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Use of a three-factor interpretive optimisation strategy in the development of an isocratic chromatographic procedure for the screening of diuretics in urine samples using micellar mobile phases

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Abstract

Screening of diuretics in urine is feasible through direct injection of the samples into the chromatographic system and isocratic reversed-phase liquid chromatography (RPLC) with micellar–organic mobile phases of sodium dodecyl sulfate (SDS) and 1-propanol. The surfactant coverage of the chromatographic column makes the addition of organic competing amines less necessary than in conventional aqueous–organic RPLC to achieve well-shaped peaks. Also, the range of elution strengths of micellar mobile phases required to elute mixtures of hydrophobic and hydrophilic diuretics is smaller. This allows the isocratic separation of the diuretics within adequate analysis times. An interpretive methodology is applied to optimise the resolution of a mixture of 15 diuretics of diverse polarity and acid–base behaviour (althiazide, amiloride, bendroflumethiazide, benzthiazide, bumetanide, canrenoic acid, chlorthalidone, ethacrynic acid, furosemide, piretanide, probenecid, torasemide, triamterene, trichloromethiazide and xipamide), using pH and concentrations of surfactant and organic modifier in the mobile phase as separation factors. Twelve diuretics were resolved in 25 min using 0.055 *M* SDS–6.0% 1-propanol at pH 3.0. The mixture of 15 diuretics was also resolved with two mobile phases showing complementary behaviour: 0.05 *M* SDS–5.6% 1-propanol at pH 5.4 and 0.11 *M* SDS–5.4% 1-propanol at pH 4.2. The results were applied to the analysis of urine samples with limits of detection similar to those usually reported for aqueous–organic RPLC, taking into account that the samples were injected without any previous treatment to separate or preconcentrate the analytes.

Keywords: Optimization; Mobile phase composition; Micellar liquid chromatography; Diuretics

1. Introduction

Diuretics have long been used to lower blood pressure in hypertensive patients, or control body fluid and electrolyte homeostasis in diseases such as congestive heart failure, chronic renal failure or

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cirrhosis [1,2]. The initial response to diuretics is a negative sodium and water balance. This activates several hormones such as the renin–angiotensin–aldosterone system, or the sympathetic nervous system which tend to compensate for the changes in sodium and water. This neurohormonal response may have important clinical implications. Thus, the activation of the renin–angiotensin–aldosterone cascade appears to be responsible for the difference between responders and non-responders to diuretic therapy and for the development of side-effects such as hypokalaemia, metabolic alkalosis or hyponatraemia.

Owing to the wide use of diuretics, there is a need to develop simple analytical methods to monitor the compounds in physiological samples. An extensive published work makes reference to reversed-phase liquid chromatography (RPLC) analysis of samples containing single diuretics [3]. Several methods have also been described for the screening of groups of diuretics. Isocratic elution was used to resolve diuretics of diverse nature [4–6]. Gradient elution was preferred by other authors due to the wide variety of chemical structures, functional groups, and consequently, polarities of the compounds that show diuretic activity [7–10]. Chromatographic separation was usually achieved using C_{18} columns and mobile phases of acetonitrile containing an acidic aqueous buffer in the pH range 3–7 [11].

In the reported RPLC procedures for diuretics, the chromatographic run is usually preceded by a heavy and time-consuming liquid–liquid or solid-phase extraction to remove the harmful proteinaceous material from the sample [11]. In recent years, the interest in the use of micellar mobile phases in RPLC has grown, since this provides an easy and economical solution to the direct injection of physiological samples by solubilising the protein components and coating the analytical column with surfactant monomers to avoid clogging [12]. In addition, surfactant monomers and micelles displace drugs bound to proteins, releasing them for partitioning to the stationary phase. Early work in micellar RPLC was however reluctant of its screening capability due to the low efficiencies found for some compounds, although it was soon demonstrated that the addition of an organic modifier to the surfactant solution can largely improve the peak shape [13]. We have reported recently the usefulness of this technique for the analysis of basic drugs, such as β-blockers [14], phenethylamines [15], and amino acids [16], which show high efficiencies at low pH without the need of adding an amine compound or using special columns.

In micellar RPLC procedures, the optimisation of the separation conditions is usually carried out by using mobile phases at fixed pH, which is selected in turn performing several previous runs at fixed concentrations of surfactant and organic modifier at varying pH. The accurate and reproducible elution behaviour of compounds with micellar mobile phases has permitted the development of two-factor (surfactant and modifier) optimisation strategies that expedite the experimental work [17,18]. When the eluted compounds show an acid–base behaviour in the working pH of the column, the examination of the retention at varying pH may be convenient. This can be made using the two-factor strategies at several pH levels (e.g., 3, 5 and 7). However, the best separation may still be ignored when it is located at an intermediate pH value, and the search of the optimal pH can result in an undesirable, and perhaps unsuccessful, large number of experiments.

More complex strategies which consider simultaneously the three factors, pH and concentrations of surfactant and modifier, have also been reported in micellar RPLC [19–21]. The reliability of the predictions performed with such approaches depends on the accurate description of the elution behaviour of the chromatographed compounds, which is especially troublesome using the pH as a factor. Problems in these descriptions are also found in two-factor optimisations (pH and concentration of organic modifier) in aqueous–organic RPLC [22], owing to the rapid change in retention of acidic compounds with pH and the variation in acid–base behaviour at varying mobile phase composition.

In previous work we reported the separation, in urine samples, of six diuretics (amiloride, bumetanide, chlorthalidone, furosemide, probenecid and triamterene) with a mobile phase of 0.042 *M* sodium dodecyl sulfate (SDS)–4.0% 1-propanol at pH 4.5 [23], and seven diuretics (amiloride, bendroflumethiazide, bumetanide, furosemide, hydroflumethiazide, piretanide and triamterene) with 0.055 *M* SDS–8.0% 1-propanol at pH 3.0 [24]. In this

work, the resolution of a mixture of 15 diuretics showing diverse acid–base behaviour, with mobile phases of SDS and 1-propanol at varying pH, is studied. A three-factor optimisation strategy [21] developed in our laboratory for micellar RPLC, based on a mechanistic elution model [25], is used. The results are applied to the analysis of urine samples directly injected into the chromatographic system.

2. Experimental

2.1. Reagents

SDS (99% purity, Merck, Darmstadt, Germany), sodium dihydrogenphosphate, disodium hydrogenphosphate (for analysis, Panreac, Barcelona, Spain), HCl, NaOH (Probus, Badalona, Spain), and 1-propanol (for analysis, Scharlau, Barcelona, Spain), were used to prepare the mobile phases. The chromatographic system was cleaned with methanol (for analysis, Scharlau).

Stock standard solutions of 10–40 μg/ml of althiazide, benzthiazide, bumetanide, canrenoic acid, furosemide, probenecid, triamterene and trichloromethiazide (Sigma, St. Louis, MO, USA), amiloride (ICI-Farma, Madrid, Spain), bendroflumethiazide (Davur, Madrid, Spain), chlorthalidone (Ciba-Geigy, Barcelona, Spain), ethacrynic acid (Merck, Sharp and Dohme, Madrid, Spain), piretanide (Cusí, Barcelona, Spain), torasemide (Boehringer Mannheim, Barcelona, Spain), and xipamide (Lácer, Sardenya, Barcelona, Spain) were prepared. The diuretics, except those of Sigma, were kindly donated by the pharmaceutical laboratories indicated. Table 1 shows the structural formulae. The compounds were dissolved in a few ml of ethanol (for analysis, Prolabo, Paris, France), with the aid of an ultrasonic bath (Selecta, Model 617, Barcelona, Spain), and conveniently diluted with 0.10 *M* SDS solution. The solutions of althiazide, trichloromethiazide and furosemide were protected from light with aluminium foil and kept in the dark at 4°C. Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

The following pharmaceutical formulations were administered to seven healthy volunteers (diuretic and manufacturer in parenthesis): Aldactacine (althiazide, Searle, Evreux, France), Betadipresan-diu (bendroflumethiazide, Fides-Rottapharm, Almàcera, València, Spain), Diflux (amiloride and furosemide, Volpino, Buenos Aires, Argentina), Diurex (xipamide, Lácer), Fordiuran (bumetanide, Boehringer Ingelheim, Barcelona, Spain), Higrotona (chlorthalidone, Novartis Farmacéutica, Barcelona, Spain), Isobar (triamterene, Jacques Logeais, Issy-Les-Moulineaux, France), Perbilen (piretanide, Hoechst Ibérica, Barcelona, Spain), and Rulún (trichloromethiazide, Lácer).

