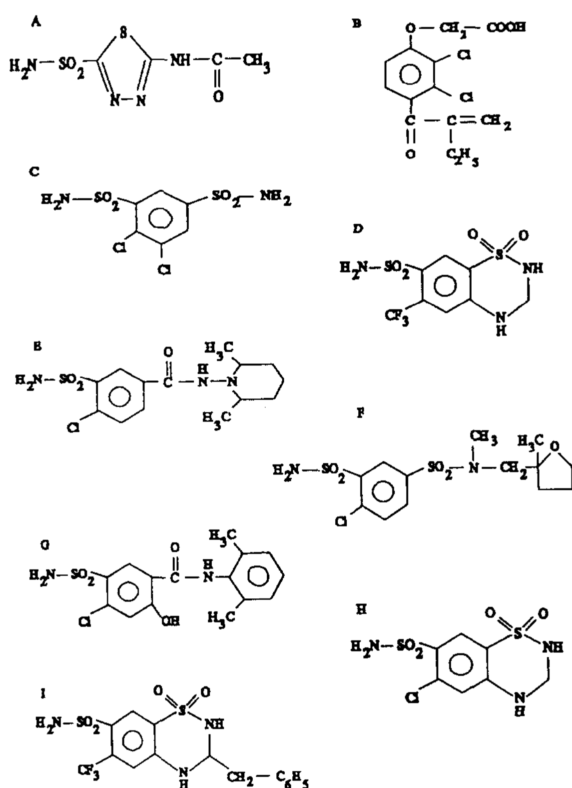

Fig. 1. Structures of diuretics. A = Acetazolamide; B = ethacrynic acid; C = dichlorphenamide; D = hydroflumethiazide; E = clopamide; F = mefruside (I.S.); G = xipamide; H = hydrochlorothiazide; I = bendroflumethizide.

determination of individual diuretics by HPLC with UV detection [4–10] and with fluorescence detection [11–14]. Screening procedures by HPLC with UV detection [15–17] and with mass spectrometry (MS) detection [18,19], have also been reported.

Gas chromatography (GC) coupled with MS is a rapid and specific technique for the determination and identification of these drugs in urine [20,21].

The polar nature of probenecid and most diuretics makes the determination of these drugs by direct GC impossible. For this reason previous derivatization is needed; such derivatization is based on methylation of the sulfonamide group and other groups containing O- or N-bonded hydrogen atoms of the diuretics.

This work describes a comparison among three different GC–MS screening procedures which use different ways of methylation, for the determination and identification of 20 diuretics and 2 uricosuric agents in spiked urine. In the first one the methyl derivatives are obtained using iodomethane in acetone in an alkaline medium [22,23], together with previous solid–liquid extraction. The second one is based on an extractive alkylation method using iodomethane in toluene and tetrahexylammonium hydrogensulphate as the phase transfer reagent, in a liquid–liquid extraction [24–26]. The last method is flash methylation by pyrolysis of tetraalkylammonium salts in the injector of the gas chromatograph using trimethylanilinium (TMA) as the derivatization agent as well as a previous solid–liquid extraction [27–29].

Positive urine samples taken from athletes are also analysed using the three derivatization procedures and are also compared.

2. Experimental

2.1. Reagents and chemicals

All reagents were of analytical grade. The reference drug samples acetazolamide, althiazide, bendroflumethiazide, benzbromarone, bumetanide, chlorothiazide, clopamide, chlorthalidone, dichlorphenamide, ethacrynic acid, furosemide, hydrochlorothiazide, hydroflumethiazide, indapamide, methyclothiazide, piretanide, probenecid, triamterene, trichlormethiazide and xipamide were from Sigma (St. Louis, MO, USA); polythiazide from Pfizer (Brussels, Belgium); mefruside was kindly provided by Bayer (Leverkusen, Germany). Methanol and acetone were obtained from Scharlau (Barcelona, Spain); nanograde toluene, silver sulphate, potassium carbonate, iodomethane and sodium hydroxide from Merck (Darmstadt, Germany); Amberlite XAD-2 and tetrahexylammonium hydrogensulphate from Serva (Heidelberg, Germany); and trimethylanilinium hydroxide from Regis (Morton Grove, IL, USA).

