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High-performance liquid chromatographic determination of diuretics in urine by micellar liquid chromatography

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ABSTRACT

The use of micellar liquid chromatography for the determination of diuretics in urine by direct injection of the sample into the chromatographic system is discussed. The retention of the urine matrix at the beginning of the chromatograms was observed for different sodium dodecyl sulphate (SDS) mobile phases. The eluent strengths of a hybrid SDS–methanol micellar mobile phase for several diuretics were compared and related to the stationary phase/water partition coefficient with a purely micellar mobile phase. The urine band was appreciably narrower with a mobile phase of 0.05 *M* SDS–5% methanol (v/v) at 50°C (pH 6.9). With this mobile phase the determination of bendroflumethiazide and chlorthalidone was adequate. Acetazolamide, ethacrynic acid, furosemide, hydrochlorothiazide and probenecid were overlapped by the urine matrix, and the retention of amiloride and triamterene was too long.

INTRODUCTION

Diuretics, which are some of the most extensively used drugs, are administered in the treatment of congestive heart failure and hypertension, among other diseases. However, diuretics are also misused in sport by competitors to reduce weight quickly, to dilute urine to prevent the detection of another drug and to control the retention of water produced by anabolic steroids. Not only for ethical grounds, but also because of serious health risks, use of these compounds was prohibited by the International Olympic Committee [1].

Numerous procedures have been described for the determination of diuretics in physiological fluids using reversed-phase liquid chromatography (RPLC) with hydro-organic phases [2–13]. However, the analyses usually require a time-consuming sample preparation step to remove proteins, which could clog the chromatographic system.

Several approaches have been used for the direct injection of physiological fluids into a chromatographic system, without a separation step. Among these are special columns [14–16] and eluents capable of solubilizing serum proteins, such as triethylammonium acetate buffers with silica packings [17]. Micellar eluents, containing anionic or non-ionic surfactants [18–22], have also been used.

In a previous report [23], micellar liquid chromatography (MLC) with a hybrid mobile phase of 0.05 *M* SDS–3% propanol was shown to be appropriate for the evaluation of diuretics in pharmaceuticals. This paper describes the use of MLC for the determination of diuretics in urine.

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EXPERIMENTAL

Reagents

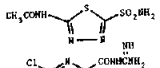
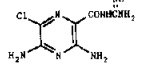
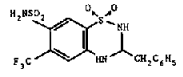
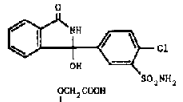
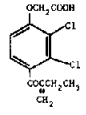
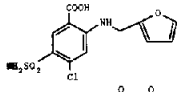
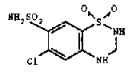
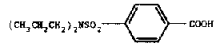
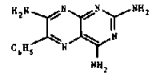
Mobile phases were prepared by mixing an aqueous SDS solution (99% purity; Merck, Darmstadt, Germany) with various amounts of alcohol. Methanol (for HPLC) and *n*-propanol (analytical grade) were from Panreac (Barcelona, Spain), and 1-pentanol (analytical grade) was from Merck. Barnstead nanopure, deionized water (Sybron, Boston, MA, USA) was used throughout. Mobile phases and samples were vacuum-filtered through 0.47-μm nylon membranes from Micron (Westboro, MA, USA). Stock solutions of 100 μg/ml of the diuretics in 0.1 *M* SDS were prepared. Most of the diuretics investigated were kindly donated from several pharmaceutical laboratories. Table I lists the structures of the diuretics and their origins.

Apparatus

The HPLC system consisted of a Hewlett-Packard (Palo Alto, CA, USA) HP 1050 chromatograph with an isocratic pump, a programmable UV–VIS detector and an integrator, HP 3396A. Samples were injected through a Rheodyne valve (Berkeley, CA, USA) with a 20-μl loop. A Spherisorb ODS-2 analytical C_{18} column (5 μm particle size, 12.5 cm × 4 mm I.D.) and a C_{18} precolumn of similar characteristics (2 cm × 4 mm I.D.) from Hewlett-Packard were used. A similar column (12 cm × 4.6 mm I.D.) and precolumn (3.5 cm × 4.6 mm I.D.) from Scharlau (Barcelona, Spain) were also used. A thermostatic bath (±1°C) was used.

