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Fast screening method for diuretics, probenecid and other compounds of doping interest☆

Rosa Ventura*, Teresa Nadal, Pilar Alcalde, J. Antonio Pascual and Jordi Segura

Departament de Farmacologia i Toxicologia, Institut Municipal d'Investigació Mèdica, Universitat Autònoma de Barcelona, Doctor Aiguader 80, 08003 Barcelona (Spain)

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ABSTRACT

A method to detect the presence of diuretics, probenecid and other agents of doping interest (morazone, mesocarb, caffeine) in urine was developed. The extraction procedure was optimized to obtain adequate recoveries of the compounds under study. The best results were achieved using alkaline extraction (pH 9.5) with ethyl acetate and the salting-out effect (sodium chloride). The extracts were analysed by HPLC with diode-array detection, using a high-speed reversed-phase column and a mobile phase containing acetonitrile and 0.1 *M* ammonium acetate (adjusted to pH 3), with gradient elution. Under these conditions, the time of analysis per sample was 12 min. Extraction recoveries, detection limits and results of the reproducibility assays are presented. The method allows the detection of the compounds in urine during at least 24 h after a therapeutic dose of the drug. Quantitative results obtained from excretion studies are also presented.

INTRODUCTION

Diuretics and probenecid are included in the list of compounds banned in sport by the Medical Commission of the International Olympic Committee since 1988. Hence, screening procedures to detect their presence in urine are required.

Although some diuretics are extensively metabolized (*e.g.*, spironolactone, excreted in urine as canrenone and other metabolic products [1,2]), most of them are excreted unchanged in urine to variable extents [3–11]. Therefore, procedures to screen for diuretics and probenecid in human urine can be designed to detect the suspected parent compounds.

The group of diuretics includes compounds with wide differences in molecular structures and physico-chemical properties. Basic (potassium-sparing diuretics, such as amiloride and triamterene), neutral (aldosterone antagonists, such as canrenone and spironolactone), weakly acidic (carbonic anhydrase inhibitors, such as acetazolamide and diclofenamide; thiazides and related agents, such as chlorthalidone) and strongly acidic compounds (loop diuretics, such as furosemide, bumetanide, piretanide and etacrynic acid) are to be considered, and these compounds cover a wide range of liposolubilities. Consequently, it is difficult to develop a common screening procedure for all these substances. Further, the presence of polar functional groups makes their determination by gas chromatography difficult without prior derivatization, which in most instances is a time-consuming step.

Most of the procedures described in the literature are high-performance liquid chromatographic (HPLC)-based methods. Tisdall *et al.* [12] and Fullinfaw *et al.* [13] reported HPLC methods to

* Corresponding author.

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screen only for acidic diuretics. Other workers [14–16] described HPLC screening methods using two extraction procedures (in acidic and basic conditions) to obtain adequate recoveries for all the compounds. Nevertheless, Cooper *et al.* [15] and Tsai *et al.* [16] reported poor detection limits that would make the detection of some of the compounds difficult after a normal ingestion of the drug. Park *et al.* [17] described a unified HPLC method to screen for diuretics using solid-phase or liquid–liquid extraction procedures.

In this work, the extraction procedure and HPLC separation were optimized for fourteen diuretics and probenecid in order to afford a unified and rapid methodology. The method can also be used to screen for other diuretics and compounds belonging to other groups of banned substances (*e.g.*, stimulants such as mesocarb and morazone) and to determine caffeine.

EXPERIMENTAL

Chemicals and reagents

The compounds studied were supplied by the following pharmaceutical manufacturers: acetazolamide (Laboratorios Wassermann, Barcelona, Spain); amiloride, etacrynic acid and probenecid (Merck, Sharp & Dohme España, Alcalá de Henares, Madrid, Spain); furosemide and piretanide (Hoechst Ibérica, Barcelona, Spain); bendroflumethiazide (Laboratorios Davur, Madrid, Spain); triamterene (Laboratorios Jorba, Madrid, Spain); spironolactone and canrenone (Searle Ibérica, Madrid, Spain); bumetanide (Boehringer Ingelheim, Barcelona, Spain); chlorthalidone and hydrochlorothiazide (Laboratorios Ciba-Geigy, Barcelona, Spain); diclofenamide (Laboratorios Frumtost-Zyma, Barcelona, Spain); and benzthiazide (A.H. Robins, Richmond, VA USA). 7-Propyltheophylline, used as internal standard (I.S.), was synthesized from theophylline (Sigma Química, Alcobendas, Madrid, Spain) and propyl iodide (Merck, Darmstadt, Germany) in alkaline medium.

