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Approach to the analysis of diuretics and masking agents by high-performance liquid chromatography–mass spectrometry in doping control

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ABSTRACT

The application of thermospray and plasmaspray high-performance liquid chromatography–mass spectrometry to the analysis of diuretics and probenecid has been investigated. The latter method gave better ionization efficiency than the former, and its response was optimized by altering the solvent composition: best results were obtained with water–methanol–acetonitrile–trifluoroacetic acid. Using different proportions of these solvents, three isocratic systems were developed to separate the compounds under study. The principal characteristic of plasmaspray positive-ion mass spectra was a protonated molecular ion and very little fragmentation was evident. In the negative ionization mode, the plasmaspray method gave mass spectra showing more fragmentation, which resulted in additional structural information. The ability of trifluoroacetic acid to form negative cluster ions precluded its use as a mobile phase component. The minimum detectable amounts determined by the analysis in the positive-ion mode was compound-dependent, but generally *ca.* 10–150 ng. In many cases the compounds could be detected in urine extracts.

INTRODUCTION

Diuretics and probenecid have been included in the list of banned compounds since the 1988 Olympic Winter and Summer Games. Diuretics are misused to reduce the body weight in sports with weight categories, and to increase the urine flow leading to a reduction in the concentration of other doping agents [1–4]. Also, the use of "carbonic anhydrase inhibitor" diuretics leads to an alkaline urine that reduce the excretion of basic drug metabolites. Probenecid is an uricosuric agent misused in sport to reduce the excretion of anabolic steroids [5].

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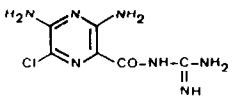
TABLE I

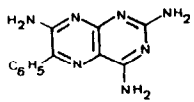
CLASSIFICATION OF DIURETICS AND PROBENECID ACCORDING TO THEIR ACID-BASE PROPERTIES

Basic compounds

1 Amiloride

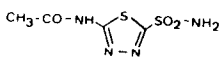


2 Triamterene

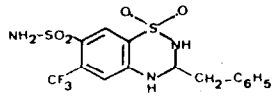


Weak acid compounds

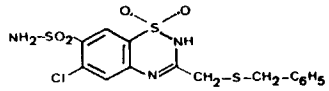
4 Acetazolamide



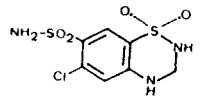
5 Bendroflumethiazide



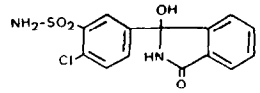
6 Benzthiazide



7 Hydrochlorothiazide

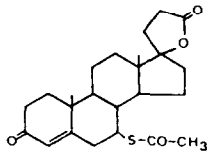


8 Chlortalidone



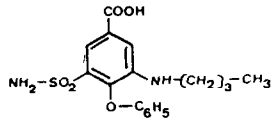
Neutral compounds

3 Spironolactone

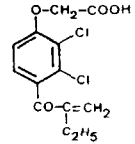


Strong acid compounds

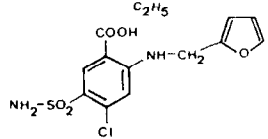
9 Bumetanide



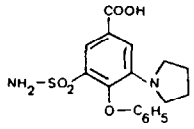
10 Etacrynic acid



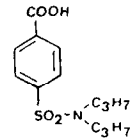
11 Furosemide



12 Piretanide



13 Probenecid



Diuretics are a pharmacological group that includes compounds with a wide variety of molecular structures and important differences in physico-chemical properties. For these reasons, the screening procedure for diuretics and probenecid is currently high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [6–9]. The confirmation of positive cases is usually performed by gas chromatography(GC)–mass spectrometry (MS) after methylation of the urine extracts [6,7].

During the past five years the introduction of HPLC–MS interfaces [10–23] has led to consider the analysis of diuretics and masking agents by this technique. The investigations described here report the analysis of these compounds by HPLC–MS using a commercial thermospray (TSP)/plasmaspray (PSP) source. The results have been analysed keeping in mind the major requirements of the doping control laboratories, especially specifity, sensitivity and turn-around time.