2.2. Apparatus

The spectra of the diuretics were obtained with a UV–visible diode array spectrophotometer (Hewlett-Packard Model 8452, Palo Alto, CA, USA), and the pH was measured with a potentiometer provided with a combined Ag/AgCl/glass electrode (Crison Model micropH 2001, Barcelona, Spain).

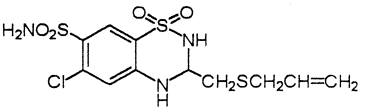
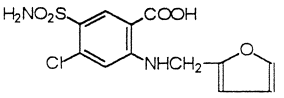
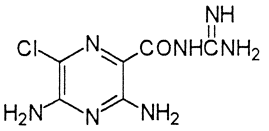
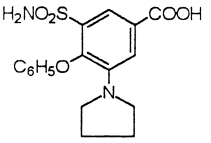
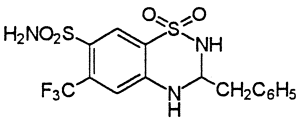
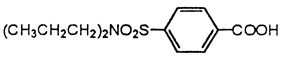
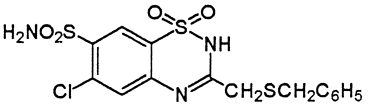
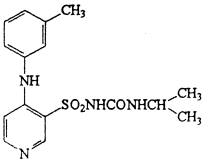
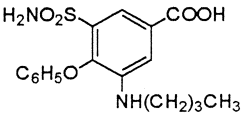
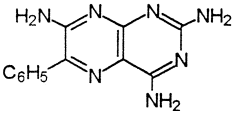
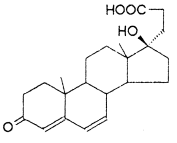
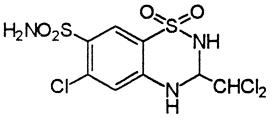
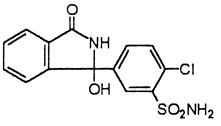
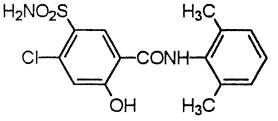
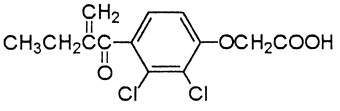
A chromatograph (Hewlett-Packard Model HP 1050) equipped with an isocratic pump and an autosampler (Model HP 1100) was used. An ODS-2 column (5 μm particle size, 125 mm×4.6 mm I.D.) was placed after a 30-mm long guard column of similar characteristics (Scharlau). Injection of the solutions into the chromatograph was made through a valve (Rheodyne, Cotati, CA, USA) provided with a standard capillary having a maximal volume of 100 μl. The injection volume was 20 μl, the flow-rate 1 ml/min, and the dead time 0.99 min. The SDS mobile phases and the diuretic solutions were filtered through 0.45-μm nylon membranes (Micron Separations, Westboro, MA, USA).

Monitoring of chromatographic separation was performed with a UV–visible detector (Hewlett-Packard Model HP 1050) set at 274 nm. The signal was acquired by a personal computer connected to the chromatograph through an integrator (Hewlett-Packard Model HP 3396A), using the PEAK-96 software (Hewlett-Packard, Avondale, PA, USA).

2.3. Sample preparation

The analyses were performed with 1-ml urine samples, which were diluted for most diuretics in a 1:25 factor with 0.10 *M* SDS at pH 3. No other

Table 1
Structure and protonation constants of diuretics

Compound	Structure	Log *K*	Compound	Structure	Log *K*
Althiazide		ND	Furosemide		3.8, 7.5[a]
Amiloride		8.7[a]	Piretanide		4.1[a]
Bendroflumethiazide		9.0[a]	Probenecid		3.4[d]
Benzthiazide		6.0[a]	Torasemide		6.9[c]
Bumetanide		3.6, 7.7[a]	Triamterene		6.2[d]
Canrenoic acid		4.6[b]	Trichloromethiazide		7.3, 8.6, 10.6[d]
Chlorthalidone		9.35[a]	Xipamide		4.8, 10[d]
Ethacrynic acid		3.5[a]			

[a] Ref. [11].
[b] Ref. [26].
[c] Ref. [27].
[d] Ref. [28].
ND, No data found.

treatment than filtration (which was carried out directly into the autosampler vials) was made before injection into the chromatograph.

3. Mathematical treatment

The elution behaviour of the diuretics with mobile phases of surfactant and alcohol at variable pH was described using the following equation [25]:

$$k = \frac{K_{AS} \cdot \frac{1}{1+K_{AD}\varphi} + K_{HAS} \cdot \frac{1}{1+K_{HAD}\varphi} \cdot K_H[H^+]}{\left(1+K_{AM} \cdot \frac{1+K_{MD}\varphi}{1+K_{AD}\varphi} \cdot [M]\right) + \left(1+K_{HAM} \cdot \frac{1+K_{HMD}\varphi}{1+K_{HAD}\varphi} \cdot [M]\right)} \quad (1)$$

where k represents the retention factor, [M] is the concentration of surfactant forming micelles (total concentration of surfactant minus critical micellar concentration), φ the volume fraction of organic modifier, $[H^+]$ the proton concentration, K_H the protonation constant of the compound; K_{AS} and K_{AM} are constants that measure the association of the basic species to the stationary phase and micelles, respectively, and K_{AD} and K_{MD} quantify linear modifications in the equilibria of this species towards bulk water and micelles, respectively, due to the presence of organic solvent in the micellar solution. The constants K_{HAS}, K_{HAM}, K_{HAD} and K_{HMD} correspond to the acidic species.

The resolution of the peaks in a chromatogram was evaluated with:

$$R = \prod_{i=1}^{p} r_i = \prod_{i=1}^{p} \left(1 - \frac{w_i'}{w_i}\right) \quad (2)$$

where w_i represents the total area of peak i and w_i' the overlapped area of that peak with the chromatogram formed by the remaining peaks. The elementary value, r_i, is a measurement of the peak purity. The proximity of the combined value, R, to 1 indicates the performance of the separation. Peak shapes, which are required to calculate w_i and w_i', were predicted using a linearly-modified Gaussian model [29].

The optimal mobile phase composition that resolves the mixture of diuretics was achieved through the simulation of chromatograms corresponding to 9261 (21^3) mobile phases arranged in a regular distribution containing 21 levels for each experimental factor (concentrations of surfactant, organic modifier and proton). Next, all those mobile phase compositions leading to retention values greater than 35 min were discarded (the remaining mobile phases were 5292). Peak purities (r_i) were calculated for each one of the n compounds in each mobile phase, giving rise to a set of n vectors. For a conventional optimisation (i.e., achievement of a single optimal mobile phase), the combined resolution vector is calculated by multiplying all peak purity vectors element-by-element. The maximal R value indicates the optimal resolution and the position in the vector the corresponding mobile phase composition.