Water used in the HPLC mobile phase was of Milli-Q grade (Millipore Ibérica, Barcelona, Spain). Methanol, acetonitrile and ethyl acetate were of HPLC grade. Diethyl ether was of analytical-reagent grade and distilled before use. Other reagents were of analytical-reagent grade. Ammonium chloride buffer was prepared by dissolution of 28 g of ammonium chloride in 100 ml of deionized water and adjusting the pH to 9.5 with concentrated ammonium solution.

Standard solutions

Stock solutions were prepared by dissolving the compounds in methanol (1 mg/ml). Triamterene stock solution was prepared with methanol and concentrated hydrochloric acid (1:1, v/v). Working solutions were prepared by 1:10 dilution of the stock solutions with methanol (100 μg/ml). All solutions were stored in the dark at 4°C.

Extraction procedures

Four basic and two acidic procedures were tested (Table I).

Basic extractions

Procedure B1. A 5-ml urine sample was pipetted into a centrifuge tube and 50 μl of the I.S. working solution were added. The urine was made alkaline with 200 μl of ammonium chloride buffer (pH 9.5) and extracted with 6 ml of diethyl ether. After mixing (rocking at 40 movements/min for 20 min) and centrifugation (5 min at 800 *g*), the organic layer was separated and taken to dryness under a stream of nitrogen at 40°C.

Procedure B2. This was identical with B1 but ethyl acetate was used as organic solvent for extraction.

Procedure B3. This was identical with B2, but sodium chloride (2 g) was added before the organic solvent to promote the salting-out effect.

Procedure B4. This was identical with B3, but the alkalinized and salted urines were extracted twice with 6 ml of ethyl acetate. After mixing and centrifugation the combined organic layers were taken to dryness under a stream of nitrogen at 40°C.

TABLE I

EXTRACTION PROCEDURES TESTED

Parameter	Procedure					
	B1	B2	B3	B4	A1	A2
Urine volume (ml)	5	5	5	5	5	5
Extraction pH	pH 9.5	pH 9.5	pH 9.5	pH 9.5	pH 2.5	pH 2.5
Salt	No	No	NaCl (2 g)	NaCl (2 g)	No	No
Extraction solvent (ml)	Diethyl ether (6)	Ethyl acetate (6)	Ethyl acetate (6)	Ethyl acetate (2 × 6)	Diethyl ether (6)	Ethyl acetate (6)

Acidic extractions

Procedure A1. A 5-ml urine sample was pipetted into a centrifuge tube, acidified (pH 2.5) with 100 μl of a 40% phosphoric acid solution and extracted with 6 ml of distilled diethyl ether. After mixing (rocking at 40 movements/min for 20 min) and centrifugation (5 min at 800 *g*), the organic layer was separated and taken to dryness under a stream of nitrogen at 40°C.

Procedure A2. This was identical with A1, but ethyl acetate was used as organic solvent.

In all instances the residues were reconstituted with 200 μl of a mixture of deionized water–acetonitrile (85:15, v/v) and aliquots of 20 μl were analysed by HPLC.

High-performance liquid chromatography

Chromatographic analysis was carried out using a Series II 1090L liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array detector. The instrument was linked to an HP 9000/300 workstation (Hewlett-Packard).

The column was Ultrasphere ODS with 3-μm particle size (7.5 × 0.46 cm I.D.) (Beckman, San Ramon, CA, USA).

The mobile phase was a mixture of 0.1 *M* ammonium acetate solution (adjusted to pH 3 with concentrated phosphoric acid) and acetonitrile with gradient elution. The acetonitrile content (initially 10%) was increased to 15% in 2 min, to 45% in 3 min, to 60% in 3 min, maintained there for 1 min, decreased to the initial conditions in 1 min and stabilized for 2 min before the next injection. The flow-rate was 1 ml/min. The aqueous component of the mobile phase was filtered through a 0.45-μm filter (Reactivos Scharlau, Barcelona, Spain) before use.