EXPERIMENTAL

Chemical and reagents

The compounds studied (Table I) were obtained from the respective pharmaceutical laboratories. Water used in the HPLC eluent was Milli-Q grade (Millipore Ibérica, Barcelona, Spain). Methanol, acetonitrile and ethyl acetate were of HPLC grade. Other reagents were of reagent-grade quality.

Liquid chromatographic system

Liquid chromatographic analysis was carried out with a Gilson pump Model 302 (Gilson International, Villiers-le-Bel, France) linked to a Gilson manometer Model 802C and a Rheodyne injector. An HP1040A detection system (Hewlett-Packard, Palo Alto, CA, U.S.A.) was used to optimize the chromatographic conditions.

The solvent mixtures used are listed in Table II. All were tested in the PSP mode, but only solvent A was used with TSP ionization. Flow-injection analysis was used with most of the solvents. Chromatographic analysis was done with solvents D, E and F, on a reversed-phase column (SGE 100 GL-4 $C_{18}P$, 10 × 0.4 cm I.D., 5 μm particle size; Scientific Glass Engineering, Victoria, Australia). The flow-rate was 0.8 or 1 ml/min.

Mass spectrometric system

Analyses were performed using a ZAB2-SEQ mass spectrometer with a PDP-11-250J data system (VG Analytical, Manchester, U.K.). This instrument was fitted with a TSP/PSP source directly coupled to the liquid chromatograph.

The source temperature was maintained at 250°C, and the probe temperature was between 240 and 260°C. Other operating parameters of the interface and mass spectrometer (plasma voltage, ion repeller, ion energy, Y focus, multiplier, etc.) were optimized daily to obtain the maximum response for the solvent ions.

TABLE II

COMPOSITION OF THE SOLVENTS USED IN THE HPLC–MS ANALYSIS

Values are % (v/v).

	Solvent						
	A	B	C	D	E	F	G
Deionized water	–	–	60	99	85	70	50
Ammonium acetate[a]	85	50	–	–	–	–	–
Acetonitrile	15	50	12	0.3	4.5	15	50
Methanol	–	–	28	0.7	10.5	15	–
TFA	–	–	0.5	0.5	0.5	0.5	–

[a] 0.05 *M* ammonium acetate aqueous solution adjusted to pH 2.1 with trifluoroacetic acid (TFA).

The instrument resolution was *ca.* 1200. Calibration was performed using a mixture of poly(ethylene glycol)s. Both positive- and negative-ion modes were tested.

Data were acquired by scanning the magnet between m/z 200 and 550 to avoid the high background resulting from solvent cluster ions at lower masses. The scan-rate was set to 3 s/decade with an interscan delay of 0.5 s.

Samples

Standard solutions. Compounds were usually handled as methanolic solutions. The minimum detectable quantities (MDQ) were measured by analysis of decreasing amounts of the compounds (1000, 500, 200, 150, 100, 50 and 10 ng) as methanolic solutions in the respective chromatographic system. They have been calculated as the amount required to generate a base-peak signal-to-noise ratio between 5 and 10 in a full-scan mode.

Biological samples. Urine samples were obtained from healthy male volunteers (clinical trial number 88/135 approved by Hospital del Mar Ethical Committee and Dirección General de Farmacia y Productos Sanitarios, Ministry of Health of Spain) after administation of a single therapeutic oral dose. Samples were analysed after administration of the following drugs: amiloride (dose: 5 mg; urine collected from 0 to 24 h after intake); triamterene (25 mg, 0–24 h; 25 mg, 0–8 h); spironolactone (50 mg, 0–24 h); acetazolamide (250 mg, 8–24 h); hydrochlorothiazide (25 mg, 0–24 h); chlortalidone (50 mg, 0–8 h); bumetanide (1 mg, 0–8 h); etacrynic acid (50 mg, 0–2 h); furosemide (50 mg, 0–24 h; 50 mg, 0–8 h); and probenecid (250 mg, 0–24 h). Blank and spiked (0.5 μg/ml) urines were also studied.