TABLE I

DIURETICS STUDIED IN THIS WORK

Diuretic	Structure	Origin
Acetazolamide		Cyanamid Ibérica
Amiloride		Ici-Farma
Bendroflumethiazide		Davur
Chlorthalidone		Ciba-Geigy
Ethacrynic acid		Merck, Sharp & Dohme
Furosemide		Lasa
Hydrochlorothiazide		Galloso Wellcome
Probenecid		Sigma
Triamterene		Sigma

The dead volume (V_M) was estimated after ten replicate injections of an aqueous solution of potassium iodide and measurement of the absorbance at 254 nm (V_M = 0.77 ml for the Hewlett-Packard column and V_M = 0.83 ml for the Scharlau column, at a flow-rate of 1 ml/min. Optimization was also performed by monitoring at 254 nm. The efficiency was calculated as the number of theoretical plates, according to the equation of Foley and Dorsey [24] for skewed peaks.

RESULTS AND DISCUSSION

Previous considerations

Direct injection of a urine sample in a chromatographic system, with an SDS mobile phase, has the drawback of the strong urine matrix band at the beginning of the chromatogram. This blank signal can overlap the peaks of the drugs to be determined. Optimization of the mobile phase should take into account not only the retention of the compound to be analysed, but also the retention of the matrix.

The position of the urine band was observed for different mobile phases. A varying SDS concentration (>0.03 *M*) in the mobile phase did not appreciably modify the band, which extended up to 8–10 min, with a series of small peaks at longer retention times. This unabled the determination of compounds with $k' < 12$. The small peaks of the urine blank were reduced at an increased detection wavelength and depended on the subject, time and method of conservation of the sample.

The addition of an alcohol has been observed to produce an overall increase in eluent strength. A smaller effect was observed with the urine band. Thus, with 5% methanol, 3% propanol or 1% pentanol added to the SDS micellar mobile phase, it extended only to 5–6 min, which permitted the analysis of compounds with $k' > 7$. The urine band was also affected by increased temperatures. At 50°C, with a hybrid mobile phase, it extended only to 3–4 min. Thus, in these conditions, compounds with $k' > 4.5$ may be determined.

On the other hand, a change in the retention of different drugs, when the composition and temperature of the mobile phase were modified, depended on the nature of the drug. In a previous paper [23], a 0.05 *M* SDS solution was found to be appropriate for the preparation of hybrid micellar mobile phases. It was also observed that, in a hybrid micellar system with added alcohol, elution of diuretics was accelerated in the order pentanol > propanol > methanol. For the determination of diuretics in urine, it should be consid-

TABLE II

INFLUENCE OF THE CONCENTRATION OF METHANOL (v/v) IN A 0.05 *M* SDS MICELLAR MOBILE PHASE ON THE CAPACITY FACTORS, EFFICIENCIES AND PEAK SYMMETRIES [24]

When the temperature is not indicated, it is room temperature. A 12.5 cm × 4 mm I.D. Spherisorb ODS-2 column and a 2 cm × 4 mm I.D. precolumn were used. *B* and *A* are the distance between the centre of the peak and the tailing or leading edge of the peak, respectively, measured at 10% of the peak height.

Diuretic	0% Methanol			2% Methanol			4% Methanol			5% Methanol			5% Methanol at 50°C		
	k'	N	B/A	k'	N	B/A	k'	N	B/A	k'	N	B/A	k'	N	B/A
Acetazolamide	1.8	920	1.4	1.5	1122	1.3	1.6	1585	1.1	1.3	1300	1.5	0.8	1110	1.6
Hydrochlorothiazide	1.8	1100	1.2	1.6	1180	1.1	1.6	1210	1.6	1.3	1140	1.7	1.0	800	1.6
Furosemide	3.0	150	2.2	2.5	600	1.1	–	–	–	1.5	1170	1.8	0.3	40	2.0
Ethacrynic acid	6.2	65	4.4	5.0	149	3.0	3.9	90	4.5	2.9	270	2.9	1.6	560	3.2
Probenecid	6.9	52	3.9	6.2	80	1.9	4.3	50	3.9	3.3	60	3.2	1.6	240	2.5
Chlorthalidone	10.7	1290	1.0	9.8	1190	3.7	9.5	1620	1.5	8.8	2150	1.1	7.3	2590	1.2
Bendroflumethiazide	15.4	980	1.4	14.4	1130	1.1	13.8	1620	1.2	12.5	780	2.6	11.9	2410	1.1
Amiloride	33.7	111	3.7	32.7	135	3.7	30.9	180	4.0	29.5	240	3.7	30.4	470	2.7
Triamterene	78.0	–	–	71.2			65.8	190	3.0	60.0	–	–	54.2	120	3.4