The detector was set to monitor the signals at 240, 270, 290, 300, 318 and 350 nm. In addition, the full ultraviolet spectrum between 200 and 400 nm of the detected peaks was stored in the data system and plotted at the end of each run.

Recovery studies

Water samples spiked with 2 μg/ml of the compounds (for acetazolamide, 5 μg/ml) were subjected to the extraction procedures. The extraction recoveries were calculated by comparison of the peak area of each compound obtained from these extracted samples with that of a solution in deionized water–acetonitrile (85:15, v/v) at a concentration equivalent to 100% recovery.

Extraction recoveries were studied statistically using a one-way ANOVA test. Differences between procedures were considered significant when $p < 0.01$.

Excretion studies

Excretion studies involving drug administration and urine collections were performed with healthy male volunteers under the authorization of the Hospital del Mar Ethical Committee (Barcelona, Spain) and the Spanish Ministry of Health (assay No. 88/135). Compounds were administered as single therapeutic doses and urines were collected for a period up to 24 h.

Volunteers were under medical supervision throughout the study.

RESULTS

The extraction recoveries obtained for some of the compounds under study using the different procedures are compared in Fig. 1.

Under acidic conditions (procedures A), amiloride (basic compound) is not extracted. Hydrochlorothiazide and acetazolamide (weakly acidic compounds) are best recovered using ethyl acetate (procedure A2). In contrast, diethyl ether (procedure A1) gives higher yields for chlortalidone, furosemide and bumetanide. The extraction of the other compounds is not significantly affected by the solvent used at acidic pH.

Under basic conditions (procedures B), ethyl acetate (procedure B2) gives significantly higher recoveries than diethyl ether (procedure B1). The salting-out effect (procedure B3) also helps in enhancing the extraction yields for most of the compounds. The use of two consecutive extractions (procedure B4) leads to an increase in the recoveries, which is especially important for strongly acidic compounds. It is worth noting that weakly acidic compounds are better extracted under basic conditions (procedure B), except acetazolamide. The extraction recoveries for the compounds under study obtained using procedure B4, their detection limits and the intra- and inter-day reproducibilities are listed in Table II.

A chromatogram of a methanolic solution of some diuretics and probenecid is presented in Fig. 2. The separation of the compounds is achieved in less than 8.5 min. Additionally to the compounds used to optimize the methodology, retention times of other diuretics and other compounds of doping interest are listed in Table III. Although some of the compounds elute at similar retention times, all of them are easily distinguishable by their UV spectra [18]. Relative standard deviations (R.S.D.s) of the relative retention time (RRT) calculated using fresh mobile phase preparations during 8 days range from 0.24 to 1.94%.

