Analysis of Urine Samples Containing Cardiovascular Drugs by Micellar Liquid Chromatography with Fluorimetric Detection

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Abstract

A simple direct injection chromatographic procedure with fluorimetric detection is successfully applied to the determination of mixtures of 4 diuretics (amiloride, bendroflumethiazide, piretanide, and triamterene) and 6 β-blockers (acebutolol, atenolol, labetalol, metoprolol, nadolol, and propranolol), which are usually administered in combinations for the treatment of hypertension, in urine samples. The procedure makes use of C_{18} columns and micellar mobile phases of sodium dodecyl sulphate (SDS), propanol, and phosphate buffer at pH 3. The adequate resolution of most drugs is obtained with a chemometrics approach where the retention is modeled as a first step using the retention factors in only 5 mobile phases. Afterward, an optimization criterion that takes into account the position and shape of the chromatographic peaks is applied. A mobile phase of 0.11M SDS–8% propanol could resolve mixtures of 8 drugs and was adequate for the analysis of the combinations of diuretic and β-blocker usually prescribed. However, a mobile phase of larger elution strength, such as 0.15M SDS–15% propanol, is preferred for the analysis of mixtures of amiloride–metoprolol, amiloride–labetalol, and triamterene–propranolol. The method is sensitive enough for the routine analysis of diuretics and β-blockers at therapeutic urine levels with limits of detection in the 0.5–28-ng/mL range. Urinary excretion studies show that the detection of most drugs is possible up to 24–72 h after their ingestion.

Introduction

Diuretics and β-blockers are administered in combination because of their additive effect in the treatment of hypertension (1–3). The simultaneous prescription of these drugs is necessary when appropriate control of the arterial pressure is not possible with each of them separately. β-Blockers reduce the arterial pressure, the cardiac rhythm, and the contractility of myocardium (and consequently, the cardiac waste). However, some blocking agents administered alone retain considerable amounts of water and electrolytes. The role of diuretics is to enhance renal excretion, which produces a further reduction of the arterial pressure.

Several associations of diuretics and β-blockers are used throughout the world. Here, the development of a rapid and simple procedure for analysis of urine samples containing both drugs is shown. The investigated compounds were the diuretics amiloride, bendroflumethiazide, piretanide, and triamterene and the β-blockers acebutolol, atenolol, labetalol, metoprolol, nadolol, and propranolol. Amiloride and bendroflumethiazide are administered in combination with any one of the cited β-blockers, whereas piretanide is usually associated with acebutolol or atenolol and triamterene is associated with propranolol.

High-performance liquid chromatography (HPLC) analysis of pharmaceutical formulations where a diuretic and a β-blocker are combined has been reported. Thus, combinations of bendroflumethiazide and nadolol (4) and amiloride and atenolol (5) were analyzed with reversed-phase columns using methanol–acetate buffer eluents. Bendroflumethiazide and atenolol were also appropriately separated with a micellar mobile phase of sodium dodecyl sulphate (SDS) and propanol at pH 3 (6).

Although there are several reports in the literature about analytical liquid chromatographic procedures that monitor the separate intake of diuretics and β-blockers, only a recent reference on the determination of a mixture of triamterene and metoprolol in physiological fluids was found (7). In the procedure, a column-switching technique with a new packing material, LiChrospher RP-18 alkyl-diol-silica (ADS) was tested. The method allowed direct injection of the physiological fluids; however, protein denaturation or solid-phase extraction (SPE) was required to enhance the sensitivity of the procedure and the lifetime of the ADS column.

Other separation techniques have been utilized to analyze combinations of diuretics and β-blockers in urine. Triamterene and propranolol were determined by thin-layer chromatography with ethyl acetate–methanol–25% aqueous NH_3 (8:1:1) as mobile phase (8); bendroflumethiazide, triamterene, and acebutolol by capillary zone electrophoresis with pulsed-laser fluorescence detection (9); and amiloride, metoprolol, and labetalol by capillary isotacophoresis after SPE (10).

Micellar liquid chromatography (MLC) provides an interesting

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method for the direct injection of physiological samples by solubilizing the protein components via the surfactant coating of the analytical column. In addition, the surfactant monomers appear to displace the drug bound to the protein, releasing it for partitioning to the stationary phase. The compatibility with conventional reversed-phase column packings is particularly attractive.

The report of Armstrong and Nome (11), where the retention of solutes in a reversed-phase column with micellar mobile phases was explained through a three-phase partitioning model, was the true starting point of MLC. Much of the work done since then has addressed the better understanding of the interactions of the solutes inside the chromatographic column. Several experimental variables (type and concentration of surfactant and organic modifier, pH, ionic strength, and temperature) can be used to better control the retention of solutes and increase the efficiency of the chromatographic peaks. Recently, the partitioning theory in MLC has been extended to include the effect of organic modifiers (12,13) and acid-base equilibria (14) on the retention. The stable and reproducible behavior of micellar mobile phases allows the accurate prediction of the retention of solutes with a model that can further be used to optimize the separation of mixtures of solutes (15).

Previously, procedures that made use of SDS–propanol mobile phases to analyze mixtures of the diuretics amiloride, bendroflumethiazide, bumetanide, chlorthalidone, furosemide, hydroflumethiazide, piretanide, spironolactone, triamterene, and xipamide (16,17) or the β-blockers acebutolol, atenolol, celiprolol, labetalol, metoprolol, nadolol, and propranolol (18) in urine samples were developed by us. Fluorimetric monitoring of the drugs showing native fluorescence allowed a selective and sensitive detection.

The optimization of the experimental conditions for the determination of combinations of diuretics and β-blockers in urine samples requires a particular investigation. The use of micellar mobile phases and the direct injection of the urine sample simplify and largely expedite the establishment of the optimal conditions of analysis and the performance of the procedures.

Table I. Structures and Protonation Constants of the Fluorescent Diuretics and β-Blockers

Compound	Structure	log *K*
Acebutolol	$CH_3CH_2CH_2CONH$–(ring, $COCH_3$)–$OCH_2CH(OH)CH_2NHCH(CH_3)_2$	9.2*
Amiloride	Cl, N, $CONHC(=NH)NH_2$, H_2N, N, NH_2	8.7†
Atenolol	$(CH_3)_2CHNHCH_2CH(OH)CH_2O$–(ring)–$CH_2C(=O)NH_2$	9.6*
Bendroflumethiazide	H_2NO_2S, F_3C, SO_2, NH, N H, CH_2–(ring)	9.0†
Labetalol	H_2NOC, HO–(ring)–$CH(OH)CH_2NHCH(CH_3)CH_2CH_2$–(ring)	8.7, 7.4*
Metoprolol	$CH_3OCH_2CH_2$–(ring)–$OCH_2CH(OH)CH_2NHCH(CH_3)_2$	9.7*
Nadolol	OH, OH, $OCH_2CH(OH)CH_2NHC(CH_3)_3$	9.4*
Piretanide	COOH, N, SO_2NH_2, OC_6H_5	4.1†
Propranolol	$OCH_2CH(OH)CH_2NHCH(CH_3)_2$	9.5*
Triamterene	H_2N, N, N, NH_2, C_6H_5, N, N, NH_2	6.2†

* Protonation constants taken from reference 20.
† Protonation constants taken from reference 21.