Alternatively, two or three optimal complementary mobile phases (CMPs), which altogether resolve the mixture were obtained. Although a full description of the methodology used to obtain the composition of the CMPs was previously reported [30], some details are next outlined. First, all possible distributions of the n compounds in two or three subsets (for two and three CMPs, respectively) are obtained. The combined resolution vector for the compounds assigned to each subset (for a particular distribution) is calculated and the mobile phase giving maximal resolution found, as in a conventional single mobile phase optimisation. The maximal values for each one of the two or three subsets are then multiplied to obtain a global value representing the resolution for that compound distribution, linked to the two or three corresponding CMPs. This process is carried out for all possible distributions that can be made with the n compounds. The maximal global resolution indicates the best combination of mobile phases that would resolve the mixture, the optimal CMPs.

In this work, 15 compounds were considered which results in 13 283 and 2 375 101 different distributions of the compounds for two and three CMPs, respectively. The high number of distributions makes an exhaustive systematic search of the optimal mobile phase impractical. The use of a genetic algorithm (GA) is a faster solution. In this approach, compound distributions are encoded, by identifying them to chromosomes. Let us consider a simple example with five compounds with a distribution represented by a chromosome such as [1 1 2 2 1], which means that two CMPs will be searched

and compounds 1, 2 and 5 should be resolved with the first CMP, whereas compounds 3 and 4 should be resolved with the second CMP. The global resolution linked to a given chromosome will be the product of combined resolutions for the compounds resolved with each CMP. The GA heuristically changes the chromosome by maximising this product up to convergence.

All software used for peak measurement, data treatment, simulation and optimisation, was developed by the authors using QuickBasic Extended (Microsoft, 1985–1989) and Visual Basic 5.0 (Microsoft, 1987–1997). The chromatographic data were treated with MICHROM (Marcel Dekker, 2000) [31]. Laboratory-built routines written in MATLAB 4.2c (Mathworks) were used for the search of the optimal CMPs through the application of GAs.

4. Results and discussion

4.1. Effect of pH on the retention of diuretics

The mixture of diuretics consisted of four compounds (althiazide, amiloride, bendroflumethiazide and chlorthalidone), which do not show any acid–base behaviour in the working pH range of the C_{18} column, and 11 compounds that are protonated at different pH values (benzthiazide, bumetanide, canrenoic acid, ethacrynic acid, furosemide, piretanide, probenecid, torasemide, triamterene, trichloromethiazide and xipamide) (Table 1). The acidic strengths in aqueous medium of these compounds (log protonation constant, log *K*) decreased in the order: probenecid (3.4), ethacrynic acid (3.5), bumetanide (3.6), furosemide (3.8), piretanide (4.1), canrenoic acid (4.6), xipamide (4.8), benzthiazide (6.0), triamterene (6.2), torasemide (6.9), and trichloromethiazide (7.3).

The change in retention factors with pH in a micellar mobile phase of 0.05 *M* SDS–4.0% propanol is shown in Fig. 1 for some diuretics. The retention of althiazide, amiloride, bendroflumethiazide and chlorthalidone did not change in the pH range 3–7. Benzthiazide, triamterene and trichloromethiazide suffered a partial deprotonation at pH 7, and ethacrynic acid was only partially protonated at pH 3. Finally, a sigmoidal decrease in *k* values with pH was observed for bumetanide, canrenoic acid, furosemide, piretanide, probenecid, torasemide and xipamide. For these diuretics, the retention was very low at pH>6 where the basic species dominates. The greater retention of most diuretics in acidic medium results from the preferential attraction of the protonated species towards the surface of the stationary phase modified by the adsorption of surfactant monomers with respect to the micelles.

Log *K* values were calculated using the *k* vs. pH data at three concentrations of SDS and increasing volume fraction of propanol in the mobile phase, for the diuretics that show a sigmoidal acid–base behaviour (bumetanide, ethacrynic acid, furosemide, piretanide, probenecid, torasemide and xipamide). As shown in Table 2, log *K* is shifted towards higher pH when the concentration of SDS is increased, whereas it decreases at increasing volume fraction of the alcohol. The greater changes in log *K* are observed upon addition of 1% propanol to pure micellar mobile phases (without alcohol). Also, the protonation constants are usually greater in micellar medium with respect to the aqueous non-micellar medium (Table 1). This indicates that the pH region where the protonated (the most retained) species dominates is increased in the micellar chromatographic system.

The efficiencies (*N*) of the chromatographic peaks (measured at 10% of peak height [32]) of some diuretics were enhanced in acidic medium. Thus, for a mobile phase of 0.05 *M* SDS–6.0% propanol, the efficiencies at pH 7 and 3 were: althiazide (1425, 1515), amiloride (225, 600), bendroflumethiazide (1930, 1950), benzthiazide (970, 1600), bumetanide (320, 2600), canrenoic acid (270, 980), chlorthalidone (1210, 1325), ethacrynic acid (270, 3670), furosemide (240, 1960), piretanide (230, 2480), probenecid (300, 3420), torasemide (1055, 2440), triamterene (170, 700), trichloromethiazide (525, 1160), and xipamide (130, 3300). As observed, the efficiencies of neutral and basic diuretics did not change with pH, and the greatest enhancements corresponded to the most acidic compounds. In aqueous–organic RPLC, the addition of amines to the eluents is usual to improve peak shape by association with the free silanol groups on the stationary phase [6,33]. Amines seem to be less necessary in micellar RPLC [14–16]. The hydro-