Samples were extracted by a procedure already described [24]. Briefly, urine samples (5 ml) were rendered alkaline (pH 9–10) and sodium chloride (2 g) was added. The samples were twice extracted with 6 ml of ethyl acetate, and the

combined organic layers were dried under nitrogen. The residues were then dissolved with 200 μl of acetonitrile–water, and an aliquot (10–20 μl) was analysed by HPLC–PSP-MS.

RESULTS

Mass spectral data

When the solvent contained ammonium acetate, a characteristic TSP mass spectrum showed a protonated molecular ion $[M+H]^+$ and adduct ions such as $[M+NH_4]^+$, $[M+H+CH_3CN]^+$, and $[M+NH_4+CH_3CN]^+$, with no fragmentation. In general, adduct ions were more abundant than $[M+H]^+$ ions, although the reproducibility was low.

PSP mass spectra were similar when either mobile phase was used (with and without ammonium acetate). They were generally characterized by a $[M+H]^+$ ion with little fragmentation. Adduct ions of the $[M+H]^+$ ion with mobile phase components were generated for some compounds, mainly when solvents with ammonium acetate were used. Ions obtained using solvents without ammonium acetate are listed in Table III. The reproducibility of the PSP mass spectra was good, except for compounds **7**, **8** and **11**.

Negative ionization mode mass spectra were also evaluated. Cluster ions from TFA were observed at *m/z* 227 $[CF_3COOH \cdot CF_3COO]^-$ and *m/z* 341

TABLE III

POSITIVE- AND NEGATIVE-ION PLASMASPRAY MASS SPECTRA

Positive- and negative-ion PSP mass spectra of diuretics and probenecid. Italicized values are the base peaks. Solvents: C for positive-ion mode; G for negative-ion mode. For other conditions see text.

Compound	MW	Positive-ion mode		Negative-ion mode	
		$[M+H]^+$	Other	$[M-H]^-$	Other
Amiloride (**1**)	229.04	*230* (*231*[a])		*228*	
Triamterene (**2**)	253.10	*254*		*252*	
Spironolactone (**3**)	416.20		*341*, 382		339, *373*, 381
Acetazolamide (**4**)	221.98	*223*		*221*	
Bendroflumethiazide (**5**)	421.03	*422*	343	420	*279*, 303, 318, 381, 401
Benzthiazide (**6**)	430.98	*432*	353	430	*307*, 331, 340
Hydrochlorothiazide (**7**)	296.97	298	*286*	296	255, 261, 284, *286*
Chlortalidone (**8**)	338.01	339 (340[a])	*321*[a], *323*, 353[a], 364, 382[a]	*337*	320
Bumetanide (**9**)	364.10	*365*	286	*363*	270, 283
Etacrynic acid (**10**)	302.10	*303*		301	*209*, 222, 243, 266
Furosemide (**11**)	330.00	331	*251*	329	*294*
Piretanide (**12**)	362.09	*363*	284	361	268, *286*
Probenecid (**13**)	285.10	*286*		*284*	

[a] Occasionally observed.

$[(CF_3COOH)_2 \cdot CF_3COO]^-$ saturated the detector response, precluding the use of TFA as a mobile phase component. Ions obtained with PSP in negative ionization mode using a solvent without TFA (solvent G) are shown in Table III. In general, negative ion mass spectra were characterized by a deprotonated molecular ion $[M-H]^-$ with more fragmentation than positive-ion spectra.

Comparison between TSP and PSP responses

Using solvent A (containing ammonium acetate), the ratios of the peak areas obtained for the base-peak ion recording using PSP and TSP ionizations were 12 for compound **10** (*m/z* 320, $[M+NH_4]^+$), 19 for compound **9** (*m/z* 365, $[M+H]^+$) and 26 for compound **6** (*m/z* 449, $[M+NH_4]^+$). When the signal-to-noise ratios were considered, the differences were of lower magnitude but still favoured PSP. Additionally, the stability of the response using TSP was found to be more dependent on instrumental parameters, such as the temperature of the vaporizer.

