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Improved detection limits for screening of diuretics by coupled liquid chromatography and ultraviolet–visible spectrophotometry

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ABSTRACT

Experimental conditions have been studied in order to improve the sensitivity for the analysis of diuretics and probenecid in urine samples by high-performance liquid chromatography with ultraviolet detection. Sample clean-up and chromatographic parameters have been optimized to obtain a suitable sensitivity for the detection or quantification of each diuretic using an HP-Hypersil ODS-C_{18} column (5 μm, 250 mm × 4 mm I.D.), taking into account the pharmacological properties of each compound. The reliability of this method was tested by analysing urine samples after a minimum single-dose administration of chlorthalidone and probenecid.

INTRODUCTION

The identification and quantification of diuretics in biological fluids is of interest because of the potential of therapeutic drug monitoring in optimizing patient care and in control of doping. In the past few years, diuretics have been misused and abused in sports. Gas chromatography (GC) coupled with mass spectrometric (MS) detection is the most reliable method for diuretic characterization. GC, however, is limited by the low volatility of diuretics and by the requirement of prior derivatization. Because of the time and cost involved, high-performance liquid chromatography (HPLC) coupled with UV detection is usually the technique of choice for the screening of diuretics, the confirmation of positive samples being usually performed by GC–MS.

In a previous study, we evaluated the effects of a number of variables that affect the liquid chromatographic separation of synthetic mixtures of diuretics [1]. We have also compared conventional liquid–liquid extraction and solid-phase extraction in different non-polar packings for the separation of diuretics from urine [2].

This paper describes the optimization of the experimental conditions to obtain a suitable sensitivity for both determination and identification purposes, taking into account the pharmacokinetic behaviour of each compound. Representative diuretics belonging to the different pharmacological groups were studied. Probenecid, a uricosuric agent, was also included because it has a weak diuretic activity and has been used in sports to decrease the urinary excretion of anabolic steroids.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 1040A liquid chromato-

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graph, equipped with a diode-array detector linked to a data system (Hewlett-Packard HPLC Chem Station, Palo Alto, CA, USA) was used for data acquisition and storage. The system was coupled to a quaternary pump (Hewlett-Packard, 1050 Series) with a 25-μl sample loop injector.

The column was an HP-Hypersil ODS-C_{18}, particle size 5 μm, 250 mm × 4 mm I.D. (Darmstadt, Germany). The detector was set to collect a spectrum every 640 ms (over the range 200–400 nm) and all the assays were carried out at ambient temperature. The identity of each compound was established by comparing the retention times and UV spectra in real samples with those previously obtained by injection of standards.

Reagents

All the reagents were of analytical grade. Methanol and acetonitrile were of HPLC grade from Scharlau (Barcelona, Spain). Water was distilled, deionized and filtered through 0.45-μm Nylon membranes (Teknokroma, Barcelona, Spain). Diuretic standard solutions were prepared by dissolving the pure compounds in methanol: amiloride hydrochloride, hydrochlorothiazide and chlorthalidone from ICI-Pharma (Pontevedra, Spain), acetazolamide from Cyanamid Ibérica (Madrid, Spain), triamterene, bendroflumethiazide, ethacrynic acid and probenecid from Sigma (St. Louis, MO, USA), furosemide from Lasa (Barcelona, Spain), cyclothiazide and bumetanide from Boehringer Ingelheim (Barcelona, Spain) and spironolactone from Searle Ibérica (Madrid, Spain). The internal standard was β-hydroxymethyltheophylline from Sigma. Propylamine hydrochloride from Fluka (Buchs, Switzerland), sodium dihydrogenphosphate monohydrate from Merck (Darmstadt, Germany), sodium acetate trihydrate, disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$), sodium bicarbonate and potassium carbonate from Probus (Barcelona, Spain) and HPLC-grade ethyl acetate from Scharlau were also used.

Standard solutions

The standard solution of each diuretic was prepared by dissolving 50 mg of the pure compound in 25 ml of methanol (2000 μg/ml); the triamterene standard solution was prepared by dissolving 100 mg of the pure compound in 250 ml of methanol (400 μg/ml). The internal standard was prepared by dissolving 250 mg of the pure compound in 250 ml of methanol (1000 μg/ml). All the solutions were stored in the dark at 2°C.