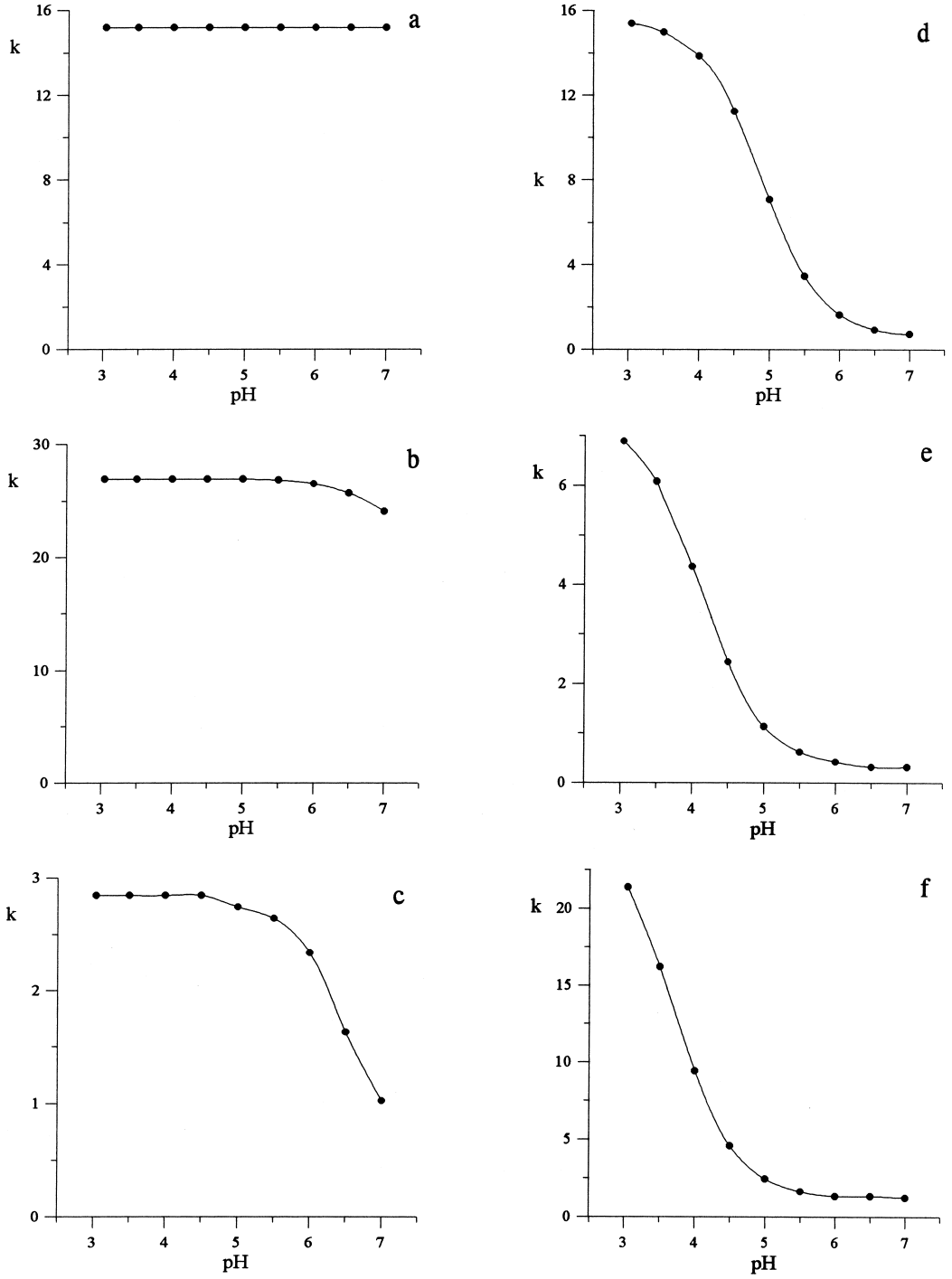


Fig. 1. Effect of pH on the retention of the diuretics: (a) amiloride, (b) triamterene, (c) trichloromethiazide, (d) bumetanide, (e) furosemide, and (f) ethacrynic acid. Mobile phase composition: 0.05 *M* SDS–4.0% propanol.

Table 2
Protonation constants of several diuretics at increasing concentration of SDS and propanol

Compound	SDS (*M*)	Propanol (%, v/v)						
		0	1	2	3	4	5	6
Bumetanide	0.05	ND[a]	5.35±0.10	5.10±0.11	4.96±0.11	4.87±0.13	4.80±0.07	4.72±0.14
	0.10	ND	5.68±0.10	5.43±0.05	5.26±0.10	5.14±0.13	5.03±0.19	4.93±0.10
	0.15	ND	5.94±0.06	5.63±0.10	5.41±0.08	5.27±0.09	5.18±0.03	5.08±0.03
Ethacrynic acid	0.05	5.02±0.12	4.11±0.08	3.95±0.09	3.89±0.07	3.83±0.07	3.80±0.05	3.78±0.03
	0.10	5.31±0.11	4.35±0.08	4.21±0.10	4.06±0.09	4.00±0.03	3.98±0.10	3.95±0.05
	0.15	5.42±0.03	4.48±0.08	4.31±0.07	4.16±0.09	4.13±0.03	4.10±0.07	4.08±0.03
Furosemide	0.05	4.54±0.10	4.36±0.07	4.28±0.10	4.24±0.07	4.22±0.07	4.16±0.09	4.123±0.013
	0.10	4.83±0.11	4.64±0.07	4.50±0.11	4.47±0.04	4.44±0.02	4.42±0.08	4.39±0.05
	0.15	5.03±0.08	4.86±0.09	4.68±0.04	4.66±0.14	4.62±0.03	4.59±0.06	4.57±0.09
Piretanide	0.05	5.61±0.05	5.01±0.15	4.86±0.09	4.75±0.10	4.65±0.15	4.61±0.12	4.58±0.03
	0.10	6.12±0.06	5.45±0.06	5.23±0.11	5.10±0.14	4.97±0.19	4.92±0.18	4.88±0.16
	0.15	6.42±0.05	5.78±0.04	5.39±0.10	5.25±0.08	5.14±0.15	5.05±0.05	4.99±0.04
Probenecid	0.05	5.37±0.09	4.65±0.11	4.51±0.11	4.44±0.09	4.35±0.07	4.33±0.03	4.30±0.03
	0.10	5.74±0.06	5.07±0.10	4.86±0.04	4.75±0.06	4.64±0.12	4.57±0.06	4.52±0.10
	0.15	6.00±0.08	5.27±0.05	5.09±0.05	4.98±0.08	4.91±0.08	4.81±0.11	4.74±0.04
Torasemide	0.05	5.28±0.09	5.15±0.09	5.03±0.11	4.93±0.10	4.83±0.10	4.71±0.10	4.61±0.08
	0.10	5.46±0.09	5.19±0.08	5.07±0.12	4.96±0.10	4.85±0.10	4.72±0.10	4.61±0.09
	0.15	5.55±0.09	5.24±0.09	5.11±0.08	5.00±0.09	4.87±0.09	4.74±0.08	4.61±0.08
Xipamide	0.05	ND	5.64±0.15	5.43±0.10	5.36±0.08	5.30±0.09	5.25±0.09	5.20±0.07
	0.10	ND	5.92±0.10	5.75±0.08	5.63±0.08	5.55±0.07	5.51±0.08	5.47±0.10
	0.15	ND	6.27±0.13	5.98±0.09	5.87±0.09	5.81±0.06	5.78±0.05	5.74±0.05

[a] No data obtained.

philic layer formed by the sulfate head groups of SDS above the surface of the silica makes the association kinetics, which is controlled primarily by electrostatic interactions, easier than ion-exchange processes involving the silanol groups on the silica surface. Also, the interaction of the protonated solutes with the hydrophilic layer formed by SDS reduces the penetration depth of the compounds into the bonded phase. The role of silanol groups is thus diminished.

The spectra of the diuretics in SDS solutions showed two or three absorption bands. The maximum wavelengths of the diuretics without acid–base behaviour did not change in the pH range 3–7 (althiazide, amiloride, bendroflumethiazide and chlorthalidone). The other diuretics exhibited changes of diverse importance towards lower wavelengths in one or more maxima. The mean maximum wavelengths for these compounds did not change with pH, however. The wavelengths of the three bands were 335±20, 274±12 and 223±11 nm at pH 3; 333±20, 272±12 and 224±4 nm at pH 5; and 337±18, 274±12 and 220±6 nm at pH 7. Finally, the chromatographic signal was monitored at 274 nm. At this wavelength, xipamide was satisfactorily detected although it shows a maximum at 302 nm.

4.2. Resolution of complex mixtures of diuretics

Eleven of the 15 diuretics considered in this work show acid–base behaviour in the working pH range of the C_{18} column. The dependence of their retention with the pH of the mobile phase is however diverse. The selectivity of the separation of mixtures of the diuretics was thus influenced by three factors: pH and concentrations of surfactant and alcohol, which

should be taken into account in the optimisation process. The optimisation of the resolution can certainly be made at a preselected pH level, but a full exploration is advisable in order to increase the probability of success, and also to take full advantage of the three main optimisation factors. In any case, the optimisation process is facilitated through the use of an elution model.

