Improvement of Peak Shape and Separation Performance of β-Blockers in Conventional Reversed-Phase Columns Using Solvent Modifiers

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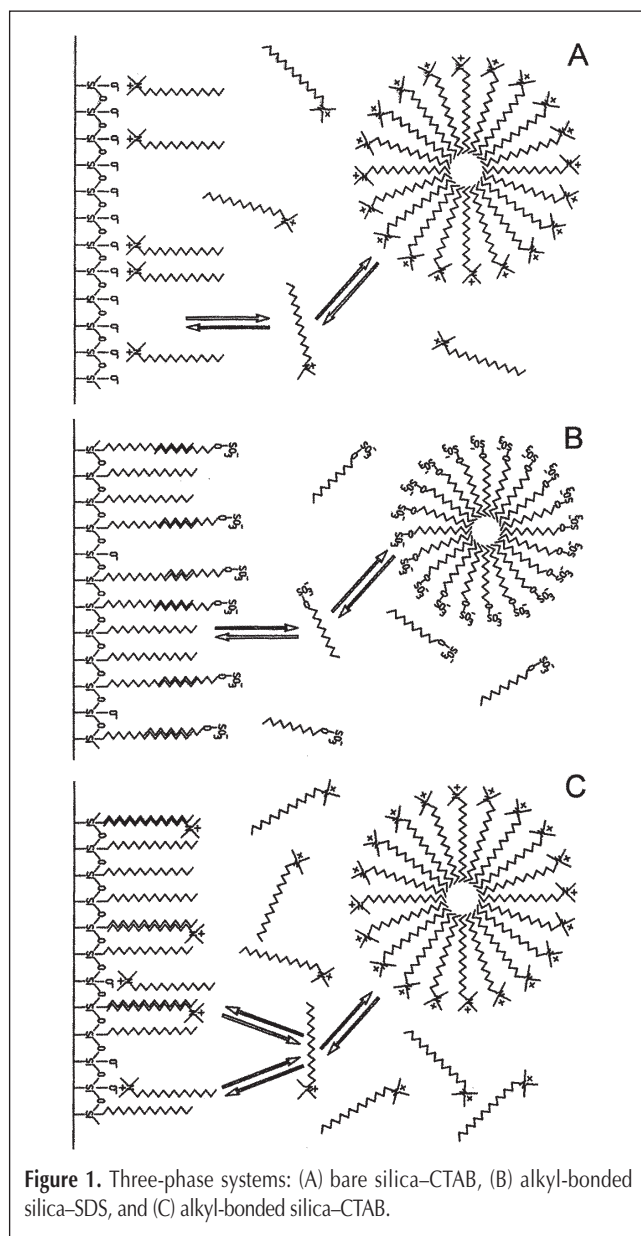
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Abstract

A comparative study of peak shape, elution behavior, and resolution of 16 β-blockers (acebutolol, alprenolol, atenolol, bisoprolol, carteolol, celiprolol, esmolol, labetalol, metoprolol, nadolol, oxprenolol, pindolol, practolol, propranolol, sotalol, and timolol) chromatographed with hybrid mobile phases of triethylamine (TEA)–acetonitrile and sodium dodecyl sulfate (SDS)–propanol is performed using conventional reversed-phase columns and isocratic elution. Both solvent modifiers (TEA and SDS) prevent the interaction of the basic drugs with the alkyl-bonded phase. However, the protection mechanisms of silanols on the packing are different. Whereas TEA associates with the silanol sites (blocking ion-exchange processes or repelling the solutes), the long hydrophobic chain of SDS is inserted in the bonded organic layer with the sulfate group protruding outside, which makes the stationary phase negatively charged. The effects of TEA, acetonitrile, SDS, and propanol on the elution strength, efficiency, peak asymmetry, and resolution are examined under an experimental design basis that is assisted by computer simulation to reach more general conclusions. The combination of improved peak shapes, larger selectivity, and a smaller range in retention among compounds of extreme polarity leads to the observation that a greater number of β-blockers can be resolved with a hybrid micellar system.