With regard to excretion studies, all the com-

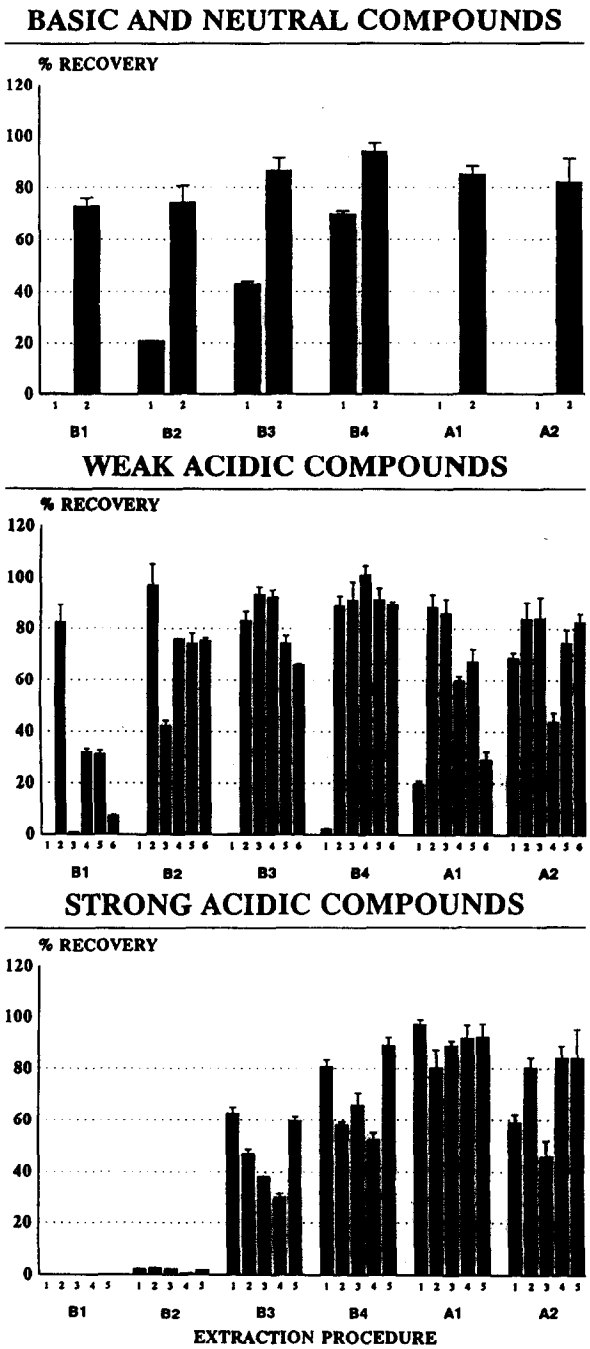

Fig. 1. Comparison of extraction recoveries obtained for some diuretics and probenecid. Extraction procedures are described under Experimental. Top: 1 = amiloride (basic); 2 = canrenone (neutral). Middle: 1 = acetazolamide; 2 = bendroflumethiazide; 3 = benzthiazide; 4 = chlorthalidone; 5 = diclofenamide; 6 = hydrochlorothiazide. Bottom: 1 = bumetanide; 2 = etacrynic acid; 3 = furosemide; 4 = piretanide; 5 = probenecid.

TABLE II

RESULTS OBTAINED USING PROCEDURE B4

Extraction recoveries, detection limits at different wavelengths and R.S.D.s obtained in intra- and inter-day reproducibility assays at the indicated concentrations.

Compound	Recovery		Detection limit		Reproducibility ($n = 8$)		
	Mean ± S.D. (%)	n	nm	μg/ml	Concentration (μg/ml)	R.S.D. (%)	
						Intraday	Interday
Basic compounds							
Amiloride	70.1 ± 1.07	4	350	0.02	1	3.7	6.7
Triamterene	67.9 ± 5.98	3	350	0.01	1	8.2	17.1
Caffeine	88.9 ± 5.27	3	270	NS[a]	3	1.6	5.9
7-Propyltheophylline (I.S.)	86.8 ± 7.05	4	270	NS	1	2.6	7.9
Neutral compounds							
Canrenone	94.0 ± 8.07	4	270	0.1	NS	–	–
Spironolactone	92.9 ± 9.66	4	240	0.1	NS	–	–
Weakly acidic compounds							
Acetazolamide	2.3 ± 0.30	4	270	2	10	4.5	13.1
Bendroflumethiazide	89.1 ± 3.81	3	270	0.05	NS	–	–
Benzthiazide	91.5 ± 7.03	4	270	0.1	0.5	8.0	8.7
Chlorthalidone	101.4 ± 3.35	4	270	0.2	1	3.2	5.8
Diclofenamide	91.6 ± 4.55	4	290	0.2	1	2.9	5.5
Hydrochlorothiazide	89.6 ± 1.11	4	270, 318	0.1	1	2.7	7.4
Strongly acidic compounds							
Bumetanide	80.8 ± 2.62	4	270, 350	0.02	0.5	2.2	6.0
Etacrynic acid	58.3 ± 0.96	3	270	0.2	NS	–	–
Furosemide	66.2 ± 4.52	4	270, 350	0.05	0.5	2.7	6.3
Piretanide	52.5 ± 2.57	4	270, 350	0.1	0.5	2.6	5.7
Probenecid	89.3 ± 3.05	4	270	0.1	0.5	3.2	5.7

[a] NS = Not studied.