Experimental

Reagents

Sodium dodecyl sulphate (99% purity, Merck, Darmstadt, Germany), sodium dihydrogenphosphate (for analysis, Panreac, Barcelona, Spain), HCl, NaOH (Probus, Badalona, Spain), methanol, 1-propanol (for analysis, Scharlau, Barcelona, Spain), ethanol (for analysis, Prolabo, Paris, France), and triethylamine (99.5% purity, Fluka, Buchs, Switzerland) were used. The diuretics were amiloride (ICI-Farma, Madrid, Spain), bendroflumethiazide (Davur, Madrid, Spain), piretanide (Cusí, Barcelona, Spain), and triamterene (Sigma, St. Louis, MO); the β-blockers were acebutolol chlorhydrate (Italfármaco, Alcobendas,

Madrid, Spain), atenolol (Zéneca Farma, Madrid, Spain), labetalol chlorhydrate (Glaxo, Tres Cantos, Madrid, Spain), metoprolol tartrate (Ciba-Geigy, Barcelona, Spain), nadolol (Squibb, Esplugues de Llobregat, Barcelona, Spain), and propranolol chlorhydrate (ICI-Farma). Table I shows the structure of the diuretics and β-blockers.

Preparation of standard solutions of the drugs

Stock standard solutions (10 μg/mL) of the drugs were prepared. The compounds were dissolved in a few milliliters of ethanol with the aid of an ultrasonic bath (model 617, Selecta, Barcelona, Spain) and diluted to the respective amount with 0.1M SDS at pH 3 (phosphate buffer). The final contents of ethanol in the SDS solutions guaranteed the formation of micelles. Working standard solutions were prepared by the appropriate dilution with the mobile phase. The solutions were stored in the dark at 4°C. Nanopure water (Barnstead, Sybron, Boston, MA) was used to prepare these and other solutions.

Apparatus

The equipment consisted of a Hewlett-Packard (Palo Alto, CA) model HP 1050 chromatograph provided with an isocratic pump, a model HP 1100 autosampler, and a model HP 1046A fluorimetric detector. The excitation wavelength was 230 nm for all compounds, and the emission wavelength was 300 nm for atenolol, metoprolol, nadolol, and propranolol and 440 nm for

Table II. Coefficients of Equation 1 for the Diuretics and β-Blockers

Compound	c_0	c_1	c_2	c_3
Optimization with triethylamine				
Acebutolol	–0.0188	0.904	0.316	4.07
Amiloride	–0.0267	1.79	0.625	2.30
Atenolol	–0.0372	1.87	1.52	7.21
Bendroflumethiazide	–0.0642	1.32	0.741	8.77
Labetalol	–0.0031	0.226	0.0178	3.34
Metoprolol	–0.0054	0.439	0.193	3.73
Nadolol	–0.0119	0.942	0.552	6.67
Piretanide	–0.0394	1.16	0.739	5.83
Propranolol	0.0015	0.165	–0.0086	3.14
Triamterene	–0.0052	1.06	0.253	2.18
Optimization without triethylamine				
Acebutolol	–0.0030	0.385	0.138	6.52
Amiloride	–0.0137	1.19	0.301	5.51
Atenolol	–0.0415	0.993	1.08	12.97
Bendroflumethiazide	–0.0367	2.25	1.62	3.96
Labetalol	–0.0005	0.18	–0.0015	3.91
Metoprolol	–0.0025	0.205	0.0836	4.89
Nadolol	–0.0113	0.431	0.322	9.73
Piretanide	–0.0264	0.984	0.973	7.80
Propranolol	0.0001	0.0922	–0.0197	3.48
Triamterene	–0.0047	0.623	0.108	4.44

Table III. Limits of Detection for the Diuretics and β-Blockers in Aqueous Solution and Urine*

	LOD (ng/mL)	
Compound	Water	Urine
Acebutolol	27.9	27.6
Amiloride	11.1	10.6
Atenolol	3.5	3.8
Bendroflumethiazide	16.7	17.5
Labetalol	23.8	28.3
Metoprolol	15.4	19.2
Nadolol	12.3	12.3
Piretanide	12.3	12.3
Propranolol	9.4	11.8
Triamterene	1.2	2.8

* Eluted with a mobile phase of 0.11M SDS–8% propanol.

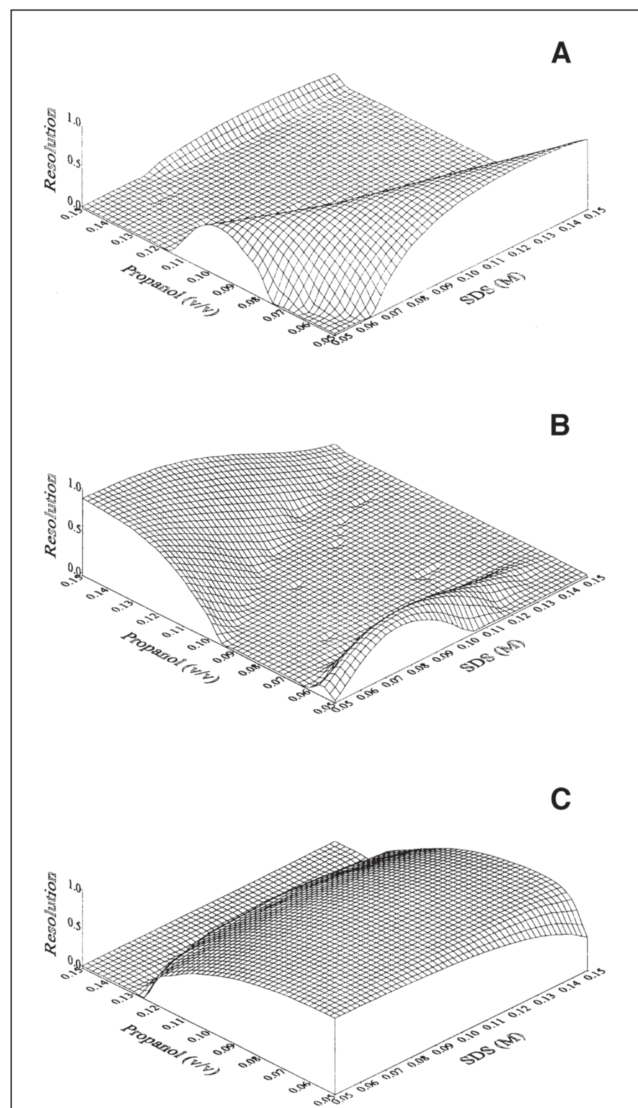

Figure 1. Global resolution diagram according to the peak-to-valley optimization criterion for the separation of diverse diuretics and β-blockers eluted with SDS–propanol mobile phases at pH 3 with amine (amiloride, bendroflumethiazide, triamterene, acebutolol, atenolol, labetalol, metoprolol, and propranolol) (A), without amine (amiloride, bendroflumethiazide, piretanide, triamterene, acebutolol, atenolol, labetalol, metoprolol, and propranolol) (B), and again without amine (amiloride, bendroflumethiazide, triamterene, atenolol, labetalol, metoprolol, nadolol, and propranolol) (C).