Introduction

Many drugs of interest contain basic nitrogens. Analysis of these compounds is very often performed by reversed-phase (RP) liquid chromatography (LC), using stationary phases based on octadecyl (C_{18})-or octyl (C_8)-modified silica. However, several problems are found such as severely low efficiencies, tailed peaks, no elution of strongly retained bases, and strong dependence of retention on sample size (1). Protonated basic compounds can interact with the RP support through several mechanisms in

Figure 1. Three-phase systems: (A) bare silica–CTAB, (B) alkyl-bonded silica–SDS, and (C) alkyl-bonded silica–CTAB.

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addition to hydrophobic partition: ion exchange on silanols, salting-out, and ion-pair formation, which depend on the nature of solute, stationary and mobile phases, pH, temperature, and ionic strength (2). It is generally accepted, however, that peak asymmetry is caused mostly by ionic interaction of the positively charged species with free silanols of the packing.

Ion exchange can be at least partially avoided by lowering the pH of the mobile phase to suppress silanol ionization. A variety of base-deactivated packings from several manufacturers are also becoming widespread. However, because of their high cost, conventional RP-LC packings are still common, and the problematic interactions of basic solutes with the siliceous supports are solved by the addition of amine modifiers [such as triethylamine (TEA)] to the mobile phase. These additives associate with silanol sites, blocking ion-exchange processes with solutes or repelling them (3).

Bare silica dynamically modified with long-chain quaternary ammonium ions added to the mobile phase [such as the cationic surfactant cetyltrimethyl ammonium bromide (CTAB)] has also been applied successfully to the analysis of basic drugs (4). At equilibrium, three phases are present in the chromatographic system: the layer of electrostatically associated quaternary ammonium ions with their hydrophobic chains acting as stationary phase, micelles, and bulk solvent in which surfactant monomers are dissolved (Figure 1A). An excellent separation of tricyclic antidepressants was found with this system, which was impossible even on a C_{18} deactivated column. This result was explained by the large number of positively charged species (the ammonium ions) in the mobile phase, which compete with the solutes for free silanols.

More recently, RP-LC with conventional C_{18} columns and hybrid micellar mobile phases of the anionic surfactant sodium dodecyl sulfate (SDS) and propanol or pentanol have been reported to yield good performance in the analysis of basic drugs such as phenethylamines (5), tetracyclines (6), and β-blockers (7). In these systems, the mobile phase contains micelles and monomers of the surfactant, as in the previously described bare silica system. However, NMR studies have indicated that on the densely grafted stationary phase, the long hydrophobic chain of SDS is inserted in the bonded organic layer with the sulfate group protruding outside (Figure 1B) (8). This makes the stationary phase negatively charged. The silanols and the C_{18} grafted moities are thus covered by surfactant monomers, which changes the stationary phase behavior. In contrast, in C_{18} columns, cationic surfactants (such as CTAB) can give rise to two kinds of interactions: hydrophobic association with the alkyl-bonded layer similar to SDS and electrostatic attraction to the residual-free silanols such as bare silica (Figure 1C). Although ammonium groups of CTAB are buried inside the C_{18} layer, the stationary phase is positively charged and repels protonated basic drugs, which would elute at short retention times or even with the void volume (9).

Tailed peaks with low efficiencies are, however, still obtained when basic compounds are chromatographed in pure SDS mobile phases. This behavior is well known and has been observed for many other compounds, especially those of low polarity. Poor wetting of the stationary phase by the aqueous micellar phase (10), together with a slow solute exit rate from the micelle and the stationary phase (11), have been suggested as the reasons for the poor mass transfer between bulk phases. The sur-

factant is known to be adsorbed on the stationary phase in amounts approximating that of the bonded hydrocarbon. The increase in film thickness seems to be the main issue responsible for the decreased efficiency (12). Routinely, peak shape is improved by the addition of a short-chain alcohol, which desorbs the surfactant out of the stationary phase and reduces the viscosity of the surfactant-C_{18} structure (13). A thinner surfactant layer adsorbed on the column permits a better diffusion of protonated solutes and is effective in preventing their association with free silanols. The interaction of the charged solutes with the hydrophilic layer of SDS reduces also their penetration depth in the bonded phase. The kinetics of solute–sulfate electrostatic association seems to be more facile than ion-exchange processes involving silanols on the silica surface (5).