In previous work, we demonstrated that Eq. (1) provides good predictions of the retention in micellar mobile phases at variable pH [25]. This equation has nine parameters (K_{AM}, K_{AS}, K_{MD}, K_{AD}, K_{AHM}, K_{AHS}, K_{HMD}, K_{HAD} and K_{H}), and requires therefore a suitable design including at least nine mobile phases. We have demonstrated previously, however, that the data from 12 to 15 mobile phases should be used to obtain more reliable parameters and decrease the prediction errors [21]. In any case, the use of a number of mobile phases larger than the number of parameters in the model permits the evaluation of the accuracy of the predictions. The experimental design used to model the elution behaviour of the diuretics consisted of 15 mobile phases distributed at three pH levels (3, 5 or 7). The composition of the mobile phases at each pH level was: 0.05 *M* SDS–2.0% propanol, 0.15 *M* SDS–2.0% propanol, 0.05 *M* SDS–6.0% propanol, 0.15 *M* SDS–6.0% propanol and 0.10 *M* SDS–4.0% propanol (four located in the corners of the rectangular factor domain and one at its centre).

The global relative prediction errors obtained with Eq. (1) are given in Table 3. The accuracy of the three-factor model is excellent, usually below 2%. It should be noted that the retention factor of the basic species of benzthiazide, triamterene and trichloromethiazide, and the acidic species of furosemide, probenecid, and especially ethacrynic acid, cannot be obtained in the studied pH range. This could result in an uncertainty in the estimation of the parameters of Eq. (1), which will be translated into large errors in the prediction of the retention in critical regions where the compounds are being protonated. However, as observed, the errors for these diuretics were similar to other compounds. On the other hand, piretanide, bumetanide and xipamide at pH 7, and canrenoic acid at pH 3, showed double peaks for the mobile phases of lowest and largest elution strengths, respectively. In the modelling process only one of these peaks was considered, which yielded good results.

Table 3
Relative global prediction errors for the diuretics (Eq. (1))[a]

Compound	ϵ (%)	Compound	ϵ (%)
Althiazide	1.5	Furosemide	0.6
Amiloride	1.8	Piretanide	1.9
Bendroflumethiazide	0.9	Probenecid	1.3
Benzthiazide	0.9	Torasemide	1.6
Bumetanide	2.0	Triamterene	1.0
Canrenoic acid	1.3	Trichloromethiazide	1.7
Chlorthalidone	0.9	Xipamide	2.3
Ethacrynic acid	0.7		

[a] Fifteen mobile phases.

We tried to resolve initially the mixture of 15 diuretics. However, at the optimal conditions found (0.05 *M* SDS–3.0% propanol at pH 5.3), the elution of torasemide required 60 min, an extreme overlapping existed between chlorthalidone–althiazide, and a partial overlapping between trichloromethiazide–probenecid–piretanide and althiazide–benzthiazide–bumetanide (Fig. 2 top and middle). An optimisation performed by limiting the retention time of the last eluting compound at 35 min (0.065 *M* SDS–3.8% propanol at pH 5.2) yielded much greater overlapping of the diuretics at the beginning of the chromatogram, except for furosemide (Fig. 2 bottom).

All the diuretics could be fully resolved individually in less than 35 min (i.e., limiting individual resolutions were $r_L > 0.99$), when found together in a mixture using specific mobile phase compositions for each diuretic, except chlorthalidone ($r_L = 0.988$) and probenecid ($r_L = 0.978$), which showed partial overlapping. This suggested the possibility of using another optimisation strategy developed in our laboratory, which leads to two or three mobile phases which complement each other to resolve all the compounds in a mixture [30]. The complementary mobile phases are selected in such a way that each one resolves optimally only some compounds in the mixture, while the remaining compounds which are resolved by the other mobile phases, can overlap among them. In previous work, this strategy was applied to problems involving one [34] or two [30] factors. We show here the application to a three-factor separation problem, and include also the condition of limiting the retention time of the last

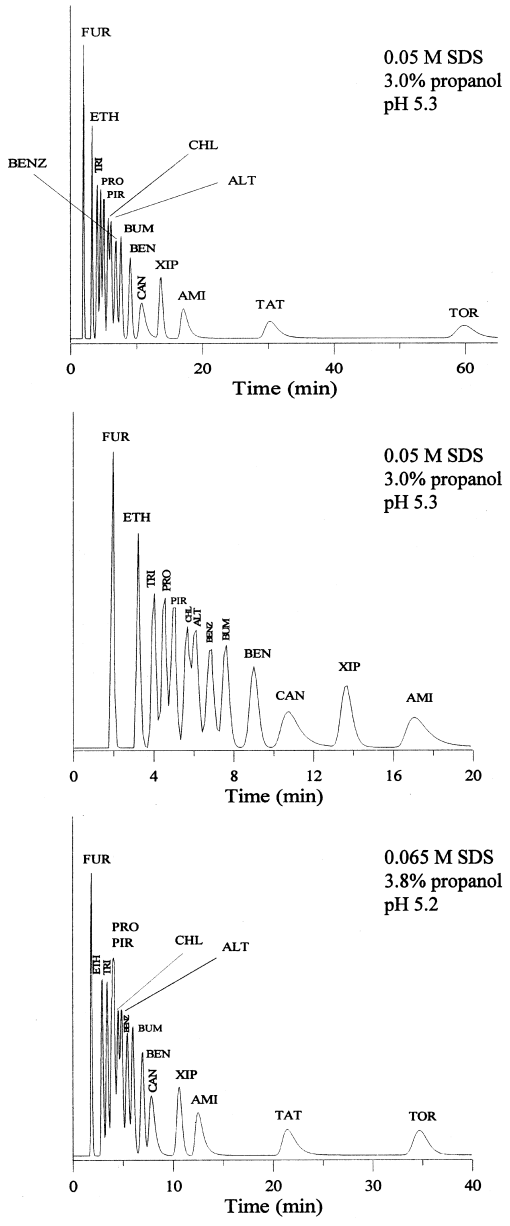


Fig. 2. Chromatograms of a mixture of 15 diuretics for the optimal mobile phase compositions obtained: (top and middle) without limiting the retention time, and (bottom) limiting the retention time at 35 min. Chromatogram (middle) is an expansion of chromatogram (top) for retention times below 20 min. Compounds: althiazide (ALT), amiloride (AMI), bendroflumethiazide (BEN), benzthiazide (BENZ), bumetanide (BUM), canrenoic acid (CAN), chlorthalidone (CHL), ethacrynic acid (ETH), furosemide (FUR), piretanide (PIR), probenecid (PRO), torasemide (TOR), triamterene (TAT), trichloromethiazide (TRI), and xipamide (XIP).

eluting compound. The use of two or more separate chromatographic analyses to resolve complex samples has also been proposed by Dolan et al., using as separation variables temperature and gradient time [35].

Fig. 3 illustrates the chromatograms of the mixture of 15 diuretics separated with two CMPs, where the analysis time of torasemide was fixed at 35 min. Furosemide, trichloromethiazide, althiazide, amiloride, torasemide and triamterene, on the one