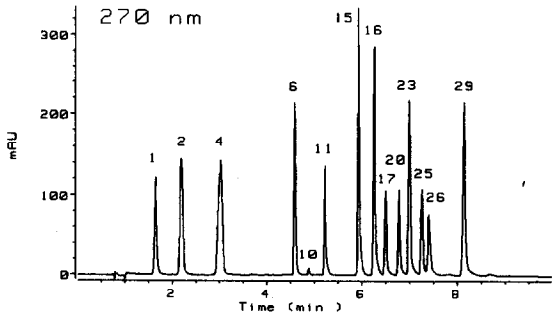

Fig. 2. Chromatogram obtained at 270 nm after analysis of a methanolic solution of some diuretics and probenecid. Peaks 1 = amiloride; 2 = acetazolamide; 4 = hydrochlorothiazide; 6 = 7-propyltheophylline (I.S.); 10 = diclofenamide; 11 = torasemide; 15 = furosemide; 16 = buthiazide; 17 = benzthiazide; 20 = piretanide; 23 = bendroflumethiazide; 25 = bumetanide; 26 = probenecid; 29 = canrenone.

pounds used to optimize the methodology were detected in urines collected from 0 to 24 h after intake of a single therapeutic dose. Quantitative results for urines from some excretion studies are listed in Table IV. Calibration equations for the determination of the compounds had correlation coefficients higher than 0.999.

Caffeine was well resolved from interfering compounds and showed a linear response in the range 2–20 μg/ml, following the equation $y = 1.054x - 0.012$ ($r = 0.9992$), where y is the peak-area ratio between caffeine and the I.S. and x is the caffeine concentration in μg/ml.

Examples of chromatograms corresponding to blank, spiked and true positive urine samples obtained either from excretion studies or from

TABLE III

ABSOLUTE AND RELATIVE (RRT) RETENTION TIMES OF THE COMPOUNDS STUDIED AND WAVELENGTHS USED TO MONITOR THEM

Compound	Retention time (min)	RRT	Wavelength (nm)
Amiloride	1.64	0.362	270, 350
Acetazolamide	2.19	0.483	270
Caffeine	2.76	0.610	270
Hydrochlorothiazide	3.01	0.664	270, 318
Triamterene	3.67	0.810	270, 350
7-Propyltheophylline (I.S.)	4.53	1	270
Morazone metabolite 1	4.54	1.002	270
Chlorthalidone	4.72	1.041	270
Morazone	4.76	1.050	270
Diclofenamide	4.85	1.070	270, 290
Torasemide	5.17	1.141	270, 290
Clopamide	5.20	1.147	270, 240
Morazone metabolite 2	5.45	1.203	270
Mesocarb metabolite	5.49	1.211	270, 350
Furosemide	5.88	1.298	270, 350
Buthiazide	6.23	1.375	270, 318
Benzthiazide	6.47	1.428	270
Cyclothiazide 1	6.60	1.457	270, 318
Cyclothiazide 2	6.70	1.479	270, 318
Piretanide	6.71	1.481	270, 350
Polythiazide	6.91	1.525	270, 318
Etacrynic acid	6.92	1.527	270
Bendroflumethiazide	6.97	1.538	270
Xipamide	7.11	1.569	270, 300
Bumetanide	7.18	1.584	270, 350
Probenecid	7.31	1.613	270, 240
Mesocarb	7.77	1.715	270, 350
Spironolactone	7.79	1.719	240
Canrenone	8.08	1.783	270

sport competitors (true doping positive cases) are shown in Figs. 3–9.

DISCUSSION

Owing to the different physico-chemical properties (acidbase behaviour, liposolubility) of the target compounds, it is difficult to develop single extraction procedures and chromatographic conditions to obtain adequate selectivity and sensitivity for all the compounds under study.

The extraction at acidic pH allows the recovery of acidic and neutral compounds but not of the basic diuretics. However, optimized basic extraction allows the recovery of all kind of compounds studied. For this reason, extraction in alkaline conditions with salting-out seems to be suitable for screening purposes to detect diuretics in urine. The basic extracts were much cleaner than the acidic extracts so an increase in signal-to-noise ratio is consequently obtained.