Amine modifiers and SDS prevent the interaction of basic compounds with alkyl-bonded phases and improve chromatographic performance. However, their interaction with the column packing is different, which is revealed in the chromatographic behavior. In this work, the elution and peak shape of 16 β-blockers of varying polarity, eluted with mobile phases of TEA–acetonitrile and SDS–propanol, are comparatively studied using a conventional C_{18} column and isocratic elution. The impact on peak resolution is also examined.

Experimental

Reagents

Sixteen β-blockers were studied: 1, atenolol; 2, practolol; 3, sotalol; 4, carteolol; 5, nadolol; 6, pindolol; 7, acebutolol; 8, celiprolol; 9, esmolol; 10, metoprolol; 11, timolol; 12, bisoprolol; 13, labetalol; 14, oxprenolol; 15, propranolol; and 16, alprenolol. Details on the manufacturers are given elsewhere (7). The drugs were dissolved in a small amount of methanol and diluted with water. The concentration of the injected solutions was 10 µg/mL.

The aqueous–organic mobile phases were prepared with acetonitrile [high-performance LC (HPLC) grade, Scharlab, Barcelona, Spain] and triethylamine (Fluka, Buchs, Switzerland) and the micellar mobile phases with SDS (Merck, Darmstadt, Germany) and 1-propanol (HPLC grade, Scharlab). All solutions were buffered at pH 3 with disodium hydrogenphosphate and HCl (Panreac, Barcelona, Spain). Nanopure water (Barnstead, Sybron, Boston, MA) was used throughout.

Apparatus and software

A model HP 1050 HPLC system (Hewlett-Packard, Palo Alto, CA) was equipped with an isocratic pump, an autosampler (Series 1100 Model G1313A), and a UV–vis detector. The β-blockers were monitored at 225 nm, with the exception of timolol (300 nm). Chromatographic runs were carried out at room temperature. The flow rate was 1 mL/min, and the injection volume was 20 µL. Duplicate injections were made. The analytical separations were performed with a 125-mm (4.6-mm i.d.) Spherisorb unendcapped ODS-2 column of 5-µm particle size (Scharlab) linked to a similar 30-mm ODS-2 guard column (Scharlab).

Data acquisition was performed with the Peak-96 software (Hewlett-Packard, Avondale, PA). Simulation and optimization of

chromatograms were carried out with home built-in routines written in MATLAB 4.2c (The Mathworks, Natlick, MA).

Results and Discussion

The chromatographic separation of a set of 16 β-blockers in a conventional C_{18} column was examined to compare the relative performance of hybrid aqueous–organic (TEA–acetonitrile) and micellar (SDS–propanol) systems. The compounds contained one or more basic nitrogens, with dissociation constants in the range $pK_a = 8.1$–9.7. The retention of β-blockers was therefore not affected by the pH inside the working range of the C_{18} column (pH = 3–7), but efficiencies and peak symmetries improved at increasing acidity of the mobile phase. The separations were thus carried out at pH 3 (phosphate buffer).

The main objective of this work was to study the resolution capability of both RP-LC modes (aqueous–organic and micellar). For this purpose, changes in elution strength and peak shape were measured for several mobile phase compositions. Given the complexity of the drug mixture and the strongly varying chromatographic behavior of the analytes with mobile phase composition, the studies were assisted by computer simulation. This permitted the observation of the chromatography of β-blockers in a wide experimental domain and achievement of more general conclusions.

Retention modeling

The mentioned study was based on modeling of the retention, which was performed using the following experimental sets: (*a*) the pure aqueous–organic system (%acetonitrile) was 20, 25, 30, 40, and 60; (*b*) the amine-modified aqueous–organic system

(%TEA:%acetonitrile) was 0.05:21.2, 0.065:18.6, 0.065:23.9, 0.10:17.5, 0.10:21.2, 0.10:25, 0.135:18.6, 0.135:23.9, and 0.15:21.2; (*c*) the pure micellar system (M SDS) was 0.075, 0.10, 0.125, and 0.15; and (*d*) the hybrid micellar system (M SDS:%propanol) was 0.075:5, 0.113:5, 0.15:5, 0.075:10, 0.112:10, 0.15:10, 0.075:15, and 0.15:15.