Table IV. Intra- and Interday Accuracies and Precisions in the Analysis of Urine Samples with 0.11M SDS–8% Mobile Phase

Compound	Found* (µg/mL)	Mean (µg/mL)
Acebutolol	0.95 ± 0.03	
(1.0 µg/mL)	0.97 ± 0.03	
	0.937 ± 0.019	0.94 ± 0.03
	0.928 ± 0.014	
	0.90 ± 0.03	
Amiloride	0.825 ± 0.009	
(0.83 µg/mL)	0.905 ± 0.006	
	0.963 ± 0.012	0.95 ± 0.09
	0.986 ± 0.016	
	1.076 ± 0.010	
Atenolol	0.320 ± 0.004	
(0.31 µg/mL)	0.318 ± 0.006	
	0.317 ± 0.005	0.310 ± 0.012
	0.302 ± 0.007	
	0.294 ± 0.005	
Bendroflumethiazide	0.680 ± 0.015	
(0.81 µg/mL)	0.65 ± 0.04	
	0.697 ± 0.008	0.69 ± 0.02
	0.693 ± 0.018	
	0.705 ± 0.009	
Labetalol	0.731 ± 0.011	
(0.72 µg/mL)	0.722 ± 0.007	
	0.71 ± 0.02	0.71 ± 0.03
	0.705 ± 0.008	
	0.66 ± 0.03	
Metoprolol	0.310 ± 0.002	
(0.32 µg/ml)	0.303 ± 0.004	
	0.307 ± 0.003	0.303 ± 0.006
	0.299 ± 0.005	
	0.294 ± 0.004	
Nadolol	0.280 ± 0.006	
(0.28 µg/mL)	0.287 ± 0.008	
	0.277 ± 0.003	0.275 ± 0.010
	0.2676 ± 0.0017	
	0.261 ± 0.003	
Piretanide	0.644 ± 0.008	
(0.81 µg/mL)	0.640 ± 0.010	
	0.697 ± 0.012	0.67 ± 0.03
	0.685 ± 0.012	
	0.680 ± 0.017	
Propranolol	0.317 ± 0.006	
(0.31 µg/mL)	0.316 ± 0.006	
	0.3111 ± 0.0015	0.309 ± 0.008
	0.303 ± 0.005	
	0.300 ± 0.004	
Triamterene	0.1019 ± 0.0011	
(0.15 µg/mL)	0.1342 ± 0.0014	
	0.1314 ± 0.0017	0.124 ± 0.014
	0.1176 ± 0.0011	
	0.1356 ± 0.0007	

* The intraday values corresponded to 5 injections of the samples.

acebutolol, amiloride, bendroflumethiazide, labetalol, piretanide, and triamterene. The flash frequency of the xenon lamp was 220 Hz, and the response time was 4000 ms. The photomultiplier gain used for the optimization procedure was 2^7 for nadolol and triamterene; 2^8 for amiloride, atenolol, metoprolol, and propranolol; 2^9 for bendroflumethiazide and piretanide; and 2^{10} for acebutolol and labetalol. The slits were 2 mm × 2 mm before the flow cell, 4 mm × 4 mm after the flow cell, and 4 mm × 4 mm before the photomultiplier tube. The cutoff filter removed all light below 280 nm.

The flow rate was 1.0 mL/min and the injection volume was 20 µL. The dead time was taken as the mean value of the first deviation of the baseline obtained in each chromatogram after the injection of the micellar solutions of the analytes (t_0 = 1.05 min). The signal was acquired with a PC computer connected to the chromatograph through a model HP 3396A integrator using the PEAK-96 program (Hewlett-Packard, Avondale, PA). The chromatographic data were treated with MICHROM (19).

Columns and mobile phases

An ODS-2 column (120 mm × 4.6 mm i.d., 5-µm particle size) was connected to a 30-mm guard precolumn of similar characteristics (Scharlau, Barcelona, Spain). The analytical column was washed weekly with 60 mL of water to eliminate the surfactant and afterwards flushed with 60 mL of methanol (HPLC grade, Scharlau).

The micellar mobile phase recommended for the determination of mixtures of diuretics and β-blockers contained 0.11M SDS, 8% propanol, and 0.01M NaH_2PO_4 at pH 3. A mobile phase of 0.15M SDS–15% propanol at pH 3 of larger elution strength was also used to diminish the retention of some drugs. Mobile phases with added triethylamine were also considered in the optimization studies. The pH was fixed after the addition of triethylamine to the micellar SDS solution and before adding propanol. The mobile phases were filtered through 0.45-µm nylon membranes (Micron Separations, Westboro, MA).

It was observed that once triethylamine was utilized, mobile phases without the amine resulted in changed retention times with respect to the same mobile phases employed before the addition of triethylamine to the system. This was probably due to an irreversible modification of the stationary phase where triethylamine was associated with the remaining monomers of surfactant adsorbed on the alkyl-bonded phase or the free silanol groups on the column.

Urine samples

The analyses were performed with 1 mL of the urine samples that were diluted (1:25 factor) with the mobile phase before injection. The aqueous and urine solutions of the drugs were injected into the chromatograph without any other treatment than filtration, made directly in the autosampler vials through 0.45-µm cellulose acetate membranes (Micron Separations). The filters were previously conditioned by passing 10 mL of the drug solutions through. The optimization of the procedure was performed with spiked urine samples containing accurately known amounts of the drugs.