Optimization of PSP response

Signal-to-noise ratios calculated from the base-peak ion recording ranged from 3 to 40 times higher using solvent C (without ammonium acetate and with methanol as the main organic modifier) than with solvent B (with ammonium acetate and acetonitrile), depending on the compound analysed.

Three HPLC isocratic systems were developed to separate diuretics and probenecid using a reversed-phase column and the same components of solvent C

TABLE IV

RETENTION TIMES AND MINIMUM DETECTABLE QUANTITIES

Retention times and minimum detectable quantities (MDQ) obtained by HPLC–PSP-MS analysis. For solvent descriptions see Table II. For other conditions see Experimental.

HPLC eluent	Compound	Retention time (min)	MDQ (ng)
Solvent D	Acetazolamide (**4**)	3.9	< 10
	Hydrochlorothiazide (**7**)	4.3	150
Solvent E	Amiloride (**1**)	2.1	50
	Chlortalidone (**8**)	2.5	100
	Triamterene (**2**)	4.0	50
	Furosemide (**11**)	6.3	500
	Benzthiazide (**6**)	7.2	50
	Bendroflumethiazide (**5**)	9.2	–
Solvent F	Probenecid (**13**)	3.2	50
	Bumetanide (**9**)	3.7	50
	Etacrynic acid (**10**)	4.8	50
	Spironolactone (**3**)	9.4	50

(mobile phases D, E and F). Compounds analysed in each isocratic system and their retention times are listed in Table IV.

Figs. 1–3 compare the HPLC–PSP-MS analysis with HPLC–UV analysis under the same chromatographic conditions. As can be seen, the interface did not significantly degrade the chromatographic resolution, although peaks were broadened.

Sensitivity

In order to estimate the sensitivity of the HPLC–PSP-MS systems, MDQ for diuretics and probenecid were measured in scan mode. The data in Table IV show that the MDQ was compound-dependent and ranged from less than 10 ng for acetazolamide to 500 ng for furosemide. Most of the compounds had a MDQ of *ca.* 50 ng.

Analysis of real urine extracts

Fig. 4 shows the results obtained by HPLC–PSP-MS analysis of a spiked urine (0.5 μg/ml). Urine samples from excretion studies (tested and detected as positive by a routine HPLC–UV system [9,24] were analysed by HPLC–PSP-MS. All were

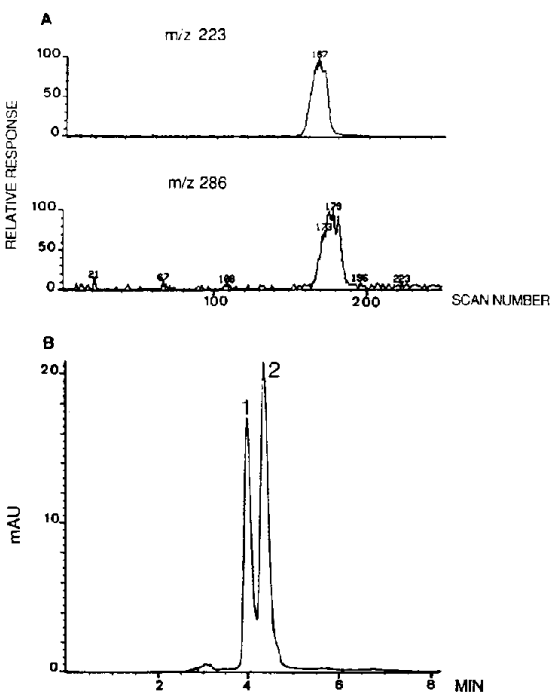


Fig. 1. (A) Recordings of *m/z* 223 for acetazolamide and *m/z* 286 for hydrochlorothiazide obtained by HPLC–PSP-MS analysis. (B) Chromatogram of the same two compounds obtained with UV detection at 270 nm; peaks: 1 = acetazolamide; 2 = hydrochlorothiazide. HPLC eluent, solvent D. For other conditions see Experimental.