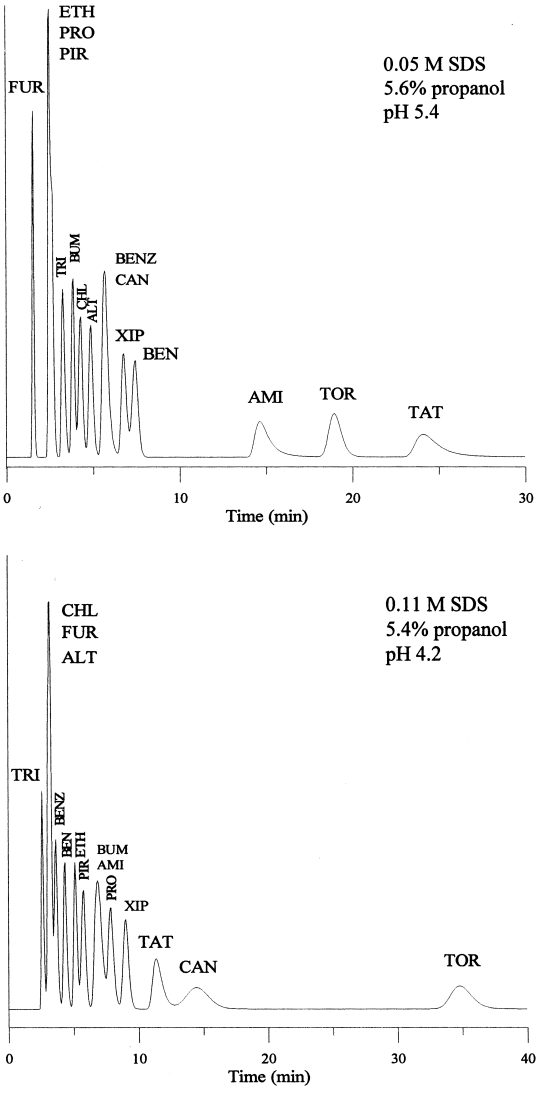


Fig. 3. Chromatograms of a mixture of 15 diuretics eluted with two optimal CMPs. See Fig. 2 for peak identity.

hand, and bendroflumethiazide, xipamide, triamterene, canrenoic acid and torasemide, on the other, were fully resolved with 0.05 *M* SDS–5.6% propanol at pH 5.4 (Fig. 3 top) and 0.11 *M* SDS–5.4% propanol at pH 4.2 (Fig. 3 bottom), respectively. Partial overlapping still existed for bumetanide, chlorthalidone, xipamide and bendroflumethiazide eluted with the first mobile phase, and for trichloromethiazide, benzthiazide, ethacrynic acid, piretanide and probenecid with the second mobile phase. This means that using both mobile phases, all the diuretics are fully or partially resolved.

The separation is obviously enhanced using three CMPs. Fourteen diuretics were fully resolved in these conditions: furosemide, trichloromethiazide, althiazide, xipamide, bendroflumethiazide, amiloride, torasemide and triamterene with 0.05 *M* SDS–5.8% propanol at pH 5.4 (Fig. 4 top), bumetanide, canrenoic acid, benzthiazide, bendroflumethiazide, torasemide, amiloride and triamterene with 0.05 *M* SDS–2.6% propanol at pH 6.6 (Fig. 4 middle), and bendroflumethiazide, ethacrynic acid, piretanide, probenecid, xipamide and torasemide with 0.14 *M* SDS–3.8% propanol at pH 4.4 (Fig. 4 bottom). Chlorthalidone could also be resolved sufficiently with 0.05 *M* SDS–5.8% propanol at pH 5.4.

The separation of a mixture of 12 diuretics (the 15 indicated above except canrenoic acid, probenecid and torasemide) was finally considered. Torasemide showed much greater retention than the other diuretics, probenecid usually overlapped with piretanide, and canrenoic acid showed two peaks at the selected mobile phase, which made the analysis of the mixtures of 15 diuretics difficult. Frequent changes in the elution order were found by changing the composition of the mobile phase at several pH values, which can be problematic in the application of an optimisation strategy. At pH 3, bendroflumethiazide–furosemide and amiloride–piretanide, and at pH 4, benzthiazide–furosemide, amiloride–piretanide and ethacrynic acid–bumetanide changed their elution order. At pH 5 and 6, the retention of other diuretics also reversed. These changes in elution order are however irrelevant with the optimisation procedure applied in this work.

The three-factor approach yielded three regions of maximal resolution with the following optimal compositions: 0.05 *M* SDS–3.0% propanol at pH 5.2 ($R=0.588$), 0.05 *M* SDS–4.2% propanol at pH 4.2 ($R=0.589$), and 0.055 *M* SDS–6.0% propanol at pH 3.0 ($R=0.544$). The optima at pH 5.2 and 4.2 were

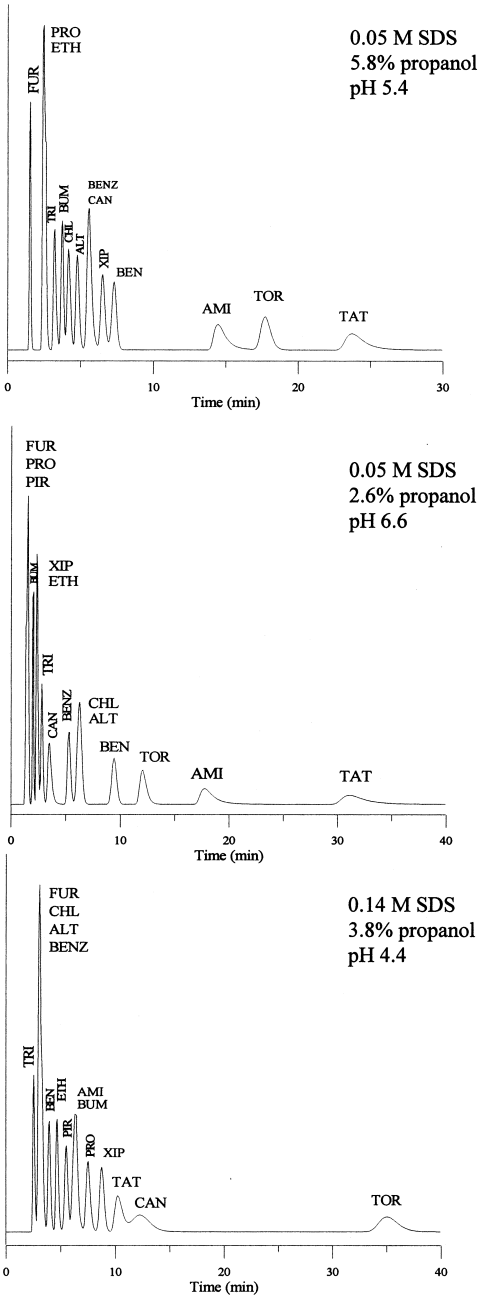


Fig. 4. Chromatograms of a mixture of 15 diuretics eluted with three optimal CMPs. See Fig. 2 for peak identity.

however poorly robust since a small change in pH produced significant changes in the relative positions of the peaks of some diuretics. The predicted and experimental chromatograms of the diuretics in aqueous solution and urine matrix for the mobile phase at pH 3.0 are compared in Fig. 5. The errors in the prediction of the retention for some diuretics are due to the strong dependence of the retention with pH. This is a problem not exclusive of micellar RPLC but found in any chromatographic technique where the retention is affected by pH. In previous work, the high accuracy of the predictions made in micellar mobile phases at fixed pH has been extensively demonstrated [36].