The solvent percentage in the pure aqueous–organic system [set (*a*)] ranged from 20% to 60%. Because the amine accelerated the elution, mobile phases containing this modifier required a narrower range of acetonitrile (17.5–25%) to get similar variation in retention [set (*b*)]. In both pure and hybrid micellar systems, the concentration of SDS was made to range between 0.075 and 0.15M. The retention of some solutes in the pure micellar system was too long, but a greater concentration of surfactant was not convenient because of the high viscosity of the mobile phases and the deterioration of peak shape. The long retention times in the pure micellar system made modeling of the retention of some solutes difficult.

In both RP-LC systems, the range of retention factors (*k*) was rather wide. For the least retained solute in the strongest mobile phases, $k = 0.43$ (atenolol, 0.15% TEA–25% acetonitrile) and 2.0 (atenolol, 0.15M SDS–15% propanol). For the most retained in the slowest mobile phases, $k = 51$ (propranolol, 0.1% TEA–17.5% acetonitrile), 78 (alprenolol, 20% acetonitrile), 111 (alprenolol, 0.075M SDS–5% propanol), and $k > 120$ (alprenolol, 0.075M SDS).

The retention behavior in pure aqueous–organic systems is classically described using linear or quadratic relationships between log *k* and the volumetric fraction of solvent. On the other hand, pure micellar systems are often modeled according to the Armstrong equation (14), which can be transformed to give a linear relationship between 1/*k* and the molar concentration of micellized surfactant. In previous work, hybrid micellar retention
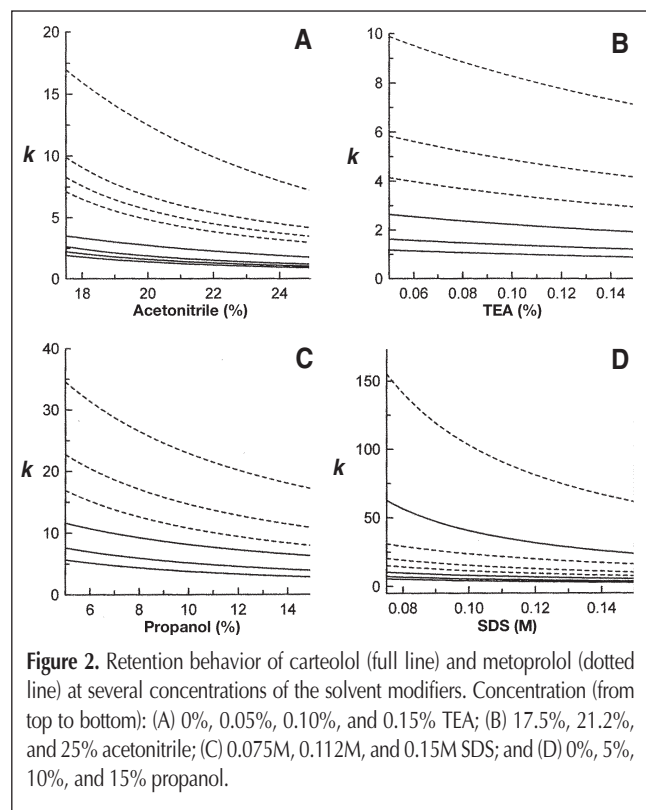

Table I. Global Mean Errors Obtained in the Description of the Retention of 16 β-Blockers Eluted with Hybrid Aqueous–Organic and Micellar Mobile Phases