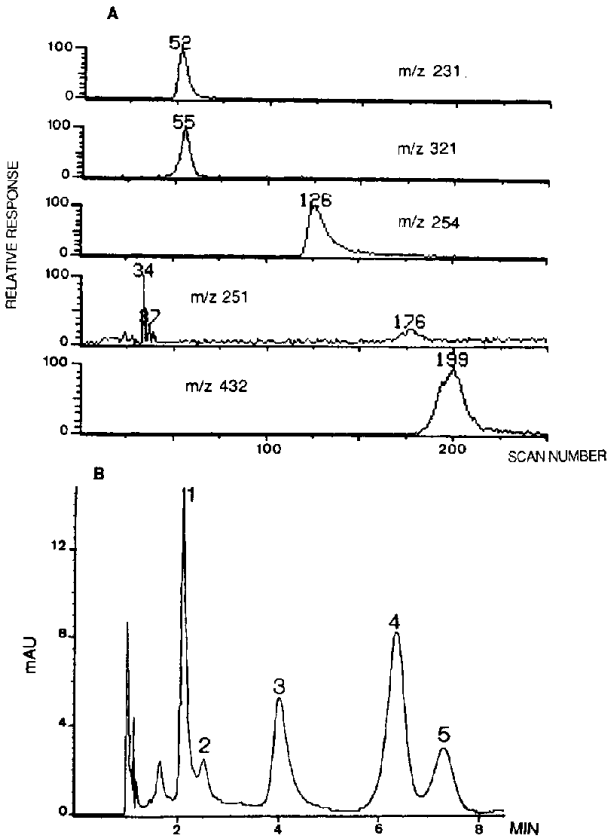


Fig. 2. (A) Recordings of *m/z* 231 for amiloride, *m/z* 321 for chlortalidone, *m/z* 254 for triamterene, *m/z* 251 for furosemide and *m/z* 432 for benzthiazide, obtained by HPLC–PSP-MS analysis. (B) Chromatogram of the same five compounds obtained with UV detection at 270 nm; peaks: 1 = amiloride; 2 = chlortalidone; 3 = triamterene; 4 = furosemide; 5 = benzthiazide. HPLC eluent, solvent E. For other conditions see Experimental.

confirmed as positive, except those obtained after furosemide ingestion. Results of the HPLC–PSP-MS analysis of samples collected after chlortalidone, probenecid and spironolactone intake are presented in Figs. 5–7.

DISCUSSION

HPLC-MS parameters

In the routine HPLC method used to screen for diuretics and probenecid, separation is carried out with gradient elution in a reversed-phase column and a mobile phase containing ammonium acetate and acetonitrile [9,24]. Using an isocratic solvent with the same components (solvent A) PSP and TSP ionizations

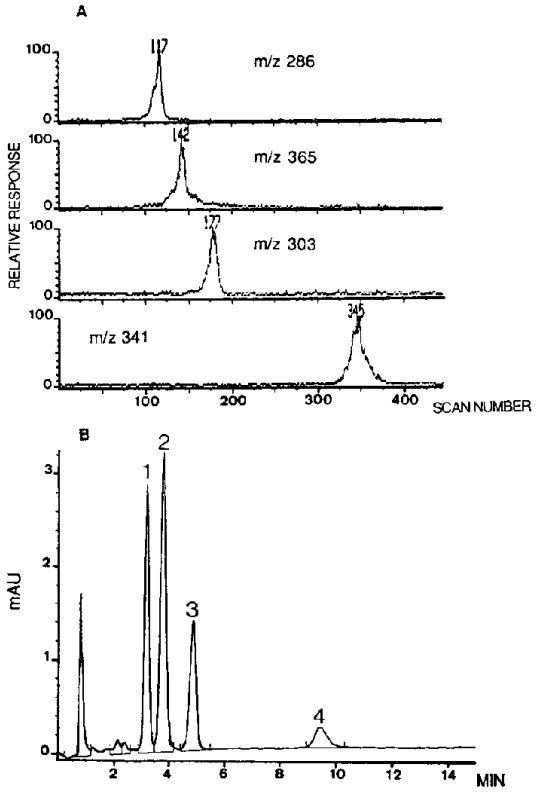


Fig. 3. (A) Recordings of *m/z* 286 for probenecid, *m/z* 365 for bumetanide, *m/z* 303 for etacrynic acid and *m/z* 341 for spironolactone obtained by HPLC–PSP-MS analysis. (B) Chromatogram of the same four compounds obtained at 270 nm; peaks: 1 = probenecid; 2 = bumetanide; 3 = etacrynic acid; 4 = spironolactone. HPLC eluent, solvent F.