Table 1
Diuretics: number of methyl groups, retention times (t_R), k' values and characteristic ions with their relative abundances

Diuretic	No. of methyl groups	t_R (min)	k'	Characteristic ions, m/z (relative abundance, %)
Acetazolamide	3	3.62	0.684	249 (100), 43 (63), 108 (42), 83 (39), 264 (24)
Probenecid	1	3.90	0.814	270 (100), 135 (72), 199 (58), 271 (15), 104 (14)
Ethacrynic acid	1	4.02	0.870	261 (100), 263 (64), 243 (45), 45 (30), 316 (8)
Dichlorphenamide	4	5.15	1.39	44 (100), 253 (78), 255 (50), 108 (39), 360 (6)
Benzbromarone	1	6.29	1.92	278 (100), 438 (71), 173 (71), 440 (38), 439 (22)
Hydroflumethiazide	4	6.30	1.93	387 (100), 236 (56), 215 (54), 344 (50), 252 (46)
Chlorothiazide	3	6.55	2.05	44 (100), 248 (71), 275 (46), 169 (28), 277 (22)
Furosemide	3	6.95	2.23	81 (100), 372 (22), 96 (10), 339 (5)
Clopamide	2	6.97	2.24	111 (100), 112 (56), 127 (50), 55 (16), 139 (4)
Mefruside (I.S.)	2	7.43	2.46	85 (100)
Chlorthalidone	4	7.67	2.57	287 (100), 363 (68), 176 (63), 255 (56), 289 (32)
Bumethanide	3	7.71	2.59	406 (100), 363 (97), 254 (96), 318 (62), 196 (23)
Piretanide	3	8.40	2.91	295 (100), 296 (26), 404 (24), 266 (19), 297 (12)
Xipamide	3	8.72	3.06	276 (100), 277 (35), 168 (28), 396 (12), 233 (8)
Hydrochlorothiazide	4	9.01	3.19	353 (100), 310 (94), 218 (62), 202 (62), 288 (46)
Indapamide	3	9.01	3.19	161 (100), 132 (40), 131 (16), 407 (10)
Triamterene	6	9.15	3.26	336 (100), 322 (64), 169 (25), 309 (20), 338 (18)
Mefruside metabolite ([a])	2	9.80	3.56	99 (100), 325 (55), 327 (21), 218 (14),
Methyclothiazide	3	9.90	3.60	352 (100), 354 (51), 244 (12), 246 (4)
Trichlormethiazide	4	10.72	3.99	352 (100), 354 (45), 244 (15), 42 (15)
Polythiazide	3	11.01	4.12	352 (100), 354 (45), 244 (22), 42 (17), 246 (8)
Bendroflumethiazide	4	12.00	4.58	386 (100), 278 (25), 42 (16), 387 (15), 388 (12)
Althiazide	4	13.20	5.14	352 (100), 354 (39), 244 (22), 42 (18), 145 (11)

[a] 5-Oxomefruside.

2.2. Stock solutions

Stock solutions were prepared in methanol at a concentration of 100 μg/ml. The solutions were sealed and refrigerated at 4°C until use.

2.3. Internal standard solution

Mefruside was used as the internal standard (I.S.) and was also dissolved in methanol to 100 μg/ml.

2.4. Instrumentation

A Hewlett-Packard (HP, Palo Alto, CA, USA) Model 5890 Series II gas chromatograph connected to a Model 5971 A electron-impact (EI) mass-selective detector via a capillary direct interface was used. All chromatograms were obtained in the selected-ion mode (SIM). An HP fused-silica capillary column (25 m × 0.20 mm I.D., cross-linked 5% phenylmethylsilicone, film thickness 0.33 μm) was coupled to the ion source. The carrier gas was helium at a flow-rate of 1 ml/min and the split ratio was 10:1. The temperatures were: 280°C for the injector, 300°C for the detector, initial column temperature 230°C and final column temperature 320°C. The column temperature was increased at a rate of 35°C/min.