Mobile phase

The mobile phase was acetonitrile–0.05 *M* phosphate (pH 3) or –acetate buffer (pH 4). The acetonitrile content was increased linearly from 15% at zero time to 80% after 8 min. After 8 min the acetonitrile content was kept constant. The phosphate and acetate buffers were prepared by dissolving the appropriate amount of sodium dihydrogenphosphate monohydrate or sodium acetate trihydrate, respectively, in 500 ml of distilled and deionized water; 0.7 ml of propylamine hydrochloride was added to this solution, and the pH was adjusted by adding the minimum amount of concentrated phosphoric or acetic acid. All the solutions were prepared daily, filtered through a 0.45-μm Nylon membrane (Teknokroma), and degassed with helium before use. The flow-rate was 1 ml/min.

Urine samples

Urine samples were spiked with the appropriate amount of diuretic standard solutions, then subjected to liquid–liquid extraction or solid-phase extraction [2]. Six different Bond-Elut columns, 100 mg/1 ml, from Scharlau were evaluated for the extraction: C_{18}, C_8, C_2, cyclohexyl (CH), phenyl (PH) and cyanopropyl. After the extraction, the samples were filtered through 0.45-μm Nylon filters (Teknokroma). Finally, 5 μl of each sample were injected into the column using a Hamilton micro-syringe.

Limit of detection

The limit of detection was estimated by analysis of solutions of decreasing concentration of each diuretic in methanol. It was established as the concentration required to generate a signal-to-noise ratio of 3. The obtained values were con-

firmed by analysis of urine samples spiked with the appropriate amount of diuretics to produce a concentration, after sample treatment, equivalent to the estimated limits of detection.

Human studies

Urine samples were obtained from healthy volunteers after a single administration of the lowest recommended dose of probenecid (500 mg) or chlorthalidone (25 mg). These samples were tested using the conditions recommended for the screening of diuretics: C_{18} solid-phase extraction columns for sample clean-up, gradient elution with acetonitrile–phosphate buffer and detection at 230 nm. Chlorthalidone was quantified with a calibration graph constructed from urine samples spiked with this diuretic in the 1–8 μg/ml range.

RESULTS AND DISCUSSION

The mobile phases recommended for the elution of the different compounds tested in the two chromatographic systems proposed for screening of diuretics are shown in Table I. The chromatographic systems differed only in the aqueous component of the mobile phase solution: 0.05 *M* phosphate buffer (pH 3) or 0.05 *M* acetate buffer (pH 4), with acetonitrile as the organic component [1]. These are the most common eluents used for quantification of diuretics in biological samples [3].

Most of the diuretics tested show better sensitivity at low wavelengths. However, because of the absorption of the mobile phase, wavelengths lower than 250 nm cannot be used with acetonitrile–acetate buffer mixtures. Thus acetonitrile–phosphate buffer mixtures are recommended for screening tests. However, in this latter system, the most acidic compounds, such as ethacrynic acid or probenecid, show poor sensitivity [4,5]. We have demonstrated that acetate buffer (pH 4) can be used to increase the resolution and sensitivity of these compounds by improving their chromatographic response [1]. Therefore, this aqueous component is recommended for quantification proposes (Table I). For amiloride, acetazolamide, triamterene or bendroflumethiazide, either eluents can be used as they show peak maxima at wavelengths higher than 250 nm. Table I also shows the wavelength of maximum absorbance of each compound.

For sample treatment, solid-phase extraction is

TABLE I

OPTIMAL EXPERIMENTAL CONDITIONS FOR DETECTING DIURETICS AND PROBENECID

Compound	Aqueous component in the mobile phase[a]	Wavelength (nm)	Solid-phase extraction packing material
Amiloride	P–A	365	C_{18}
Acetazolamine	P–A	275	–[b]
Hydrochlorothiazide	P	230	C_{18}
Triamterene	P–A	370	C_8
Chlorthalidone	P	230	C_8
Furosemide	P	230	C_{18}
Cyclothiazide	P	230	C_2
Bendroflumethiazide	P–A	275	C_{18}
Ethacrynic acid	A	275	C_{18}
Bumetanide	P	230	C_2
Probenecid	A	254	C_{18}
Spironolactone	P	240	C_2

[a] P = 0.05 *M* phosphate buffer; A = 0.05 *M* acetate buffer.

[b] Liquid–liquid extraction into ethyl acetate.