Model*	%Relative error[†]																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Mean
(a) $\log k = a_0 + a_1A + a_2T$	4.4	3.0	1.9	2.9	3.0	2.0	2.9	3.0	2.4	2.8	2.7	2.9	4.7	2.5	3.0	2.7	2.9
(b) $\log k = a_0 + a_1A + a_2T + a_{12}AT$	4.4	3.0	1.7	2.9	3.0	2.0	2.9	2.9	2.3	2.7	2.7	2.8	4.4	2.4	2.7	2.2	2.9
(c) $\log k = a_0 + a_1A + a_2T + a_{12}AT + a_{11}A^2$	4.5	2.8	4.5	2.2	2.4	1.7	2.5	2.6	2.1	2.6	2.3	2.7	2.6	2.5	1.6	1.5	2.4
(d) $\log k = a_0 + a_1A + a_2T + a_{12}AT + a_{22}T^2$	4.2	2.6	1.7	3.0	3.2	2.0	3.0	2.9	2.4	2.7	2.8	2.6	3.8	2.3	2.5	2.0	2.8
(e) $1/k = a_0 + a_1A + a_2AT$	4.5	2.6	1.4	2.6	3.0	2.0	3.6	5.2	3.5	3.4	3.3	5.0	4.2	4.0	4.7	5.0	3.6
(f) $1/k = a_0 + a_1A + a_2T + a_{12}AT$	4.4	2.7	1.4	2.2	2.4	1.5	2.9	4.2	3.0	2.8	2.6	4.3	2.5	3.4	3.3	3.8	3.0
(g) $1/k = a_0 + a_1A + a_2T + a_{12}AT + a_{11}A^2$	**4.3**	**2.8**	**1.4**	**2.1**	**2.3**	**1.4**	**2.2**	**2.2**	**1.7**	**2.2**	**2.0**	**2.2**	**1.7**	**1.9**	**1.1**	**1.3**	**2.1**
(h) $1/k = a_0 + a_1A + a_2T + a_{12}AT + a_{22}T^2$	4.1	2.0	1.3	1.5	1.7	0.9	1.7	2.5	1.6	1.8	1.6	2.9	2.2	1.8	2.4	2.5	2.1
(i) $\log k = a_0 + a_1P + a_2S$	3.9	3.0	3.1	3.7	3.9	2.9	2.9	3.4	3.3	2.9	6.1	3.4	6.4	3.9	4.1	4.1	3.8
(j) $\log k = a_0 + a_1P + a_2S + a_{12}PS$	3.9	3.0	3.0	3.7	3.8	3.0	3.0	3.0	3.2	2.9	6.1	3.4	6.4	3.9	4.1	3.8	3.8
(k) $\log k = a_0 + a_1P + a_2S + a_{12}PS + a_{22}P^2$	3.7	2.9	2.8	3.7	3.7	3.0	2.9	2.9	3.0	2.9	2.9	3.2	3.0	3.1	2.0	3.9	3.1
(l) $\log k = a_0 + a_1P + a_2S + a_{12}PS + a_{11}S^2$	2.4	1.7	1.9	2.5	1.4	1.1	1.6	2.4	1.6	1.1	4.1	1.5	5.6	2.2	2.3	2.1	2.2
(m) $1/k = a_0 + a_1P + a_2S$	7.5	5.5	6.2	7.6	6.9	5.8	5.8	7.1	6.9	6.7	6.4	6.0	9.3	7.4	6.7	9.6	7.0
(n) $1/k = a_0 + a_1P + a_2S + a_{12}PS$	3.0	2.0	2.9	2.7	2.1	1.4	1.8	3.5	1.3	1.5	1.2	1.3	3.6	1.1	1.3	2.3	2.1
(o) $1/k = a_0 + a_1P + a_2S + a_{12}PS + a_{22}P^2$	**1.3**	**1.0**	**0.6**	**1.3**	**1.4**	**0.7**	**0.7**	**2.0**	**0.6**	**0.5**	**0.6**	**0.8**	**1.7**	**0.6**	**1.1**	**0.8**	**1.0**
(p) $1/k = a_0 + a_1P + a_2S + a_{12}PS + a_{11}S^2$	2.9	2.1	2.9	2.6	1.3	1.2	1.9	3.4	1.2	1.5	0.8	1.1	3.6	0.9	1.4	2.2	2.0

* A = acetonitrile, T = triethylamine, P = propanol, and S = SDS. Selected models are boldfaced.
[†] Codes 1–16 correspond to the compound numbers given in the Experimental section.