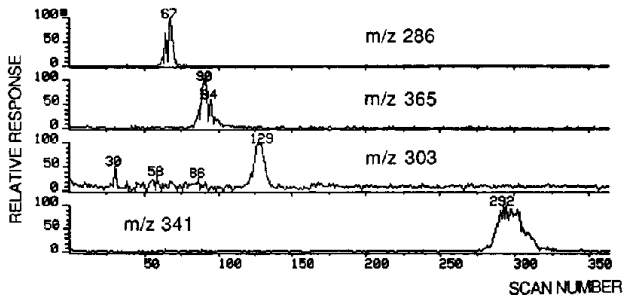


Fig. 4. Recordings of the base-peak ions of probenecid (*m/z* 286), bumetanide (*m/z* 365), etacrynic acid (*m/z* 303) and spironolactone (*m/z* 341) obtained after analysis of a spiked urine (0.5 μg/ml) by HPLC PSP-MS. HPLC eluent, solvent F. Solvent delay, 90 s. For other conditions see Experimental.

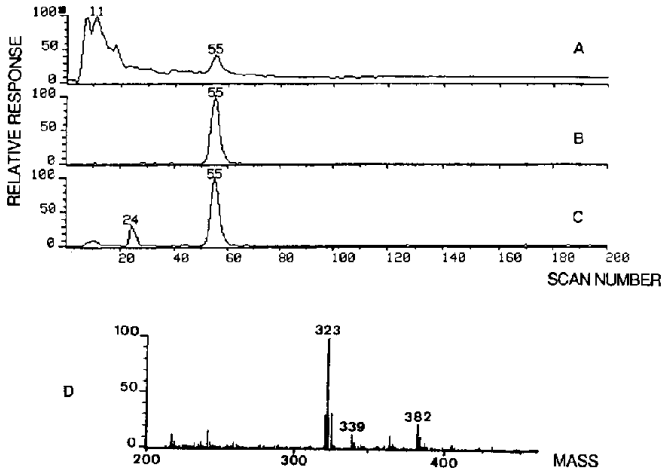


Fig. 5. HPLC–PSP-MS analysis of a urine sample collected after chlortalidone intake. (A) Total ion current recording (m/z 200–550). (B) Recording of m/z 321. (C) Recording of m/z 339. (D) Subtracted mass spectrum of the peak at the retention time of chlortalidone (2.5 min). HPLC eluent, solvent E. Solvent delay, 45 s. For other conditions see Experimental.

were compared. Results indicate that PSP gives a higher ionization efficiency for both the compounds and the background ions than TSP, and less dependence on vaporizer temperature [23].

In order to improve the PSP response another solvent was tested. A buffer is not essential to promote PSP ionization [13,19,22], and earlier experience in our