TABLE II

LIMITS OF DETECTION OBTAINED FOR DIURETICS AND PROBENECID

Compound	Limit of detection (ng/ml)	
	Screening procedure	Optimized conditions
Amiloride	30	–
Acetazolamide	60	10
Hydrochlorothiazide	10	–
Triamterene	10	–
Chlorthalidone	6	–
Furosemide	3	–
Cyclothiazide	20	–
Bendroflumethiazide	30	20
Ethacrynic acid	200	60
Bumetanide	6	–
Probenecid	200	60
Spironolactone	20	–

preferred over conventional liquid–liquid extraction procedures, because they provide higher recoveries of a majority of the compounds tested, and also because they are less time-consuming. C_{18} columns are recommended for screening tests (Table I) because, for most of the compounds tested, the efficiency of the extraction is better with this packing [2]. However, for quantification purposes, CH or PH columns could be suitable for amiloride, triamterene, chlorthalidone or furosemide because the recovery of these drugs is almost complete, whereas the retention of the biological matrix is less than that obtained with C_{18}, C_8 or C_2 packings. The retention of acetazolamide is low in these packing materials because of its high polarity; therefore, conventional liquid–liquid extraction is recommended for quantification.

Limit of detection

Table II shows the limits of detection found under the conditions proposed for the screening of diuretics: C_{18} solid-phase extraction for sample clean-up and acetonitrile-phosphate (pH 3) gradient elution and detection at 230 nm. The high values obtained for ethacrynic acid and probenecid were due to the characteristics of their chromatographic response in acetonitrile–phosphate buffer eluents, as has been previously indicated. Substitution of phosphate buffer (pH 3) by acetate buffer (pH 4) increases the sensitivity for both compounds, and the limits of detection for the other diuretics tested are still lower than the values recommended for the Medical Commission of the International Olympic Committee for control of doping (0.1 μg/ml) [6].

The limits of detection found are clearly lower than those previously obtained with HPLC–UV assays for the screening of diuretics in urine samples, which were in the range 0.1–5 μg/ml [4,5]. The sensitivity of the proposed assay is comparable with that obtained with a GC–MS method proposed by Lisi *et al.* [7]. These authors reported limits of detection for acetazolamide, hydrochlorothiazide, bumetanide and ethacrynic acid in the 0.03–0.1 μg/ml range. Recently, Ventura *et al.* [8] have applied an HPLC–MS system to the screening of different diuretics. Although the sensitivity in detecting furosemide is very poor, that obtained for a majority of diuretics is comparable with that achieved with the proposed procedure. The limits of detection obtained by Ventura *et al.* [8] for ethacrynic acid or probenecid are lower than those we have found working with phos-