2.5. Analytical procedure

Procedure 1

To 5 ml of urine 10 μl of I.S. solution (100 μg/ml) was added and then the urine was passed through a Pasteur pipette (230 mm × 7 mm) containing a 20-mm plug of Amberlite XAD-2 resin. The resin was washed with 5 ml of deion-

Table 2
Comparison of the three derivatization procedures

Pharmacological properties	Diuretic	Derivatization (%)[a]		
		Iodomethane in acetone	Extractive methylation	Flash methylation
Loop	Bumetanide	51	62	100
	Ethacrynic acid	83	100	4
	Furosemide	28	19	100
	Piretanide	40	56	100
Thiazide	Althiazide	100	0	0
	Bendroflumethiazide	100	83	22
	Chlorothiazide	100	2	2
	Hydrochlorothiazide	89	100	85
	Hydroflumethiazide	57	62	100
	Methyclothiazide	100	19	0
	Polythiazide	99	100	0
	Trichlormethiazide	100	0	0
Thiazide-type	Clopamide	5	14	100
	Chlorthalidone	6	4	100
	Indapamide	35	24	100
	5-Oxomefruside[b]	100	5	70
	Xipamide	23	1	100
Carbonic anhydrase inhibitors	Acetazolamide	100	25	87
	Dichlorphenamide	98	100	57
Potassium sparing	Triamterene	100	0	50
Uricosuric agents	Benzbromarone	64	0	100
	Probenecid	48	60	100

[a] Percentages related to the highest signal (assigned the value 100) obtained for each diuretic.
[b] Metabolite of mefruside in physiological urine.

ized water, eluted with 2 ml of methanol and evaporated to dryness.

The residue was dissolved in 200 μl of acetone; then 20 μl of iodomethane and 100 mg of potassium carbonate were added. This solution was heated in a heating block at 60°C for 3 h and 4 μl of the derivative extract were injected into the GC–MS system.

Procedure 2

To 5 ml of urine in a 15-ml glass tube 10 μl of I.S. solution (100 μg/ml), 100 μl of 10 *M* sodium hydroxide, 150 μl of 0.2 *M* tetrahexylammonium and 5 ml of 0.5 *M* iodomethane solution in toluene were added. Then the urine was shaken for 20 min, centrifuged at 1500 *g* for 10 min and the organic fraction containing the methyl derivatives was transferred to another tube. The toluene fraction was washed with 3 ml of saturated silver sulphate solution, centrifuged at 1500 *g* for 10 min, decanted to another tube and taken it to dryness. The residue was dissolved in 100 μl of toluene and 4 μl of the solution were injected into the GC–MS system.

Table 3
Analytical accuracy and reproducibility of the three derivatization procedures in spiked urine ($n = 5$)

Diuretic	Iodomethane in acetone			Extractive methylation			Flash methylation		
	$\bar{x}$	S.D.	R.S.D. (%)	$\bar{x}$	S.D.	R.S.D. (%)	$\bar{x}$	S.D.	R.S.D. (%)
Acetazolamide	32.0	3.19	9.97	7.98	0.870	10.9	27.8	2.95	10.6
Althiazide	12.1	1.19	9.83	0	–	–	0	–	–
Bendroflumethiazide	335	23.1	6.89	278	25.1	9.03	73.7	7.52	10.2
Benzbromarone	23.0	1.52	6.61	0	–	–	35.9	2.98	8.30
Bumetanide	69.4	9.49	13.7	84.3	9.39	11.1	136	12.2	8.97
Clopamide	39.6	2.58	8.16	88.5	8.31	9.39	632	35.1	5.55
Chlorthalidone	29.3	2.32	7.92	19.5	2.12	10.8	488	30.9	6.33
Chlorothiazide	37.0	3.21	8.67	0.629	0.0963	15.3	0.740	0.101	13.7
Dichlorphenamide	141	17.8	12.6	144	12.5	8.68	82.1	7.61	9.27
Ethacrynic acid	184	21.0	11.4	222	21.1	9.50	8.88	0.834	9.39
Furosemide	47.9	4.89	10.2	32.5	2.49	7.66	171	12.4	7.25
Hydrochlorothiazide	150	20.1	13.4	168	15.1	8.98	143	12.9	9.02
Hydroflumethiazide	211	24.9	11.8	229	21.0	9.17	370	13.8	3.73
Indapamide	15.1	1.41	9.34	10.4	1.10	10.6	43.2	3.72	8.61
Methyclothiazide	24.8	2.60	10.5	4.74	0.491	10.3	0	–	–
Piretanide	126	11.1	8.81	176	14.2	8.07	315	22.3	7.08
Polythiazide	98.5	9.81	9.96	99.6	9.11	9.14	0	–	–
Probenecid	1006	118.9	11.82	1258	109.0	8.664	2096	162.0	7.729
Triamterene	7.19	0.891	12.4	0	–	–	359	0.482	13.4
Trichlormethiazide	0.772	0.102	14.1	0	–	–	0	–	–
Xipamide	71.3	9.30	13.0	2.48	0.293	11.8	310	24.3	7.84

The mean has been determined by the peak area ratio of the diuretic base peak to the internal standard base peak. In all cases 0.5 μg/ml of each diuretic and the internal standard have been added. Means and standard deviations have been multiplied by 1000.