4.3. Application of the method to urine samples

The background signal of urine samples due to the proteins (wide band at the beginning of the chromatograms), and diverse endogenous compounds

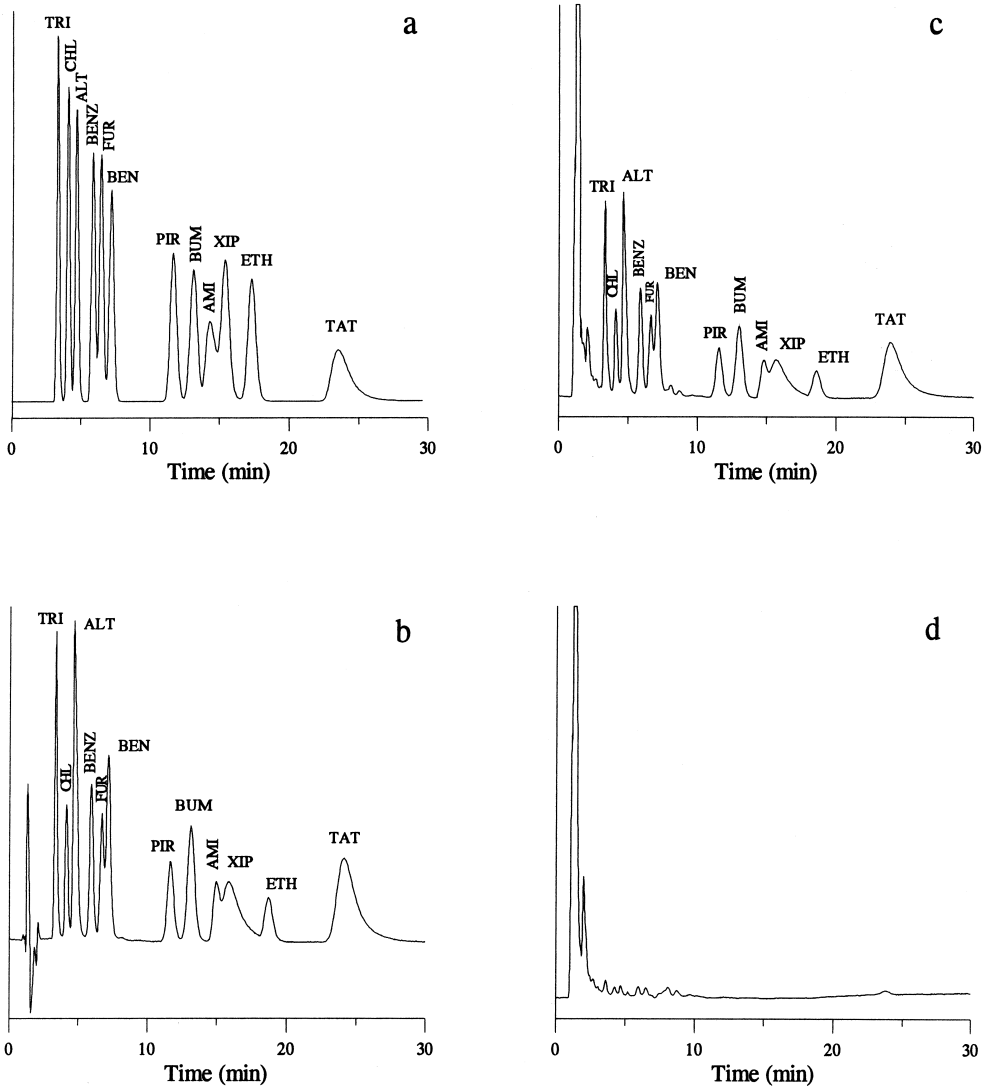


Fig. 5. Predicted (a) and experimental chromatograms in aqueous solution (b) and spiked urine sample (c); chromatogram of urine blank (d), for the diuretics eluted with 0.055 *M* SDS–6.0% propanol at pH 3.0. See Fig. 2 for peak identity.

(peaks at diverse retention times), can affect the detection of drugs. For this reason, some diuretics such as hydrochlorothiazide, hydroflumethiazide and chlorothiazide, which elute at very low retention times with the SDS–propanol mobile phases, could not be considered. Fig. 5 illustrates chromatograms of urine matrix and a spiked sample using 0.055 *M* SDS–6.0% propanol at pH 3.0.

The injection of a large number of urine samples can damage the packing material and shorten the life of the chromatographic column, or can force a frequent regeneration of the stationary phase. The analysis of the samples was consequently carried out after dilution. In these conditions, the retention times did not change at least after 200 injections. For most diuretics, the sensitivity achieved after dilution in a 1:25 factor was adequate for their detection in urine. The signals of bumetanide and chlorthalidone were however too small to be detected at very low concentrations. Injection of these diuretics was made without dilution.

Calibration curves using the areas of the chromatographic peaks were constructed in the range 1–20 μg/ml for amiloride, chlorthalidone, bendroflumethiazide, benzthiazide, bumetanide and piretanide, 0.8–16 μg/ml for ethacrynic acid, furosemide, triamterene and trichloromethiazide, 0.8–20.2 μg/ml for xipamide, and 0.2–8 μg/ml for althiazide, making duplicate injections of five solutions at increasing concentration. The regression coefficients were always $r > 0.999$.

The limits of detection (LODs, 3s criterion) were calculated from the standard deviations of seven-fold injections of urine samples spiked with the diuretics in the range 0.2–1.0 μg/ml (Table 4). The LODs were similar to those usually reported in the literature for aqueous–organic RPLC, taking into account that in this work the urine samples were injected without any previous treatment to separate or preconcentrate the analytes. The intra- and inter-day assay accuracy and precision of the procedure are given in Table 5. The analysis of the spiked samples gave values close to those expected. The relative standard deviations for the inter-day assays were in the range 0.15–2.1%.

Single oral doses of some diuretics were administered to several volunteers (amount administered is given in parenthesis): althiazide (15 mg), amiloride (20 mg), bendroflumethiazide (5 mg), bumetanide (1 mg), chlorthalidone (50 mg), furosemide (40 mg), piretanide (6 mg), triamterene (150 mg), trichloromethiazide (3 mg) and xipamide (20 mg). In each case, a sample was collected to be used as blank just before the administration of the drugs. The urine samples were refrigerated at 4°C until been analysed. The diuretics were detected in urine at least up to 24 h after oral administration. Fig. 6 shows chromatograms of urine samples containing amiloride, bumetanide, furosemide, piretanide and xipamide.

Table 4
Limits of detection for several diuretics in aqueous solution and urine samples

Compound	LOD (ng/ml)	
	Water	Urine
Althiazide	6.9	6.2
Amiloride	86	136
Bendroflumethiazide	7.1	16
Benzthiazide	18	60
Bumetanide	30	32
Chlorthalidone	49	127
Ethacrynic acid	22	40
Furosemide	6.5	15.5
Piretanide	61	61
Triamterene	64	32
Trichloromethiazide	11	73
Xipamide	18	24

5. Conclusions

Screening of diuretics in urine samples is feasible under isocratic conditions, using micellar RPLC with hybrid mobile phases of SDS and propanol, and direct injection of the samples into the chromatographic system. Conventional RPLC procedures with aqueous–organic mobile phases require the extraction of the diuretics. However, there are no single extraction conditions for all compounds of the group due to their different polarity [11]. Also, during the extraction, several endogenous compounds are co-extracted from urine owing to their polar characteristics, and chromatographed within the first minutes of elution [6]. This forces the use of different chromatographic conditions for diuretics of different polarities. The background of the matrix is a limitation to the direct injection of urine samples in