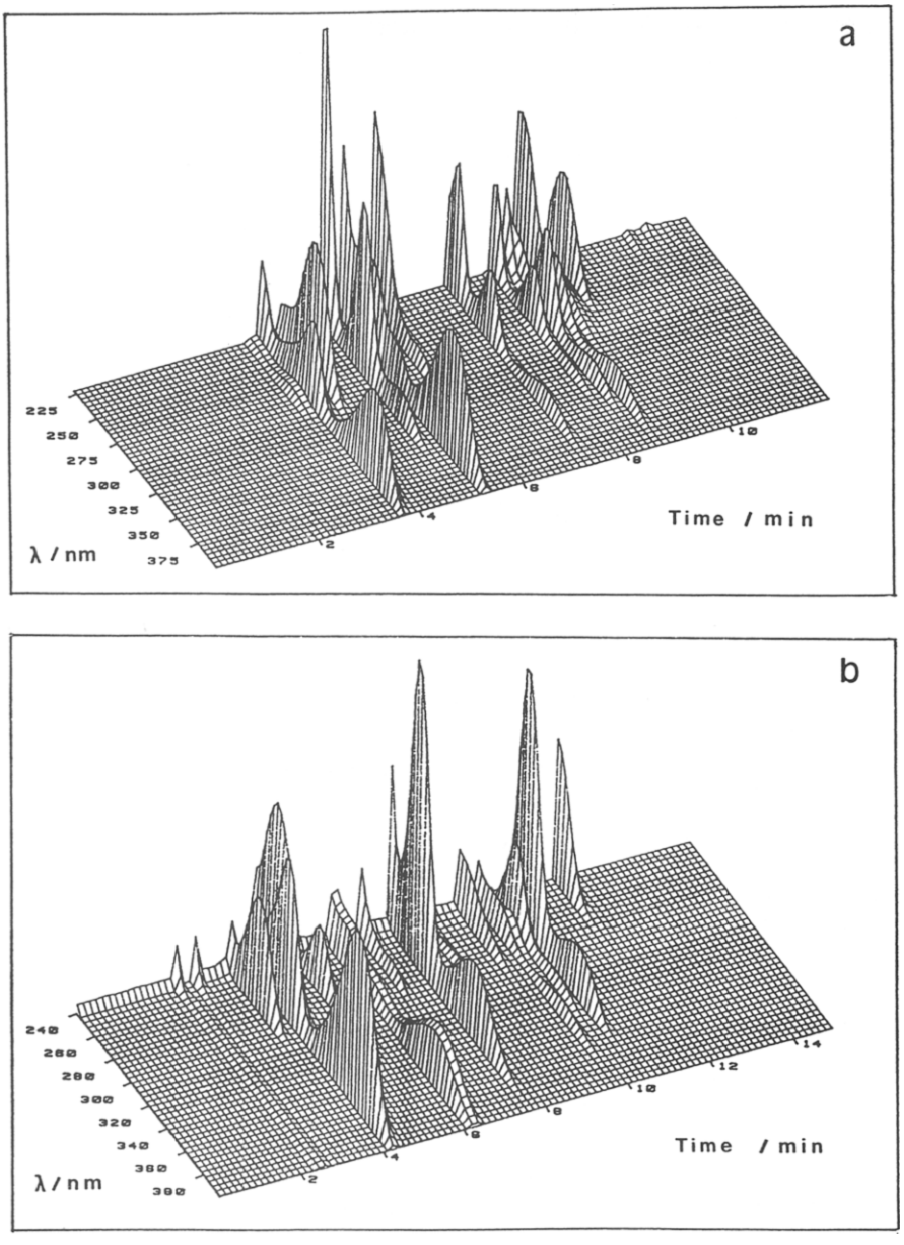

Fig. 1. A–λ–t diagrams of diuretics and probenecid using (a) phosphate buffer (pH 3) or (b) acetate buffer (pH 4) as aqueous component in the mobile phase. Retention times (min): internal standard 3.71, 3.31; amiloride 3.49, 3.93; acetazolamide 4.06, 3.95; hydrochlorothiazide 4.92, 4.90; triamterene 5.31, 5.86; chlorthalidone 5.90, 6.56; furosemide 7.31, 7.42; cyclothiazide 8.16-8.25 and 9.80-9.84; bendroflumethiazide 8.44, 10.24; ethacrynic acid 8.56, 8.11; bumetanide 8.66, 9.53; probenecid, 8.96, 9.07 and spironolactone 9.46, 11.66 for systems a and b, respectively. The amount injected of each diuretic was 0.5 μg.

phate buffers in the mobile phase, and similar to those found when using acetate buffers.

The sensitivity can be improved for some diuretics by selecting the appropriate experimental conditions. Fig. 1 shows the absorbance–wavelength–time (A–λ–t) diagrams obtained in the two studied chromatographic systems for the mixture of compounds assayed. From these dia-

TABLE III

PHARMACOKINETIC DATA OF DIURETICS AND PROBENECID

Compound	Period of activity (h)	Dose (mg)	Urine level after dosing[a] (μg/ml)
Amiloride	24	–	–
Acetazolamide	–	250	–
Hydrochlorothiazide	6–16	25	< 0.5 (48 h)
Triamterene	7–9	100	4 (7 h)
Chlorthalidone	24–72	25	1.7 (24 h)
Furosemide	6–8	40	0.4 (24 h)
Cyclothiazide	18–24	–	–
Bendroflumethiazide	> 18	5	0.2 (24 h)
Ethacrynic acid	6–8	25–100	–
Bumetanide	4–6	5	Trace (24 h)
Probenecid	–	500	–
Spironolactone	48–72	25	0.2 (24 h)

[a] Values from refs. 4 and 5.

grams one can select the appropriate mobile phase composition and detection wavelength at which both the sensitivity and selectivity are better for a particular determination. The sample clean-up can also be optimized for each diuretic according to Table I. The limits of detection obtained under the optimized conditions for diuretics, when these conditions are not the conditions proposed for the screening, are summarized in Table II. The obtained values are comparable with those found in quantification assays with UV–visible detection [3], which indicates that these conditions can be used (alone or in combination) for the quantification of diuretics and probenecid.

Human studies

Table III summarizes the time intervals at which each diuretic is effective when it is administered in therapeutic doses [9]. This table also shows the doses administered and levels determined in different previously described HPLC–UV screening procedures [4,5]. The levels determined are near to the limits of detection reported by such procedures.