The following pharmaceutical formulations were administered to young healthy volunteers to obtain the urinary excretion

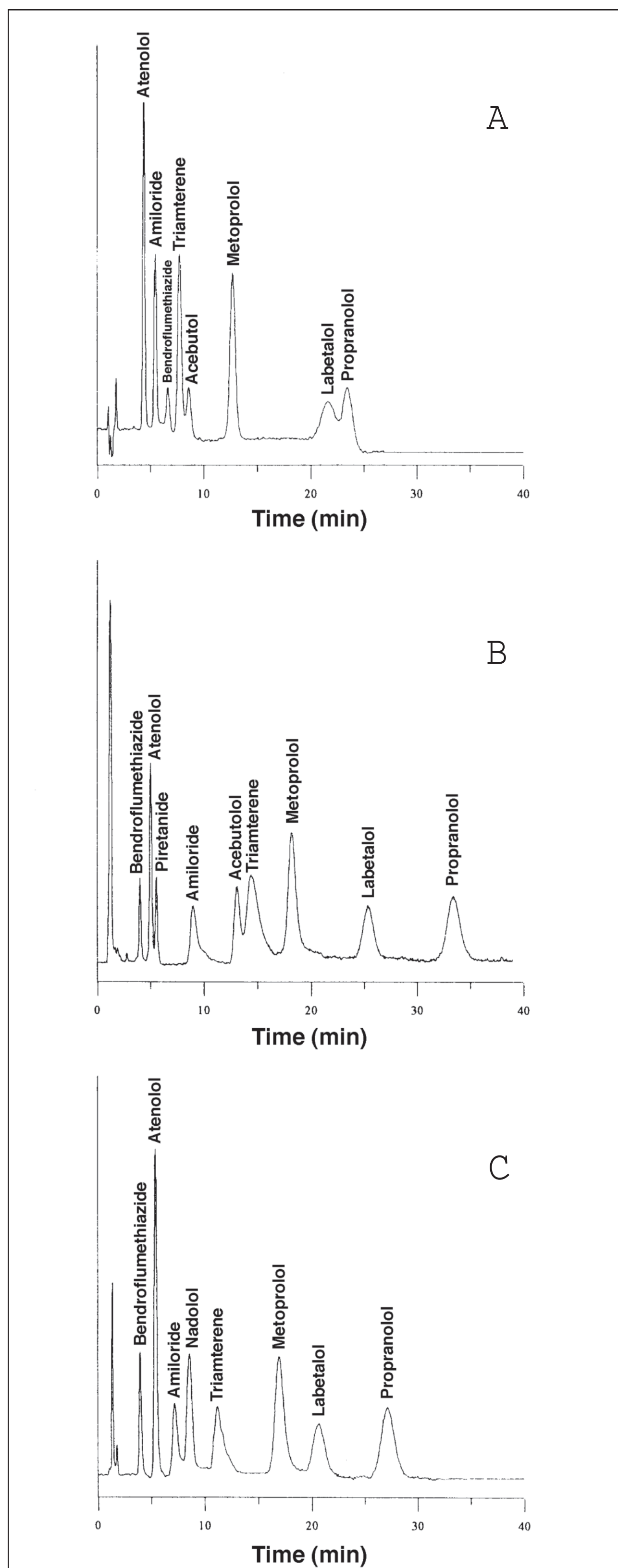

Figure 2. Experimental chromatograms of mixtures of acebutolol, amiloride, atenolol, bendroflumethiazide, labetalol, metoprolol, nadolol, piretanide, propranolol, and triamterene in aqueous solution. Mobile phases consisted of 0.12M SDS–5% propanol–0.5% triethylamine (A), 0.06M SDS–15% propanol (B), and 0.11M SDS–8% propanol (C).

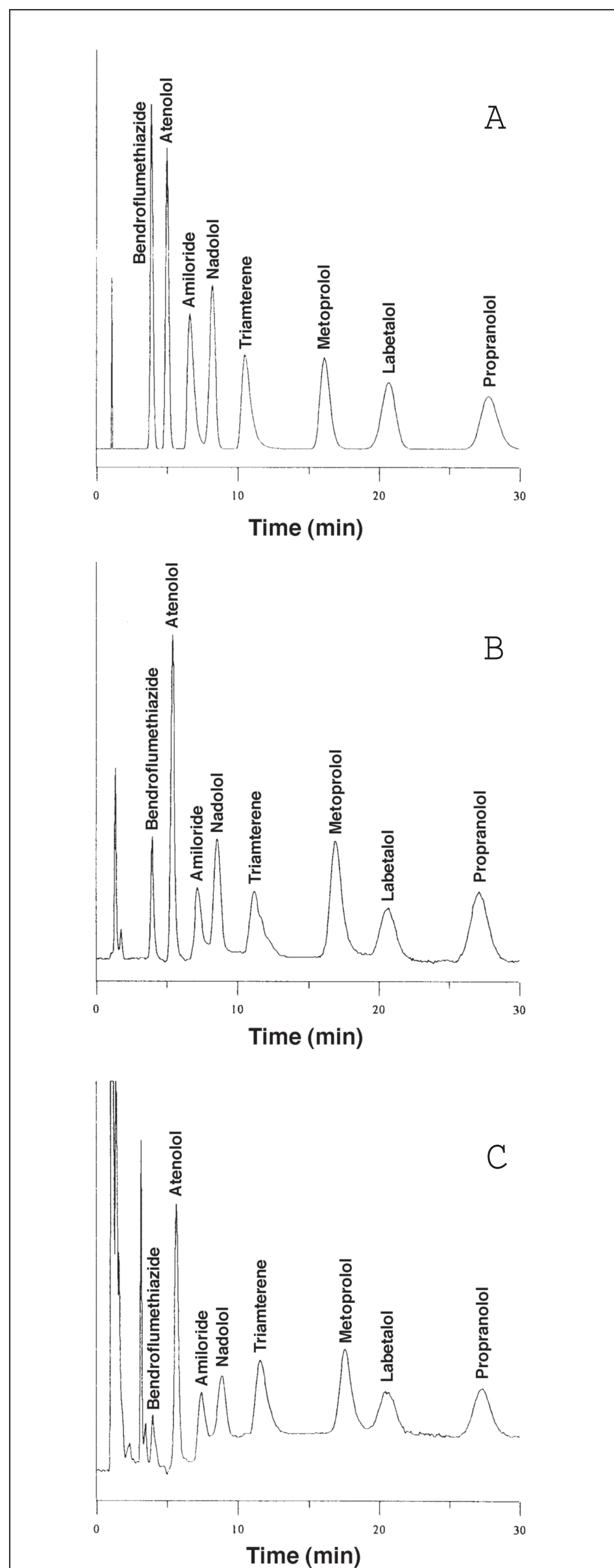

Figure 3. Predicted (A) and experimental chromatograms in aqueous solution (B) and urine (C) of a mixture of approximately 1 µg/mL amiloride, atenolol, bendroflumethiazide, labetalol, metoprolol, nadolol, and propranolol and 0.2 µg/mL triamterene for the selected mobile phase (0.11M SDS–8% propanol, pH 3).

curves of the diuretics: acebutolol (Sectral-400, Italfármaco), amiloride (Ameride, DuPont Pharma, Madrid, Spain), bendroflumethiazide and atenolol (Neatenol Diuvas, Fides-Rottapharm, Almácera, Valencia, Spain), metoprolol (Lopresor, Padró, Barcelona, Spain), propranolol (Sumial 40, Zéneca Farma), piretanide (Perbilen, Hoechst Ibérica, Barcelona, Spain), and triamterene (Salidur, Omega, Barcelona, Spain).