ered that, although a relatively large increase in eluent strength may be adequate for the most retained diuretics (amiloride and triamterene), for other diuretics (see diuretics in Table II with $k' \leq 15$ in a purely 0.05 *M* SDS mobile phase) it is not convenient to increase excessively the eluent strength, since they will overlap with the urine band. In fact, with purely micellar eluents at pH 6.9, the peaks of acetazolamide, hydrochlorothiazide, furosemide, ethacrynic acid and probenecid will overlap with the urine matrix.

The pH of the micellar mobile phase should also be considered for the optimization of retention of diuretics in a urine matrix. Cline Love and Fett [22] observed that the retention of the urine matrix with SDS mobile phases, without a modifier, was minimized and remained constant in the pH range 5.5–7.5.

Furosemide, ethacrynic acid and probenecid have a carboxylic acid group in the molecule, with pK_A *ca.* 4–5. At pH 6.9 these compounds are ionized, and electrostatic repulsion from the negatively charged surfactant monomers adsorbed on the stationary phase occurs [25,26]. Retention increases at a lower pH.

The value of pK_a in aqueous media, for the protonation of the aminosulphonyl group for acetazolamide and hydrochlorothiazide, is 7–8. Thus, at pH 6.9, the concentration of the protonated species may be important. Cline Love and Fett [22] reported an adequate retention for hydrochlorothiazide with 0.02 *M* Brij 35–0.004 *M* SDS at pH 6.5, and indicated that a lower pH would not affect the retention. The same behaviour can be expected for bendroflumethiazide and chlorthalidone, which also have an aminosulphonyl group and will be protonated at pH < 7–8.

Influence of the amount of methanol in the micellar mobile phase

The effect on the chromatographic parameters of varying the concentration of methanol in the micellar mobile phase was studied (Table II). As expected, an increasing concentration of methanol usually produced a reduction in the retention. Also, for some diuretics, an improvement in efficiency was observed. In the absence of a modifier, the retention of probenecid was practically unchanged at increasing SDS concentrations [23]. However, the addition of methanol markedly affected its retention, because the polarity of the bulk aqueous mobile phase was modified.

In Table II, the retention and efficiency with a mobile phase of 0.05 *M* SDS–5% methanol at room temperature and at 50°C are compared. As observed, the capacity factors are usually lower at the higher temperature. For some compounds, an increase in efficiency was also achieved.

Khaledi *et al.* [27] observed, with mixed mobile phases, a linear relationship between log k' and the volume fraction of alcohol in the mobile phase, Φ_{org}, for a constant micelle concentration. We observed that better linearity was achieved with k' *versus* Φ_{org} for all diuretics. In Table III, the diuretics are ordered according to the eluent

TABLE III

FITING PARAMETERS FOR THE k' *VERSUS* Φ_{org} AND log k' *VERSUS* Φ_{org} PLOTS

Diuretic	k' *versus* Φ_{org}			log k' *versus* Φ_{org}		
	Slope	Intercept	r	Slope	Intercept	r
Triamterene	−3.4	78	0.992	−0.022	1.9	0.987
Amiloride	−0.8	34	0.977	−0.012	1.5	0.974
Probenecid	−0.7	7.2	0.980	−0.063	0.87	0.965
Ethacrynic acid	−0.6	6.2	0.997	−0.061	0.80	0.986
Bendroflumethiazide	−0.5	15.5	0.958	−0.016	1.2	0.948
Chlorthalidone	−0.4	10.7	0.979	−0.016	1.0	0.976
Furosemide	−0.3	3.1	0.997	–	–	0.896

strength of the SDS–methanol mobile phase, following the k' *versus* Φ_{org} plot.