Ethyl acetate, a more polar solvent than diethyl ether, gives the best extraction recoveries for most of the compounds at alkaline pH. Weakly acidic compounds, except acetazolamide, are well extracted in the basic ethyl acetate extract owing to their high pK_a values [15–18]. The recovery of the strongly acidic compounds and acetazolamide in the basic extract is mainly achieved due to the salting-out

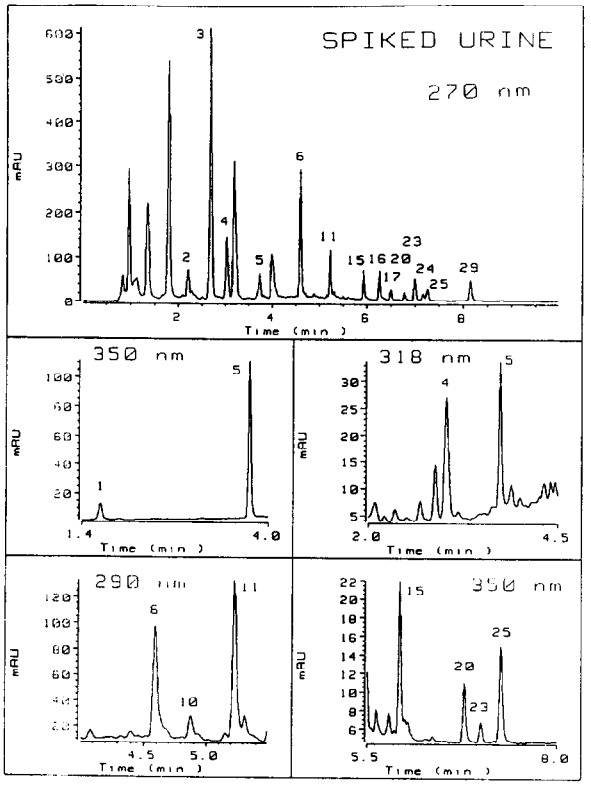

Fig. 3. Chromatograms at different wavelengths from a extract of a spiked urine containing various compounds: 1 = amiloride (0.1 μg/ml); 2 = acetazolamide (5 μg/ml); 3 = caffeine; 4 = hydrochlorothiazide (0.5 μg/ml); 5 = triamterene (0.2 μg/ml); 6 = I.S. (1 μg/ml); 10 = diclofenamide (0.5 μg/ml); 11 = torasemide (0.5 μg/ml); 15 = furosemide (0.2 μg/ml); 16 = buthiazide (0.2 μg/ml); 17 = benzthiazide (0.2 μg/ml); 20 = piretanide (0.2 μg/ml); 23 = bendroflumethiazide (0.2 μg/ml); 24 = xipamide (0.2 μg/ml); 25 = bumetanide (0.2 μg/ml); 29 = canrenone (0.2 μg/ml).

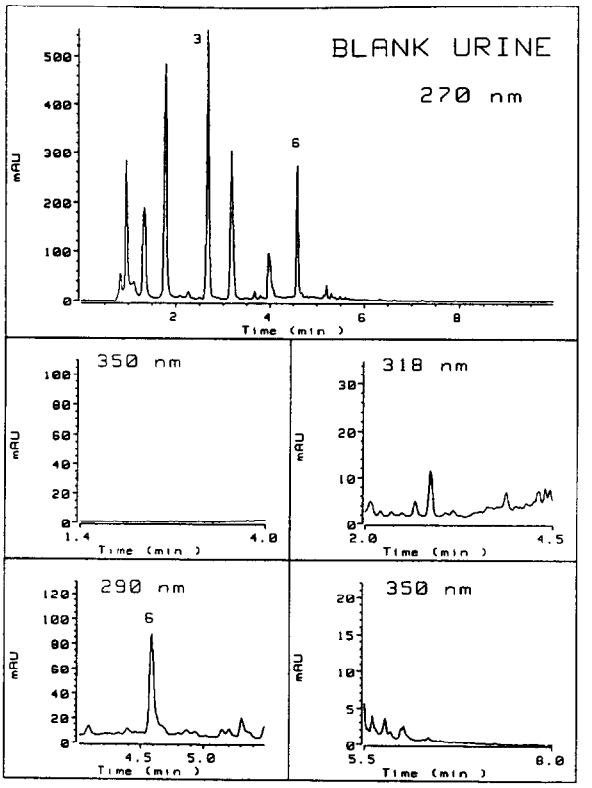

Fig. 4. Chromatogram at 270 nm and other selected wavelengths of a blank urine sample extracted using procedure B4. Peaks: 3 = caffeine; 6 = 7-propyltheophylline (I.S.).