Results and Discussion

Fluorescence of diuretics and β-blockers

Triamterene and propranolol showed the most intense fluorescence signals. Amiloride, bendroflumethiazide, atenolol, labetalol, and metoprolol were also highly fluorescent (although to a lesser extent), whereas the fluorescence intensity of piretanide and acebutolol was rather low. Several excitation maxima were observed for the diuretics and β-blockers. Measurements of the fluorescence were made near the following excitation and emission maxima: amiloride, 232 and 414 nm; bendroflumethiazide, 225 and 390 nm; piretanide, 242 and 460 nm; triamterene, 230 and 436 nm; acebutolol, 240 and 454 nm; atenolol, 225 and 300 nm; labetalol, 210 and 434 nm; metoprolol, 225 and 300 nm; nadolol, 220 and 300 nm; and propranolol, 225 and 338 nm. According to these values, the selected excitation wavelength for the chromatographic detection was 230 nm, and the emission wavelength was 440 nm for all diuretics, labetalol, and acebutolol and 300 nm for atenolol, nadolol, metoprolol, and propranolol.

The excitation and emission wavelengths scarcely changed in the pH 3–7 range. The shape and intensity of the bands of the emission spectra were also not modified, except for piretanide, triamterene, and labetalol, for which the fluorescence signal increased with pH. Only amiloride and acebutolol showed fluorescence enhancements in micellar media. The enhancement factors for these diuretics in 0.05–0.1M SDS solutions were approximately 7.5. The enhancement factors were lower in the presence of propanol.

Optimization of mobile phase composition

Selection of pH

Table I indicates the protonation constants of the diuretics and β-blockers (log *K*) in aqueous solution. The micelles of the anionic surfactant should increase the stability of the protonated species of the drugs and, consequently, the log *K* values. Among the studied drugs, piretanide showed the strongest acidity. Although the log *K* value for this compound increases in micellar solution, its anionic basic species will still dominate in neutral medium. Therefore, in this medium, the retention of piretanide should decrease because of the repulsion with the negatively charged heads of the monomeric surfactant adsorbed on the stationary phase.

The other diuretics (amiloride, bendroflumethiazide, and triamterene) and all β-blockers with log *K* values greater than 6 in water are protonated in the whole working pH range of the C_{18} column (2.5–7.5), as shown by their constant retention with decreasing pH. At pH 7, piretanide eluted at the dead time, whereas the other diuretics were adequately retained. Nevertheless, an interesting effect was observed: the efficiencies of the chromatographic peaks of all β-blockers, piretanide, and triamterene increased when the pH was decreased, probably because of the protonation of the free silanol groups in the alkyl-bonded column material. It was thus decided to select pH 3 for the separation of the drugs because of the higher retention of piretanide and the increased efficiencies of β-blockers.

Selection of the concentration of surfactant and modifier

The retention of diuretics and β-blockers on a C_{18} column with pure micellar eluents (without modifier) was high. Thus the addition of a small amount of organic solvent was convenient to decrease the retention times. The use of hybrid micellar mobile phases of variable concentrations of surfactant and modifier usually produces changes in the retention factors, efficiencies, and asymmetries of the chromatographic peaks. Adequate control of the concentrations of both additives is therefore necessary to achieve chromatograms showing good resolution and sufficient elution strength. In this work, propanol was selected as the modifier because of its intermediate elution strength.

Triethylamine is commonly added to conventional aqueous-organic mobile phases to reduce peak tailing. However, in the presence of the amine, the retention times changed. The retention decreased for all compounds except bendroflumethiazide and piretanide, for which the retention times were longer (especially for bendroflumethiazide). Knowing this, the adequacy of added triethylamine for the determination of mixtures of diuretics and β-blockers was also considered.

The elution order of the drugs was usually the same in mobile phases of SDS and propanol of diverse composition in the absence of triethylamine: bendroflumethiazide, atenolol, piretanide, amiloride, nadolol, triamterene, acebutolol, metoprolol, labetalol, and propranolol. Only triamterene and acebutolol inverted their order at concentrations of propanol above approximately 8%. The elution order of the drugs, however, was variable when triethylamine was added. Without triethylamine, and at low concentrations of propanol, nadolol showed two peaks corresponding to the diastereoisomers (22).

For the development of adequate chromatographic conditions, two different strategies were considered. First, the use of a unique mobile phase to analyze the mixtures of the 10 drugs (4 diuretics and 6 β-blockers) was investigated. The employment of one set of experimental conditions to determine several drugs can be advantageous, because it will permit the analysis of the samples from individuals which have taken different combinations of diuretics and β-blockers without the need to change the mobile phase composition. Second, the use of an optimal mobile phase for each combination was studied, which may be necessary in some cases to achieve lower retention times and accelerate the analyses. The development of both strategies was greatly facilitated by the capability of MLC to predict the retention of compounds using simple equations. The model employed for these predictions was as follows (12):

$$k = \frac{1}{c_0 + c_1\mu + c_2\varphi + c_3\mu\varphi} \quad \text{Eq 1}$$

where *k* is the retention factor and μ and φ are surfactant and propanol concentrations, respectively. This equation was non-

linearly fitted according to the method of Powell (23) using the retention data obtained from injections of the diuretic and β-blocker solutions in 5 mobile phases: 0.05M SDS–5% propanol, 0.15M SDS–5% propanol, 0.1M SDS–10% propanol, 0.05M SDS–15% propanol, and 0.15M SDS–15% propanol (all of them containing 0.01M phosphate at pH 3 in the absence and presence of triethylamine). Above 0.15M SDS, the efficiencies were too low. Above 15% propanol, the retention times were too low and the integrity of micelles was not guaranteed.

Table II shows the coefficients in Equation 1 for each drug, permitting the prediction of mobile phase composition for any desired retention time and a simple way of optimizing the separation of mixtures. It must be noted that, in the analysis of physiological fluids, the retention times of the endogeneous compounds and the protein band at the head of the chromatogram should also be considered when selecting the mobile phase.