As can be seen from this table, some compounds cannot be detected at times when they are still active. This can be fraudulently exploited in sports just before a competition, to hinder the detection of other illegal substances, *e.g.* spironolactone cannot be detected 24 h after dosing, but it is pharmacologically active until two to three days after it has been ingested. Similar conclusions can be obtained for chlorthalidone, triamterene and ethacrynic acid. The detection of ethacrynic acid is a very complex problem because of its short life-time of elimination and also because it is extensively metabolized; HPLC-UV methods proposed for screening have not detected it after a normal single dose administration [4]. Moreover, the sensitivity of the reported procedures for quantification of these diuretics with UV detection could not be suitable for pharmacokinetic studies [3].

With the proposed screening procedure, the sensitivity is clearly increased, making possible the detection of diuretics in doping controls for longer periods after dosing. The sensitivity is also suitable for quantification proposes, thus facilitating pharmacokinetic studies, which could be specially useful for chlorthalidone, triamterene, ethacrynic acid and spironolactone.

This procedure was validated by analysing urine extracts obtained from real samples. The urinary excretion–time profile obtained for chlorthalidone after the administration of the lowest recommended dose is shown in Fig. 2. From this

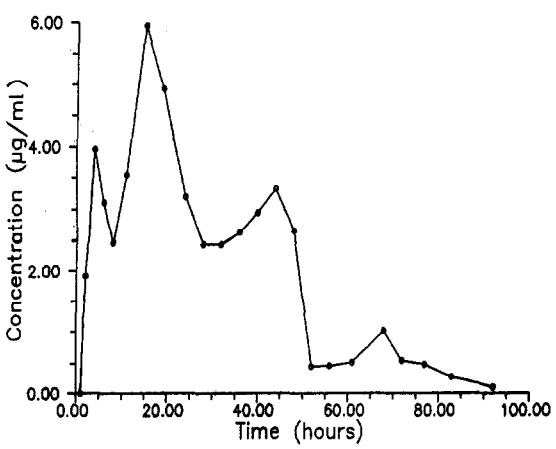

Fig. 2. Urinary excretion–time profile of chlorthalidone. Dose administered, 25 mg.

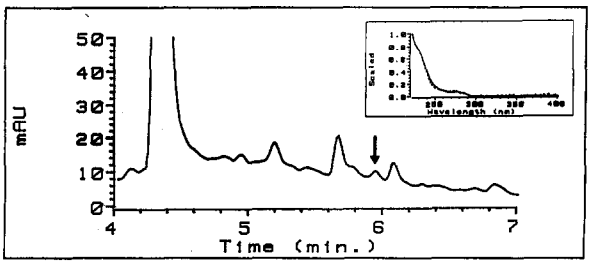

Fig. 3. Chromatogram of a urine sample obtained from a subject five days after a single dose administration of 25 mg of chlorthalidone (retention time, 5.9 min). Detection wavelength, 230 nm. Inset: a comparison of the UV spectra of the sample (– – –) and a standard of chlorthalidone (———).

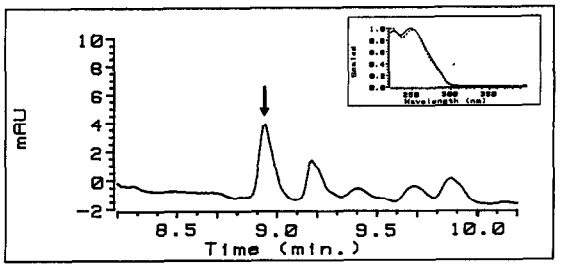

Fig. 4. Chromatogram of a positive finding of probenecid obtained 24 h after a single dose administration of 500 mg of probenecid (retention time 8.9 min). Detection wavelength, 230 nm. Inset: a comparison of the UV spectra of the sample (– – –) and a standard of probenecid (———).

figure it can be derived that, with the sensitivity of the previously reported HPLC–UV assays, this compound cannot be detected 48 h after dosing. Fig. 3 shows a chromatogram obtained five days after the administration of chlorthalidone. The chromatographic peak of this diuretic can be seen at 5.9 min, and drug confirmation can be achieved by comparing the UV absorption patterns of the sample and a standard.

Fig. 4 shows a positive finding of probenecid (obtained 24 h after dosing). Similarly, the presence of probenecid can easily confirmed by comparing the UV spectra of the sample and the standard.

CONCLUSION

The screening procedure described is sensitive enough to detect diuretics and probenecid in real urine samples. Therefore, it would be reliable for doping control and clinical laboratories.

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