Table 5
Accuracy and precision in intra- and inter-day assays

Compound (taken, μg/ml)	Found (μg/ml)[a]	Compound (taken, μg/ml)	Found (μg/ml)[a]
Althiazide (0.60)	0.588±0.003	Benzthiazide (1.96)	2.009±0.015
	0.583±0.003		2.00±0.02
	0.581±0.005		2.00±0.02
	0.565±0.002		1.95±0.03
	0.563±0.003		1.99±0.03
	0.5589±0.0015		1.993±0.011
Mean (μg/ml)	0.573±0.012	Mean (μg/ml)	1.99±0.02
Amiloride (1.78)	1.808±0.014	Bumetanide (2.04)	2.037±0.007
	1.809±0.007		2..034±0.005
	1.802±0.007		2.035±0.007
	1.804±0.005		2.041±0.006
	1.801±0.006		2.039±0.005
	1.801±0.008		2.040±0.003
Mean (μg/ml)	1.804±0.004	Mean (μg/ml)	2.038±0.003
Bendroflumethiazide (1.54)	1.524±0.006	Chorthalidone (2.06)	2.173±0.017
	1.519±0.006		2..169±0.011
	1.515±0.007		2.17±0.02
	1.513±0.013		2.149±0.011
	1.503±0.011		2.156±0.010
	1.494±0.013		2.171±0.009
Mean (μg/ml)	1.511±0.011	Mean (μg/ml)	2.165±0.010
Ethacrynic acid (2.20)	2.217±0.010	Triamterene (1.60)	1.687±0.007
	2.212±0.004		1.681±0.006
	2.213±0.010		1.682±0.006
	2.212±0.010		1.679±0.004
	2.209±0.008		1.680±0.004
	2.208±0.005		1.676±0.007
Mean (μg/ml)	2.212±0.003	Mean (μg/ml)	1.681±0.004
Furosemide (1.10)	1.151±0.002	Trichloromethiazide (1.10)	1.104±0.006
	1.150±0.003		1.061±0.006
	1.142±0.003		1.052±0.005
	1.135±0.008		1.0477±0.0019
	1.139±0.008		1.042±0.011
	1.136±0.006		1.035±0.018
Mean (μg/ml)	1.142±0.007	Mean (μg/ml)	1.06±0.02
Piretanide (1.56)	1.561±0.007	Xipamide (2.02)	2.060±0.006
	1.557±0.005		2..048±0.018
	1.554±0.006		2.051±0.009
	1.549±0.004		2.054±0.007
	1.556±0.009		2.056±0.004
	1.551±0.004		2.056±0.005
Mean (μg/ml)	1.555±0.004	Mean (μg/ml)	2.054±0.004

[a] Intra-day values obtained with seven-fold injections of spiked urine samples.

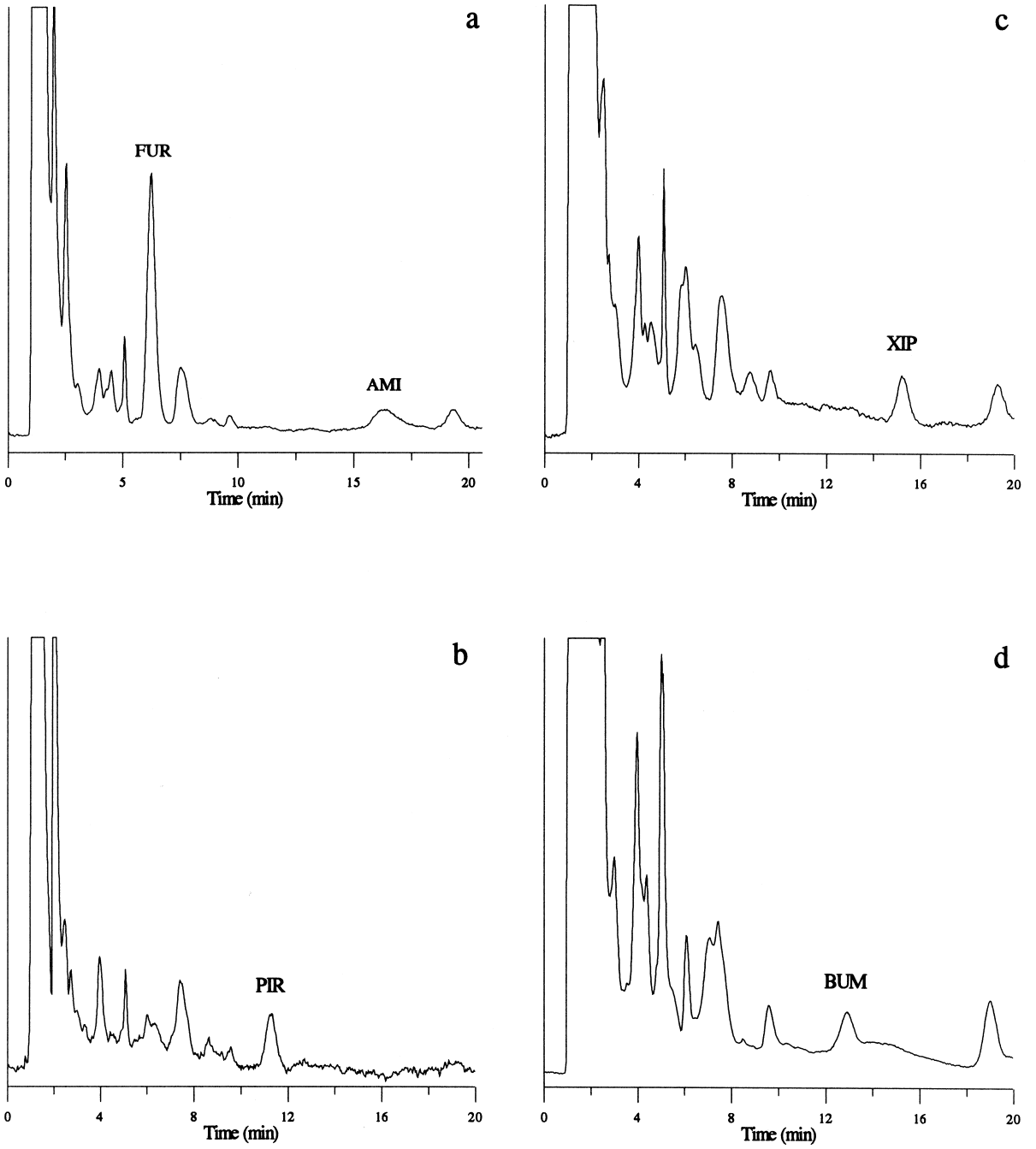


Fig. 6. Chromatograms of urine samples taken from several volunteers, which were administered the diuretics (detected concentration and time from ingestion): (a) furosemide (0.9 μg/ml, 9 h), amiloride (0.3 μg/ml, 9 h), (b) piretanide (2.0 μg/ml, 5 h), (c) xipamide (0.5 μg/ml, 7 h), and (d) bumetanide (0.6 μg/ml, 4.5 h). Mobile phase composition: 0.055 *M* SDS–6.0% propanol at pH 3.0. Urine samples were diluted in a 1:25 factor, except for piretanide and bumetanide which were injected without dilution.

micellar RPLC, but only the detection of low retained diuretics such as the thiazides hydrochlorothiazide, hydroflumethiazide and chlorothiazide is hampered due to overlapping with the protein band at the beginning of the chromatograms.

Micellar RPLC shows two additional advantages for the analysis of diuretics. Usually, good peak symmetry of weakly acidic or basic diuretics is only obtained in conventional RPLC if an organic competing amine or an ammonium salt is added to the acidic eluent [6,7,10]. The organic base prevents the diuretics to be retained by the free silanol groups of the column. The surfactant coverage of the column makes the addition of amines less necessary, since sufficiently well-shaped peaks are obtained in acidic micellar mobile phases. On the other hand, gradient elution is often required in conventional RPLC to expedite the elution of low polar diuretics in mixtures showing diverse polarities. In contrast, in micellar RPLC, the range of elution strengths required to elute hydrophobic and hydrophilic compounds is narrower. This allows the isocratic separation of the diuretics in adequate analysis times.

Finally, the predictable elution behaviour of micellar mobile phases has been once more demonstrated in spite of the rapid changes in retention with pH, which affects the reliability of the separation of compounds exhibiting acid–base behaviour in any chromatographic technique. The optimisation procedure permitted the detailed examination of a wide range of experimental conditions (pH and concentrations of surfactant and organic modifier), and succeeded in finding the optimal mobile phase composition to screen the diuretics. Twelve diuretics could be resolved in 25 min using 0.055 *M* SDS–6.0% propanol at pH 3.0. A more complex mixture of 15 diuretics could also be separated using two or three mobile phases showing complementary behaviour.

Acknowledgements

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