The optimization of the resolution of mixtures of the 10 drugs using a procedure based on the sequential variation of the composition of the mobile phase was difficult because of the changes in the elution order of the diuretics and β-blockers. However, the accurate prediction of the retention according to Equation 1 permitted the application of an interpretive procedure to predict the optimal resolution, following a criterion that utilizes the valley-to-peak ratios (15):

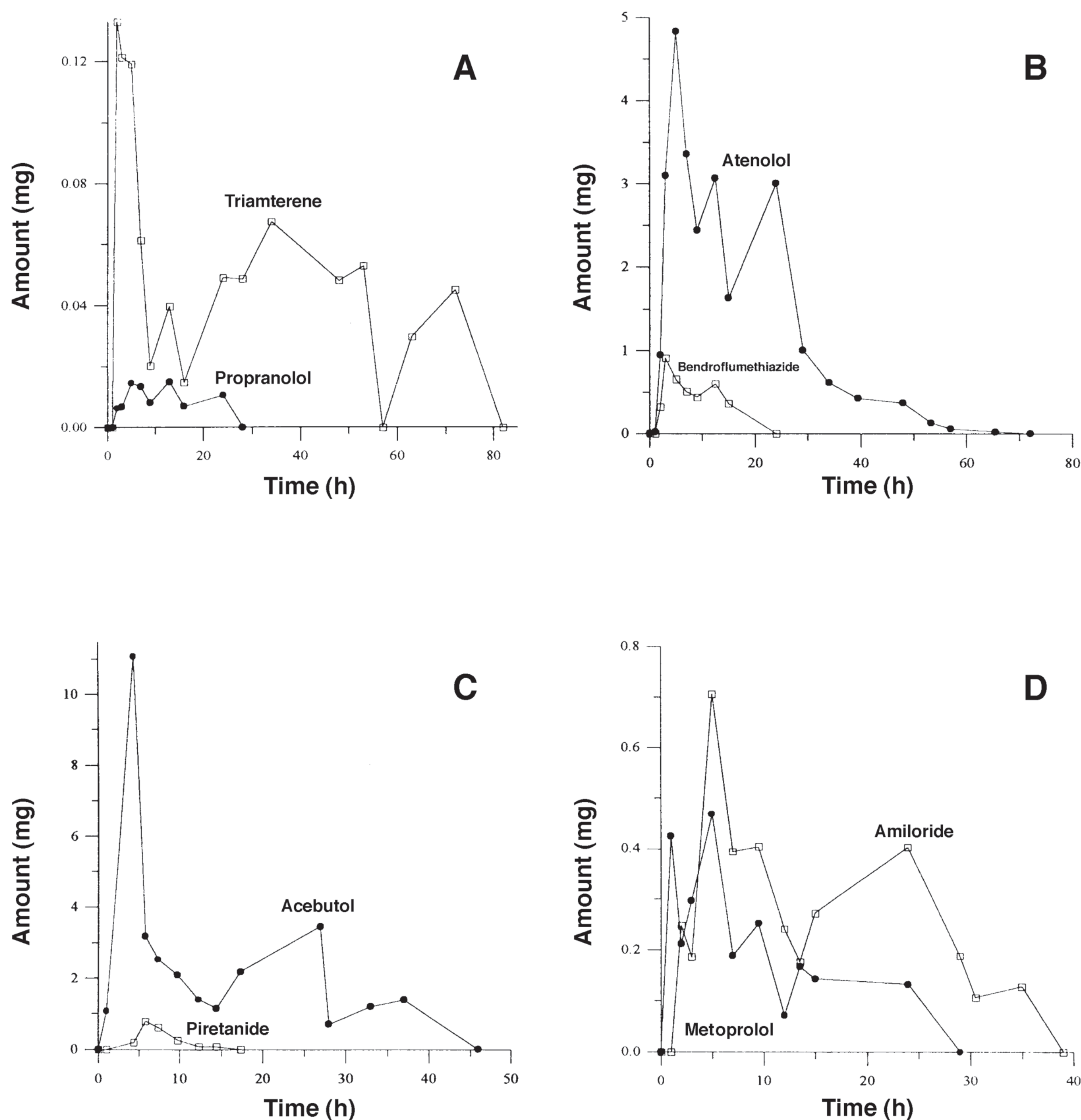

Figure 4. Study of the urinary excretion of some mixtures of diuretics and β-blockers after oral administration to healthy volunteers (oral dose given in parentheses): triamterene (25 mg) and propranolol (40 mg) (A), bendroflumethiazide (5 mg) and atenolol (100 mg) (B), piretanide (6 mg) and acebutolol (400 mg) (C), and amiloride (5 mg) and metoprolol (100 mg) (D).

$$r = \prod_{i=1}^{n-1} \frac{X_{i,i+1}}{\left(\sum \frac{X_{i,i+1}}{n-1}\right)^{n-1}} \qquad \text{Eq 2}$$

where $X_{i,i+1} = 1 - h_1/h_2$, h_1 being the height of the valley between two adjacent peaks and h_2 being the interpolated height between the maxima of both peaks measured at the abscissa of the valley. The global function of resolution, r, may vary from 0 to 1, and the proximity to unity indicates the performance of the separation. The function was maximized to obtain the optimal mobile phase.

Incorporation of the shape of the chromatographic peaks in the optimization procedure improves the results. The reliable simulation of peak shape for any mobile phase of the variable space was carried out with an asymmetrical Gaussian function where the standard deviation is a first-degree polynomial function (24):

$$h(t) = H \exp\left[-(1/2)\left(\frac{t - t_R}{s_0 + s_1(t - t_R)}\right)^2\right] \qquad \text{Eq 3}$$

where H and t_R are the height and time at the peak maximum, respectively; s_0 is the standard deviation of a symmetrical Gaussian peak describing the central region of the experimental peak; and s_1 a coefficient that quantitates its skewness. The coefficients s_0 and s_1 are related to the efficiency and asymmetry factor. These parameters were interpolated from the data obtained in the three experimental mobile phases closer to the simulated mobile phase.

Two optimization processes were carried out, one with 0.5% triethylamine in the mobile phases and the other without the amine. The selected concentration of triethylamine gave adequate efficiencies and retentions. Complete resolution of the 10 drugs was not possible in any case. In the optimization procedure carried out in mobile phases with the amine, however, enough resolution was obtained in the separation of 8 drugs. An example where piretanide and nadolol were eliminated from the mixtures is provided. For a wide range of mobile phase composition, these drugs overlapped with the peak of bendroflumethiazide, which appears associated with all the β-blockers considered. In contrast, piretanide is usually administered only with acebutolol and atenolol, and nadolol usually appears combined with only amiloride and bendroflumethiazide. Although it is not possible to distinguish between bendroflumethiazide and piretanide, the detection of bendroflumethiazide and nadolol in mixtures can still be made in two consecutive runs by using appropriate emission wavelengths (bendroflumethiazide, 440 nm; nadolol, 300 nm). Nevertheless, other mixtures containing piretanide or nadolol instead of bendroflumethiazide can be analyzed after appropriate optimization by following the proposed approach.

On the other hand, mixtures of 9 drugs (without nadolol) or 8 drugs (without piretanide and acebutolol) could be analyzed using mobile phases in the absence of triethylamine. Under optimal conditions, nadolol overlapped amiloride for the first mixture, and piretanide overlapped atenolol and acebutolol overlapped triamterene for the second mixture. However, amiloride and nadolol on one side and piretanide and atenolol on the other side could still be detected in two runs by monitoring at different emission wavelengths.