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expressed as $1/k$ was checked to properly fit a polynomial showing linear dependences in each factor (i.e., surfactant and organic solvent), which included a first order cross-term (15). However, to the our knowledge, no study about the retention modeling that simultaneously considers the amine and organic solvent concentrations in aqueous–organic systems has been reported.

Figure 2. Retention behavior of carteolol (full line) and metoprolol (dotted line) at several concentrations of the solvent modifiers. Concentration (from top to bottom): (A) 0%, 0.05%, 0.10%, and 0.15% TEA; (B) 17.5%, 21.2%, and 25% acetonitrile; (C) 0.075M, 0.112M, and 0.15M SDS; and (D) 0%, 5%, 10%, and 15% propanol.

In order to find the best retention models, the four experimental sets of data were processed in a similar way, fitting several polynomial equations relating both logarithmic and reciprocal retention factors with each modifier concentration. The experimental factors were expressed as the volumetric fraction of organic solvent or amine and molar concentration of micellized surfactant. The equations were fitted linearly using convenient weighting factors.

Table I summarizes the results for the hybrid systems. Although the best models in micellar RP-LC are well known, the prediction errors for several polynomials are shown for comparative purposes. The global errors in the table were calculated as mean deviations between experimental and predicted retention data, divided by the mean retention factor of the solute. The equations that balanced a minimal number of experiments and an accurate enough prediction capability the best, are enboldened. Note that these equations contain a quadratic term for the organic solvent (acetonitrile or propanol). As observed, the predictions were satisfactory (somewhat better for the micellar system), with mean errors usually less than 3%. More complex polynomials with additional terms may have improved the fitting of the training set, but worsened the eventual predictions in external sets because of overfitting. Additionally, more mobile phases were required.

For the aqueous–organic eluents, the mean errors and error ranges obtained using a quadratic relationship with log k were 2.6% and 1.3–4.5%, and with $1/k$, 2.6% and 1.1–3.6%. For pure micellar eluents, a simple linear relationship was checked. The mean errors and error ranges with log k were 5.2% and 2.6–8.6%, and with $1/k$, 2.1% and 0.8–3.9%.

Elution strength

Figure 2 shows the dependence of the retention factors with the

Table II. Elution Strength of the Modifiers for the Separation of the β-Blockers Using Aqueous–Organic and Micellar Mobile Phases

Compound*	Acetonitrile elution strength at TEA			TEA elution strength at acetonitrile		SDS elution strength at propanol			Propanol elution strength at SDS	
	0%	0.05%	0.15%	17.5%	25%	0%	5%	15%	0.075M	0.15M
1	–0.99	–2.46	–2.22	–1.03	–0.85	–5.38	–4.36	–4.26	–3.20	–3.12
2	–1.14	–2.77	–2.69	–1.20	–1.14	–4.32	–4.22	–2.41	–2.34	
3	–0.92	–2.68	–1.81	–1.41	–0.76	–4.38	–4.18	–2.78	–2.62	
4	–2.10	–4.87	–4.70	–1.33	–1.20	–5.96	–4.37	–4.60	–2.93	–3.10
5	–2.18	–5.13	–5.18	–1.22	–1.25	–4.08	–4.58	–3.25	–3.63	
6	–2.18	–4.07	–3.81	–1.48	–1.29	–4.41	–4.51	–2.81	–2.89	
7	–3.58	–5.84	–6.13	–1.27	–1.49	–6.55	–4.38	–4.61	–2.20	–2.37
8	–5.14	–7.06	–7.48	–1.37	–1.69	–6.96	–4.34	–4.90	–2.69	–3.11
9	–4.21	–6.18	–6.27	–1.38	–1.45	–4.36	–4.71	–3.40	–3.66	
10	–3.45	–5.31	–5.47	–1.36	–1.48	–5.64	–4.32	–4.60	–3.26	–3.46
11	–3.14	–5.47	–5.72	–1.29	–1.48	–3.16	–4.59	–4.60	–4.26	–4.28
12	–4.78	–6.30	–6.79	–1.25	–1.61	–4.64	–4.58	–3.27	–3.22	
13	–5.44	–8.00	–9.18	–0.81	–1.69	–7.26	–3.78	–5.16	–3.12	–4.15
14	–4.51	–6.07	–6.32	–1.41	–1.60	–5.48	–4.46	–4.91	–3.66	–3.99
15	–5.70	–7.31	–8.30	–1.11	–1.85	–5.42	–4.72	–5.06	–3.76	–4.02
16	–5.90	–6.99	–8.01	–1.09	–1.85	–4.70	–5.57	–3.84	–4.50	