Procedure 3

To 5 ml of urine 10 μl of I.S. solution (100 μg/ml) was added and then the urine was passed through a Pasteur pipette (230 mm × 7 mm) containing a 20 mm plug of Amberlite XAD-2 resin. The resin was washed with 5 ml of deionized water, eluted with 2 ml of methanol and evaporated to dryness.

The residue was dissolved in 25 μl of TMA and 1 μl of the solution was injected into the GC–MS system.

3. Results

Table 1 reports the retention time (t_R), the k' values and the characteristic ions used for the identification of diuretics methyl derivatives, as well as their relative abundances. There are several derivatives which coelute such as monomethylbenzbromarone/tetramethylhydroflumethiazide, trimethylfurosemide/dimethylclopamide, tetramethylchlorthalidone / trimethylbumetanide and tetramethylhydrochlorothiazide/trimethylindapamide.

As the diuretics which coelute show different mass spectra, their identification is possible in every case and there are no interferences from urinary endogenous material.

The results of the three different derivatization procedures described above for 20 diuretics and 2 uricosuric agents are reported in Table 2. The values in Table 2 are expressed as a percentage and related to the highest signal obtained for each diuretic. All the diuretics have been derivatized using procedure 1, but with TMA

derivatization of some diuretics (mainly thiazides) has not been achieved. Using procedure 2 some diuretics, such as triamterene and benzbromarone, have not been derivatized. In general, with this method the sensitivities found are lower than those obtained with the other methods.

In some cases no derivatization occurred with any of the three compared methods, even when concentrations in spiked urine have been increased above the limits usually found in physiological samples.

Representative chromatograms of spiked urine with a mixture of nine diuretics obtained by the three derivatization procedures are shown in Fig. 2. All the diuretics have been added at a concentration of 0.3 μg/ml except for acetazolamide and xipamide whose concentrations were 3 μg/ml.

As after administration of mefruside to humans, less than 1% of the dose has been found in the urine as an unchanged drug, it has been chosen as the internal standard in this work [30]. After ingestion of mefruside, the 5-oxomefruside appears in urine as the main metabolite.

The precision and accuracy have been measured using urine samples spiked at a concentration of 0.5 μg/ml. The samples have been extracted by the three derivatization procedures and subjected to GC–MS. The statistical results are shown in Table 3.

Figs. 3 and 4 illustrate two positive urine

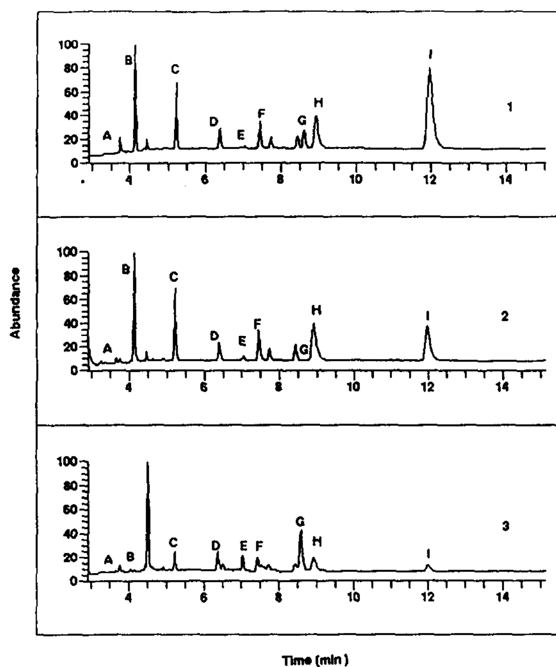

Fig. 2. Representative total ion chromatograms in spiked urine with a mixture of: A = trimethylated acetazolamide; B = monomethylated ethacrynic acid; C = tetramethylated dichlorphenamide; D = tetramethylated hydroflumethiazide; E = dimethylated clopamide; F = dimethylated mefruside (I.S.); G = trimethylated xipamide; H = tetramethylated hydrochlorothiazide; I = tetramethylated bendroflumethiazide. Chromatograms 1, 2 and 3 have been obtained using the three derivatization procedures: (1) iodomethane in acetone, (2) extractive methylation and (3) flash methylation.