Global resolution diagrams obtained with and without amine are depicted in Figure 1. The presence of 0.5% amine resulted in a very narrow maximum with good resolution (R = 0.903) and adequate retention times for 0.12M SDS–5% propanol (Figure 1A), where 8 compounds could be separated. In the absence of the amine, two optimal mobile phases existed: 0.06M SDS–15% propanol (R = 0.867) (Figure 1B) and 0.11M SDS–8% propanol (R = 0.944) (Figure 1C), where 9 and 8 compounds were resolved, respectively. Figure 2 shows the experimental chromatograms for the separation of an aqueous mixture of the diuretics and β-blockers with the 3 optimal mobile phases.

The best resolution was obtained with 0.11M SDS–8% propanol, although the analysis times were somewhat longer than those with the optimal mobile phase in the presence of amine (Figure 2). This optimal mobile phase was also far more robust than the other two considered; the resolution scarcely changed with mobile phase composition in a wide region (Figure 1). For this reason, this mobile phase was selected to perform the analysis of urine samples. In Figure 3, simulated and experimental chromatograms in aqueous solution and urine sample spiked with a mixture of the same drugs are shown. The agreement between the chromatograms is excellent.

The mobile phase of 0.11M SDS–8% propanol was adequate for the separation of each individual combination of diuretic and β-blocker usually administered. The retention times were below 10 min for the mixtures of the 4 diuretics with the β-blockers acebutolol, atenolol, and nadolol. On the other hand, it is convenient to decrease the retention times for the mixtures of the diuretics with metoprolol, labetalol, and propranolol. However, this was not possible for the combinations containing bendroflumethiazide (which is very often administered), because this diuretic showed a very low retention near the protein band at the beginning of the chromatograms of urine samples. The retention was decreased for the mixtures of amiloride–metoprolol, amiloride– labetalol, and triamterene–propranolol by using a mobile phase of 0.15M SDS–15% propanol. The retention times for amiloride, triamterene, labetalol, and propranolol changed from 7.1, 11.2, 20.6, and 27.1 min (0.11M SDS–8% propanol) to 4.3, 6.4, 9.5, and 12.2 min (0.15M SDS–15% propanol), respectively.

Analysis of urine samples

Urine background

Because of the facility in obtaining the samples, urine is preferred over other physiological fluids in controlling the intake of drugs. When the direct injection of urine is performed in the chromatographic system, the wide band at the head of the chromatogram and the peaks of endogeneous compounds at diverse retention times can seriously affect the detection of the least retained drugs. Dilution of the urine sample before its injection is convenient to reduce the width of the protein band.

Furthermore, the injection of a large number of urine samples can shorten the life of the column or enforce a frequent regeneration of the stationary phase in order to avoid the change in retention times because of the adsorbed matrix. This also made

the injection of diluted samples advisable. Therefore, it was decided to carry out the analysis after dilution of the samples. For most diuretics and β-blockers, the sensitivity achieved after dilution to a factor of 1:25 was adequate for their detection in urine, at least up to 24–72 h after ingestion of the drugs. No change in retention times were observed after at least 50 consecutive injections of diluted urine into the C_{18} column.

Figures of merit

Calibration curves were constructed for each drug using the measured areas of the chromatographic peaks at 6 increasing concentrations. The concentration ranges were as follows: 0.35–2.5 µg/mL for acebutolol, 0.17–2.1 µg/mL for amiloride, 0.044–0.9 µg/mL for atenolol, 0.1–2 µg/mL for bendroflumethiazide, 0.2–2.0 µg/mL for labetalol, 0.08–0.8 µg/mL for nadolol, 0.09–0.9 µg/mL for metoprolol and propranolol, 0.08–2.1 µg/mL for piretanide, and 0.05–0.3 µg/mL for triamterene. The curves were obtained for aqueous solutions of the analytes and for spiked urine samples diluted to a factor of 1:25. The slopes of the calibration curves in the absence and presence of urine were similar, the intercepts were usually statistically zero, and the regression coefficients were $r > 0.999$.

The limits of detection (LODs, 3 s criterion) were calculated from the standard deviation of the areas of the peaks obtained in sixfold injections in the presence and absence of urine containing the drugs at the lower concentration of the calibration curve. Table III gives the LODs achieved for the mobile phase of 0.11M SDS–8% propanol.

To enhance the sensitivities in the urinary excretion studies, the photomultiplier gain was increased to 2^9 for amiloride, propranolol, and triamterene and 2^{10} for piretanide. The retention times of amiloride, metoprolol, triamterene, and propranolol were decreased through the use of 0.15M SDS–15% propanol. The excitation and emission wavelengths were the same as in the optimization studies, except for propranolol, for which 340 nm (maximum emission wavelength for this drug) was used. The detection of this β-blocker in the urine sample required a higher sensitivity because of the extent of its degradation. The LODs for amiloride, metoprolol, triamterene, and propranolol in the new conditions were 3.7, 6.6, 0.48, and 0.51 ng/mL, respectively. The LOD for piretanide with 0.11M SDS–8% propanol and a photomultiplier gain of 2^{10} was 6.6 ng/mL.

Intra- and interday assay accuracies and precisions were obtained from fivefold injections of urine samples spiked with an intermediate concentration of the drugs in the calibration range (Table IV). The interday assay precisions (percent relative standard deviation) in spiked urine samples calculated from fivefold injections of the same sample made over a 5-day period were as follows: acebutolol, 3.2; amiloride, 9.5; atenolol, 3.9; bendroflumethiazide, 2.9; labetalol, 4.2; metoprolol, 2.0; nadolol, 3.6; piretanide, 4.5; propranolol, 2.6; and triamterene, 11.3.

Urinary excretion of combinations of diuretic and β-blocker

Urinary excretion studies were performed with 4 normal healthy volunteers who were given single oral doses of one of the following mixtures: amiloride (5 mg) and metoprolol (100 mg), bendroflumethiazide (5 mg) and atenolol (100 mg), piretanide (6 mg) and acebutolol (400 mg), and triamterene (25 mg) and propranolol (40 mg). A sample was collected just before the administration of the drugs to be used as the blank. Urine samples were taken during 24–72 h at different time intervals (in 1-h intervals during the first 3 h; in longer intervals afterwards), and the excreted volume was measured. The samples were refrigerated at 4°C until analyzed.

Figure 4 shows the amounts of acebutolol, amiloride, atenolol, bendroflumethiazide, metoprolol, piretanide, propranolol, and triamterene during urinary excretion as determined in this work. The percentages of the dose excreted unchanged for each drug were 7.8% for acebutolol, 69% for amiloride, 25% for atenolol, 76% for bendroflumethiazide, 2.4% for metoprolol, 33% for piretanide, 0.2% for propranolol, and 3.4% for triamterene.

The chromatograms of the urine samples of the volunteers that were administered piretanide and acebutolol or triamterene and propranolol showed several peaks different from those of the diuretics or β-blockers and not observed in the samples collected just before the administration of the drugs. The new peaks increased along the first samples, reduced, and finally disappeared in the samples after some hours. The peaks were attributed to diverse β-blocker metabolites (18).