Previously [23], the stationary phase/water partition coefficients, P_{SW}, of different diuretics, using a micellar mobile phase without alcohol, were obtained. Some correlation between these parameters and the eluent strength of hybrid methanolic mobile phases was observed. The more retained diuretics, triamterene and amiloride, showed larger slopes of the k' *versus* Φ_{org} plot. With a micellar eluent of SDS in the absence of alcohol, these diuretics also showed the highest P_{SW}.

At the other extreme were hydrochlorothiazide and acetazolamide, for which the eluent strength was very low (slopes for hydrochlorothiazide and acetazolamide were −0.09 and −0.07, respectively, and are not included in Table III because of poor regression). Both diuretics appeared at the beginning of the chromatograms for different SDS and methanol concentrations and, with a purely micellar mobile phase of SDS, showed the lowest values of P_{SW} ($\varnothing P_{SW}$ was 2.6 and 2.1 for hydrochlorothiazide and acetazolamide, respectively, where $\varnothing$ is the phase ratio), which indicated a low affinity for the stationary phase.

Bendroflumethiazide and chlorthalidone showed intermediate behaviour with respect to the eluent strength of the mobile phase. These diuretics presented a high P_{SW} ($\varnothing P_{SW}$ = 177 and 56, respectively). This means a high affinity for the stationary phase. The results indicated that the compounds exhibiting larger changes in retention with an increased methanol concentration are those showing the larger values of P_{SW}.

Analytical figures of merit

These results indicate that the determination of bendroflumethiazide and chlorthalidone can be performed with a 0.05 *M* SDS–5% methanol mobile phase at 50°C. Because of the wide variety of molecular structures, the wide variety of functional groups and the wide differences in pK_a values, other diuretics require other mobile phases for optimal retention and will not be considered here.

The analytical figures of merit for bendroflumethiazide and chlorthalidone were obtained with spiked urine samples, obtained from healthy subjects not receiving any medical treatment. In Fig. 1 the chromatogram of a spiked sample is shown, where the detection wavelength was 224 nm. The molar absorptivity for bendroflumethiazide was ε = 2610 and 2090 cm^{-1} l mol^{-1} at 212 and 274 nm (wavelengths of maximum absorption), respectively, and for chlorthalidone it was ε = 15 390 and 1960 cm^{-1} l mol^{-1} at 214 and 276 nm, respectively.

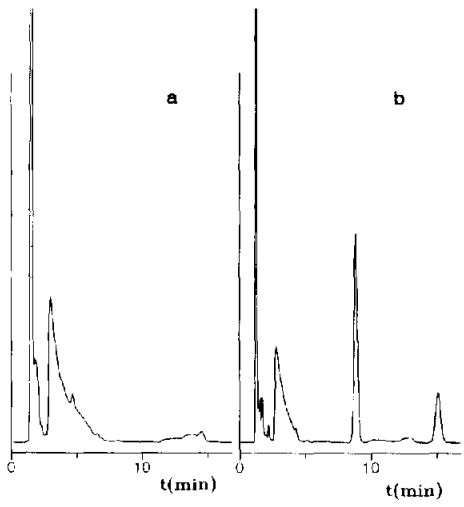


Fig. 1. Chromatograms of (a) the urine matrix and (b) a spiked sample with 20.4 μg/ml chlorthalidone (8.8 min) and bendroflumethiazide (15.2 min). The scale for the *y*-axis in the chromatogram of the urine matrix is magnified 1.5-fold with respect to that of the sample. Stationary phase, Spherisorb ODS-2, 12 cm × 4.6 mm I.D. column and 3.5 cm × 4.6 mm I.D. precolumn. Mobile phase 0.05 *M* SDS–5% methanol at 50°C. Detection wavelength, 224 nm.

The limits of detection were obtained from calibration curves obtained with mixtures of 0.1 ml of solutions of the diuretics, at various concentrations and 0.9 ml of the urine sample. These solutions were directly injected into the column. The limits of detection were in the 0.1–0.5 μg/ml range, for the optimum wavelength, which is below the concentration range required for therapeutic purposes and below the concentrations expected under typical overdosage in doping practices. The reproducibility, calculated after five replicate injections of the diuretics, was 5%.

No increase in column back-pressure and no changes of capacity factors were observed after 200 injections of urine samples. The same column was used for optimization studies, where more than 400 injections were done.

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