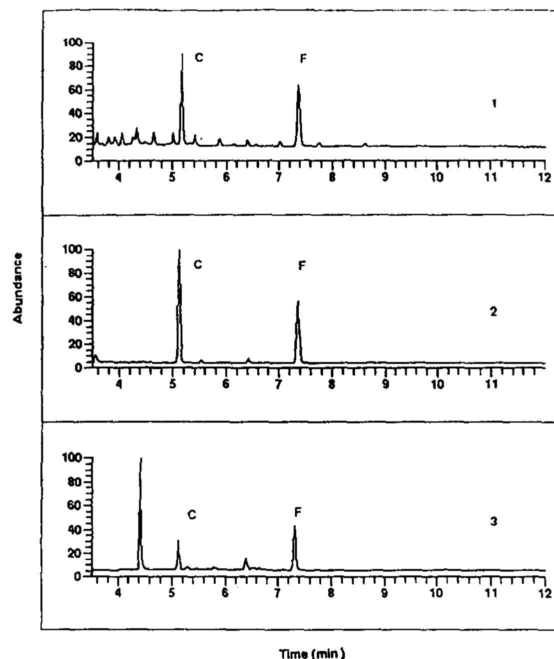

Fig. 3. Total ion chromatograms obtained from a positive urine sample containing dichlorphenamide using the three derivatization procedures: (1) iodomethane in acetone, (2) extractive methylation and (3) flah methylation. Peaks: C = tetramethylated dichlorphenamide; F = dimethylated mefruside (I.S.).

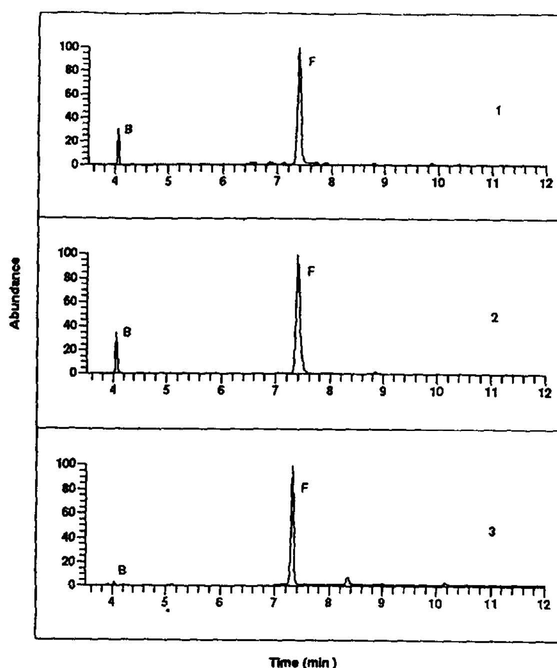

Fig. 4. Total ion chromatograms obtained from a positive urine sample containing ethacrynic acid using the three derivatization procedures: (1) iodomethane in acetone, (2) extractive methylation and (3) flash methylation. Peaks: B = monomethylated ethacrynic acid; F = dimethylated mefruside (I.S.).

samples containing dichlorphenamide and ethacrynic acid, respectively. In both cases, the signals correspond to the data in Table 2.

4. Conclusions

Screening procedures in doping analysis have to include as many drugs as possible. For this reason we propose for diuretics the derivatization using iodomethane in acetone, in a routine screening procedure. Nevertheless, the flash methylation is a reliable alternative method for a rapid confirmation in every case except for althiazide, trichlormethiazide, polithiazide and methyclothiazide. For these last two diuretics, an extractive methylation will be an alternative method; this method is not faster than flash methylation but it is faster than iodomethane in acetone.

The derivatization procedure using TMA has been applied since January 1993 to urine samples taken from athletes together with physiological samples as alternative confirmation. Over this period we have analysed about 2000 samples using iodomethane in acetone, and in more than 200 cases the flash methylation has been carried out for possible diuretics signals.

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