Conclusion

The results indicate that the MLC procedure can easily be used for the determination of mixtures of the most often prescribed fluorescent diuretics and β-blockers in urine samples, with analysis times usually below 15 min. The procedure is sensitive enough for routine analysis of the drugs at therapeutic urine levels, with LODs similar to those usually reported in the literature, taking into account that the urine sample was injected without any previous treatment to separate or concentrate the analytes. The procedure is also interesting for the detection of drug misuse in precision sports, where diuretics are taken to deliberately dilute a urine specimen in an attempt to nullify a β-blocker test.

Although a mobile phase of 0.11M SDS–8% propanol at pH 3 is recommended when performing the analysis of the mixtures of most of the diuretics and β-blockers considered, the interpretive optimization approach shown in this work offers a wide range of possible mobile phases when performing the analysis of any mixture of the diuretics and β-blockers.

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References

1. G.K. Duckett and B. Cheadle. Hypertension in the elderly: a study of a combination of atenolol, hydrochlorothiazide and amiloride hydrochloride. *Br. J. Clin. Pract.* **44:** 354–58 (1990).
2. L. Gerrard, N.M. Wheeldon, and D.G. McDevitt. Central effects of combined bendroflumethiazide and atenolol administration. A single dose study in normal subjects. *Eur. J. Clin. Pharmacol.* **45:** 539–43 (1993).
3. M. Kunneke, W. Lorenz, D. Duda, D. Kunkel, and W. Schunack. Unknown effects of clinically used drugs as bias in clinical trials: antihistaminergic activity of the antypertensive fixed-dose combination Betathiazid. *Agents Actions* **41:** C131–33 (1994).
4. S. Perlman, M. Szyper, and J.J. Kirschbaum. High-performance liquid-chromatographic analysis of nadolol and bendroflumethiazide combination tablet formulations. *J. Pharm. Sci.* **73:** 259–61 (1984).
5. R.T. Sane, V.R. Bhate, V.G. Nayak, and K.D. Ladge. Simultaneous determination of atenolol, hydrochlorothiazide and amiloride hydrochloride from marketed preparation by reversed-phase high-performance liquid chromatography. *Indian Drugs* **28:** 322–25 (1991).
6. I. Rapado Martínez, M.C. García Alvarez-Coque, and R.M. Villanueva Camañas. Performance of micellar mobile phases in reversed-phase chromatography for the analysis of pharmaceuticals containing beta-blockers and other antihypertensive drugs. *Analyst* **121:** 1677–82 (1996).
7. R. Oertel, K. Richter, T. Gramatte, and W. Kirch. Determination of drugs in biological fluids by high-performance liquid chromatography with on-line sample processing. *J. Chromatogr.* **A 797:** 203–209 (1998).
8. J. Molling, U. Leuschner, F.P. Meyer, and H. Walther. Thin-layer chromatographic detection of talinolol, propranolol and triamterene in urine. Evaluation of compliance by hytertonia patients. *Pharmazie* **41:** 301–302 (1986).
9. E. González, R. Montes, and J.J. Laserna. Pulsed-laser fluorescence detection in capillary zone electrophoresis of some banned substances in sport. *Anal. Chim. Acta* **282:** 687–93 (1993).
10. J. Sadecka and J. Polonsky. Determination of some cardiovascular drugs in serum and urine by capillary isotachophoresis. *J. Chromatogr. A* **735:** 403–408 (1996).
11. D.W. Armstrong and F. Nome. Partitioning behavior of solutes eluted with micellar mobile phases in liquid chromatography. *Anal. Chem.* **53:** 1662–66 (1981).
12. J.R. Torres Lapasió, R.M. Villanueva Camañas, J.M. Sanchis Mallols, M.J. Medina Hernández, and M.C. García Alvarez-Coque. Modelling of the retention behaviour of solutes in micellar liquid chromatography with organic modifiers. *J. Chromatogr. A* **639:** 87–96 (1993).
13. M.C. García Alvarez-Coque, J.R. Torres Lapasió, and J.J. Baeza Baeza. Description of the partitioning behavior of solutes and data treatment in micellar liquid chromatography with modifiers. *Anal. Chim. Acta* **324:** 163–73 (1996).
14. J.R. Torres Lapasió, J.J. Baeza Baeza, and M.C. García Alvarez-Coque. Description of the retention behavior in micellar liquid chromatography as a function of pH, surfactant and modifier concentration. *J. Chromatogr.* **A 769:** 155–68 (1997).
15. J.R. Torres Lapasió, R.M. Villanueva Camañas, J.M. Sanchis Mallols, M.J. Medina Hernández, and M.C. García Alvarez-Coque. Interpretive strategy for optimization of surfactant and alcohol concentration in micellar liquid chromatography. *J. Chromatogr.* **A 677:** 239–53 (1994).
16. E. Bonet Domingo, J.R. Torres Lapasió, M.J. Medina Hernández, and M.C. García Alvarez-Coque. Chromatographic monitoring of diuretics in urine samples using a sodium dodecyl sulphate-propanol micellar eluent. *Anal. Chim. Acta* **287:** 201–10 (1994).
17. S. Carda Broch, J.S. Esteve Romero, and M.C. García Alvarez-Coque. Chromatographic determination of diuretics in urine samples using hybrid micellar mobile phases with fluorimetric detection. *Anal. Chim. Acta* **375:** 143–54 (1998).
18. I. Rapado Martínez, R.M. Villanueva Camañas, and M.C. García Alvarez-Coque. Micellar liquid chromatography: a worthy technique for the determination of β-antagonists in urine samples. *Anal. Chem* **71:** 319–26 (1999).
19. J.R. Torres Lapasió, M.C. García Alvarez-Coque, and J.J. Baeza Baeza. Global treatment of chromatographic data with MICHROM. *Anal. Chim. Acta* **348:** 187–96 (1997).
20. *Comprehensive Medicinal Chemistry,* Vol. 6, C.J. Drayton, Ed. Pergamon Press, Oxford, England, 1990.
21. R. Ventura and J. Segura. Detection of diuretic agents in doping control. *J. Chromatogr. B* **687:** 127–44 (1996).
22. I. Rapado Martínez, M.C. García Alvarez-Coque, and R.M. Villanueva Camañas. Performance of micellar mobile phases in reversed-phase chromatography for the analysis of pharmaceuticals containing β-blockers and other antihypertensive drugs. *Analyst* **121:** 1677–82 (1996).
23. S.S. Rao. *Optimization: Theory and Applications.* Wiley & Sons, New Delhi, 1985.
24. J.R. Torres Lapasió, J.J. Baeza Baeza, and M.C. García Alvarez-Coque. A model for the description, simulation and deconvolution of skewed chromatographic peaks. *Anal. Chem.* **69:** 3822–32 (1997).

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