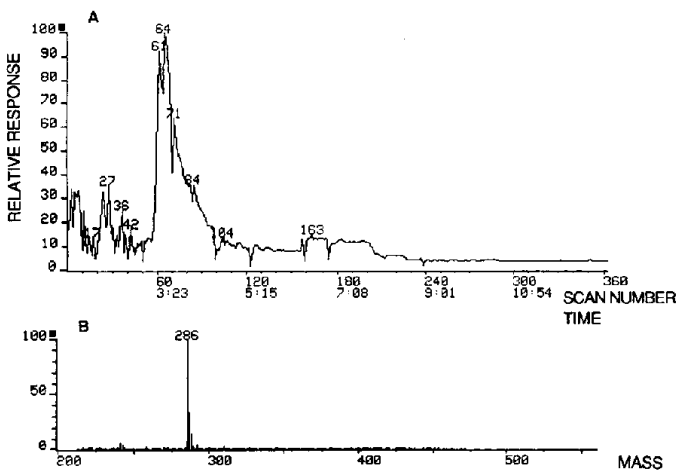


Fig. 6. HPLC–PSP-MS analysis of a urine sample collected after probenecid intake. (A) Total ion current recording (m/z 200–550). (B) Scan 61, corresponding to the peak at the retention time of probenecid (3.2 min). HPLC eluent, solvent F. Solvent delay, 90 s. For other conditions see Experimental.

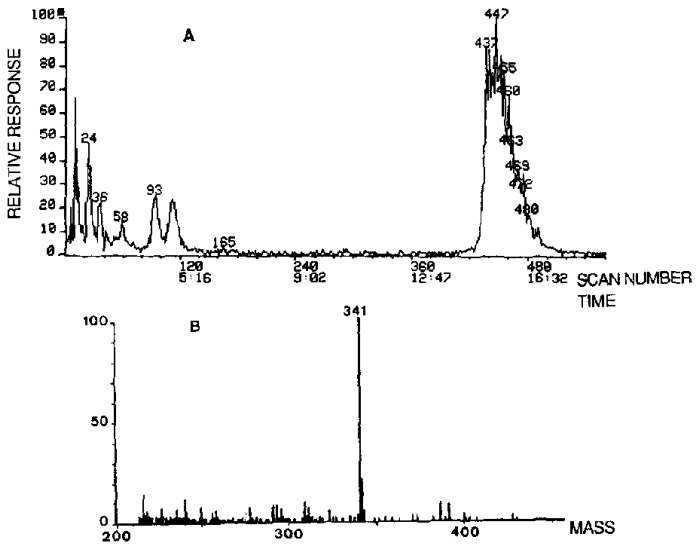


Fig. 7. HPLC–PSP-MS analysis of a urine sample collected after spironolactone intake. (A) Recording of *m/z* 341. (B) Subtracted mass spectrum of the peak at 15.5 min. HPLC eluent, solvent F. Solvent delay, 90 s. For other conditions see Experimental.

group (unpublished results) suggested that the organic modifier can influence the PSP response, as described for TSP ionization [10,12]. Thus, the PSP response using solvent C (without ammonium acetate and with methanol as the main organic modifier) was tested. Acetonitrile and TFA were also required to obtain a suitable chromatographic separation. Signal-to-noise ratios were higher in solvent C than in solvent B for all of the compounds. Since two paramcteres were changed at the same time (ammonium acetate content and organic modifier), more detailed studies are necessary to elucidate which parameter is responsible for the increase in PSP response when using solvent C.

Under our condition, HPLC–PSP-MS using gradient elution was not feasible owing to system instabilities (data not reported), although modification of the vaporizer temperature and other operating parameters during gradient elution might improve the stability [10,21]. In consequence, three HPLC isocratic systems were developed. Under these conditions, the analysis of diuretics and probenecid can be performed with a short run-time (Fig. 1–3), which may be an important factor in antidoping control. However, the number of analyses per day depends on the interface stability, and this is the main limitation of this technique for routine purposes.

Mass spectral data

As PSP positive-ion mass spectra for most of the compounds (Table III) are characterized only by a single important ion, the sensitivity can be increased by

monitoring only this peak, although limited structural information is obtained. Chan and Torok-Both [25] described a similar situation for diuretics with TSP ionization. Scanning below *m/z* 200 might be important to observe fragment ions for some compounds [20], but solvent ions can interfere in this mass range. Also, the fine adjustment of some source and probe parameters for each compound can increase the fragmentation [13].