effect (comparison of procedures B3 and B2). Procedure B4 is the method of choice for screening purposes because it appears to be the best compromise. In general terms, procedure B4 allows higher yields than other methods previously described [15–17]. Only acetazolamide has a low recovery but it can be easily detected owing to the high concentrations appearing in urine after a single therapeutic dose (Table IV).

The use of gradient elution and an ammonium buffer is needed to obtain adequate retention times and good chromatographic behaviour for all of the compounds, as reported previously [18]. The additional decrease in the retention times achieved with the 3-μm particle size column (Table III) results in a substantial decrease in the analysis time per sample as com-

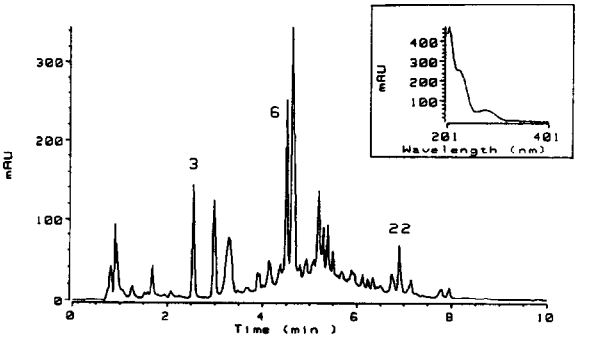

Fig. 5. Chromatogram at 270 nm of a urine sample from an excretion study of etacrynic acid and UV spectrum of the peak of etacrynic acid. Peaks: 3 = caffeine; 6 = I.S.; 22 = etacrynic acid.

TABLE IV

RESULTS OF EXCRETION STUDIES

Concentrations of the unchanged compounds detected in urines after administration of single therapeutic doses of the drugs to healthy volunteers.

Compound	Administered dose (mg)	Mean concentration in urine (μg/ml)		
		0–8 h	8–24 h	0–24 h
Amiloride	5	1.45	0.54	NS[b]
Triamterene	100	0.97	0.15	NS
Acetazolamide	250	185.56	20.83	NS
Diclofenamide	50	9.02	4.22	NS
Chlorthalidone	25	4.08	3.36	NS
	25	1.18	1.09	NS
Hydrochlorothiazide	25	10.77	2.81	NS
	50	23.02	7.87	NS
Bumetanide	1	0.13	0.15	NS
	1	0.21	0.06	NS
Etacrynic acid	50	1.57	0.61	NS
	50	3.56	0.19	NS
	50	2.91	0.33	NS
Furosemide	40[a]	5.49	0.82	NS
Piretanide	6	1.10	0.10	NS
Probenecid	250	NS	NS	26.83
	250	NS	NS	23.93

[a] Administered as furosemide xanthynol.
[b] NS = not studied.

pared with other HPLC methods described previously [15–17]. The good inter-day reproducibility of RRT and the acquisition of the full UV spectra allow the easy identification of the peaks detected.

Good reproducibility is obtained, as the intra- and inter-day R.S.D.s are in general lower than 10%. Acetazolamide was poorly recovered under the proposed conditions, thus increasing the analytical variations. For accurate quantitative or confirmatory studies, a neutral extraction using diethyl ether (recovery 21.5 ± 0.99%, n = 3) is preferred.

The detection limits obtained using the proposed method are better than those described previously [12–17], except for acetazolamide. Although 270 nm was chosen as a compromise wavelength for most of the compounds [18], the use of specific wavelengths improves the detection, especially for those appearing at short retention times (Table III).