The base peak is the $[M+H]^+$ ion, except for substances **3**, **7**, **8** and **11**. The spironolactone (**3**) base peak at *m/z* 341 can be explained by loss of the thioacetyl group at position 7; an adduct ion at *m/z* 382, corresponding to the attachment of an acetonitrile molecule, is also observed with a relative abundance lower than 10%. The base peak for furosemide (**11**) at *m/z* 251 is formed by the loss of the sulphonamide group from the $[M+H]^+$ ion. The base peaks for hydrochlorothiazide (**7**) at *m/z* 286 and chlortalidone (**8**) at *m/z* 323 were not identified. The chlortalidone peak at *m/z* 321 could arise from the loss of a water molecule from the $[M+H]^+$ ion. For chlortalidone, adduct ions of the fragments 323 or 321 with mobile phase components are observed at *m/z* 353, $[321+CH_3OH]^+$; *m/z* 364, $[323+CH_3CN]^+$; and *m/z* 382, $[323+CH_3CN+H_2O]^+$. Occasionally, *m/z* 231 and 340 can appear instead of *m/z* 230 and 339 for compounds **1** and **8**, respectively. They might be MH_2^+ ions generated because of different experimental conditions.

For compounds **5**, **6**, **9** and **12**, the ion corresponding to the $[M+H]^+$ ion minus 79 appears with a relative abundance lower than 10%. They could be formed after the loss of the sulphonamide group through a rearrangement of the resulting fragment.

The low reproducibility in some instances is likely related to different operating parameters [13], which are not controlled during the analysis.

In negative ionization mode (Table III), mass spectra showing more fragmentation were obtained. All the compounds but compound **3** show a $[M-H]^-$ ion, which is the base peak for some of them. Bumetanide (**9**) peaks at *m/z* 283 and 270 could be formed by loss of the sulphonamide group and the phenoxy moiety from the $[M-H]^-$ ion, respectively. The piretanide (**12**) peak at *m/z* 268 could also arise from loss of the phenoxy group.

Fragments generated by loss of the halogen atom from the deprotonated molecular ion appear for some compounds: *m/z* 401 for compound **5**; *m/z* 261 for **7**; *m/z* 294 for **11**; and *m/z* 266 for **10**. For compound **10**, *m/z* 243 could result from loss of the acetic moiety.

Therefore, the presence of more fragment ions in negative-ion mass spectra provides additional structural information that is useful for confirmation purposes, but chromatographic systems without TFA, which generate interfering adduct ions [26], must be developed first to separate diuretics and probenecid.

Sensitivity and analysis of urine samples

The detection capability recommended by the Medical Commission of the

International Olympic Committee for diuretics is 0.1 μg/ml [27]. Therefore, an MDQ of 50 ng (Table IV) is sufficient to detect them in urine extracts using an initial sample volume of 5–10 ml, taking into account the extraction recoveries of our procedure [9,24]. Only compounds **7**, **8** and **11** have an MDQ greater than 50 ng. When necessary, selective-ion recording may improve the MDQ for all of the compounds. It is worth noticing that amiloride (**1**) and benzthiazide (**6**), which are difficult to analyse by GC–MS, are easily detected by HPLC–PSP-MS.

A final validation of the usefulness of the HPLC–PSP-MS systems developed was accomplished by analysing urine extracts obtained from excretion studies. All the compounds but furosemide were detected. This result agrees with the MDQ evaluated for this substance. As in HPLC–UV, all the parent compounds were detected by HPLC–PSP-MS except spironolactone (**3**). Spironolactone ingestion (Fig. 7) is detected via canrenone, its urinary metabolite [28]. The mass spectrum of the chromatographic peak observed at a retention time of 15.5 min (higher than that of spironolactone) shows a base peak at m/z 341 which corresponds to the $[M+H]^+$ ion of canrenone (MW 340.20).

In summary, under our conditions HPLC–PSP-MS is sensitive enough to detect most of the compounds in real urine extracts. PSP positive-ion mass spectra of diuretics and probenecid show little fragmentation, which results in a limited structural information. In the negative-ion mode, more fragmentation was obtained. At the present time, the main problem for the application of HPLC–PSP-MS in routine doping analysis is the system stability and the need for improvements in interface performance. Even so, we conclude that HPLC–MS is an analytical tool that must be considered in the area of antidoping control.

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