Additionally to the compounds used in the optimization of the screening procedure, the method has proved to be of great value for the detection of other diuretics and very polar or thermally unstable compounds, such as mor-

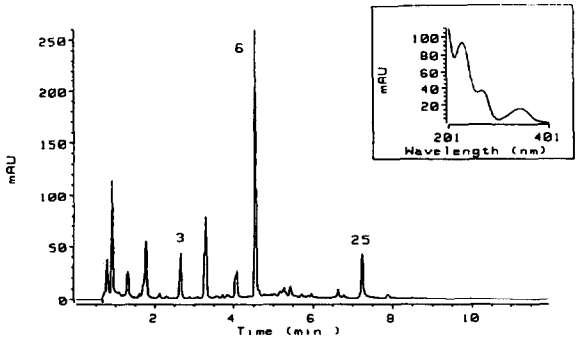

Fig. 6. Chromatogram at 270 nm of a urine sample from an excretion study of bumetanide and UV spectrum of the peak of bumetanide. Peaks: 3 = caffeine; 6 = I.S.; 25 = bumetanide.

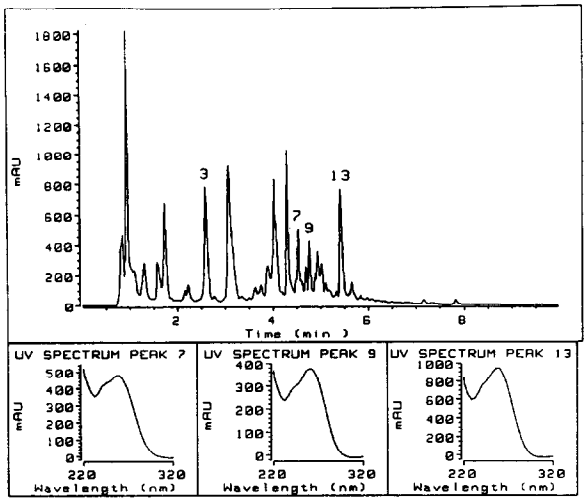

Fig. 7. Chromatogram at 270 nm of a urine sample from an excretion study of morazone and UV spectra of the peaks detected. Peaks: 3 = caffeine; 7 = morazone metabolite 1; 9 = unchanged morazone; 13 = morazone metabolite 2.

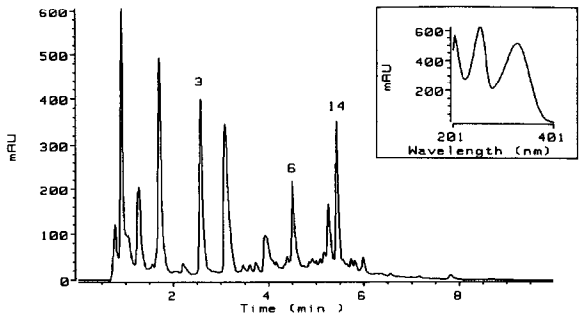

Fig. 8. Chromatogram at 270 nm of a urine sample from an excretion study of mesocarb and UV spectrum of the peak of the conjugated mesocarb metabolite. Peaks: 3 = caffeine; 6 = I.S.; 14 = conjugated mesocarb metabolite.

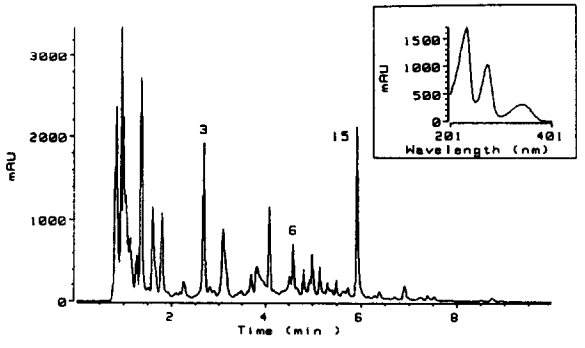

Fig. 9. Chromatogram at 270 nm of a true positive furosemide urine sample and UV spectrum of the peak of furosemide. Peaks: 3 = caffeine; 6 = I.S.; 15 = furosemide.

azone (Fig. 7) and conjugated metabolites of mesocarb (Fig. 8) [19,20].

The method developed is able to detect the ingestion of the compounds under study at least during 24 h after intake. It has been applied routinely to screen for the misuse of these compounds in the Barcelona antidoping laboratory and subjected to open and blind controls with total reliability of the results. An adaptation of this method was used at the Barcelona Olympic Games.

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