* Codes 1–16 correspond to the compound numbers given in the Experimental section.

concentrations of the modifiers in both hybrid systems for two β-blockers (carteolol and metoprolol). Acetonitrile, propanol, and SDS produced strong variations in retention, especially for the most hydrophobic compound. The reduction in retention factors (at increasing concentration of these modifiers) can be approximated to exponential or hyperbolic decays. In the micellar mobile phases, the reduction at increasing SDS is stronger at lower propanol contents. Although no data were taken for hybrid aqueous–organic mobile phases with volumetric fractions of acetonitrile of less than 17.5%, an even stronger decay was expected in this region. The effect of TEA on the retention was smaller with an almost linear trend of k versus TEA in the range 0.05–0.15%, although a strong decay took place in the 0–0.05% TEA range (Figure 2A). Consequently, in micellar RP-LC, both modifiers influence the retention strongly, whereas in the aqueous–organic system, the effect of acetonitrile is remarkably greater at the concentrations of TEA of practical interest.
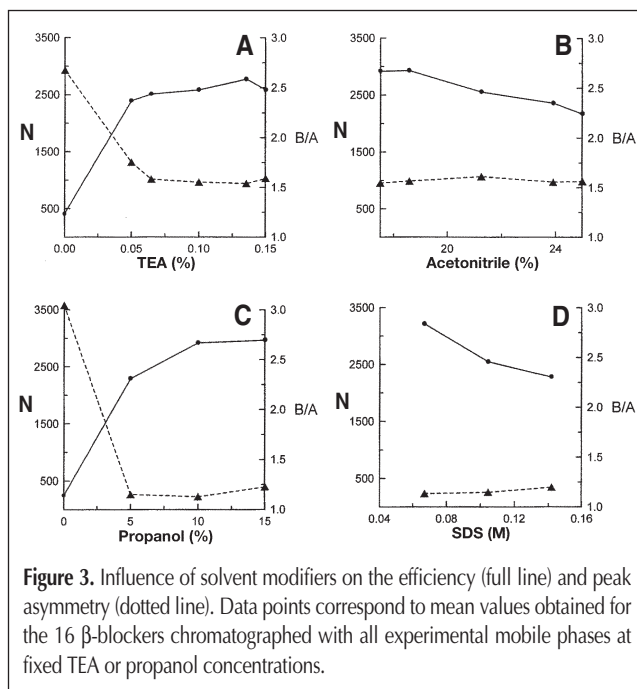
In pure aqueous–organic mobile phases, the elution strength (i.e., sensitivity of solute retention to changes in the concentra-

tion of modifier) is conventionally measured as the slope of a linear equation that relates log k with the volumetric fraction of organic solvent. Similar relationships (i.e., log k vs. acetonitrile, TEA, propanol, or SDS) were achieved in the hybrid systems by fixing the concentration of one of the modifiers in equations b and j. Table II lists the elution strength of each modifier (at fixed concentration of the other in the mobile phase) for each β-blocker in the hybrid systems. The elution strength in pure eluents is also given. The compounds were ordered according to their retention times in 0.10M SDS–15% propanol.

The elution strength of acetonitrile in the aqueous–organic

Figure 3. Influence of solvent modifiers on the efficiency (full line) and peak asymmetry (dotted line). Data points correspond to mean values obtained for the 16 β-blockers chromatographed with all experimental mobile phases at fixed TEA or propanol concentrations.

Table III. Mean Main Effects and First-Order Interactions for the Hybrid Systems

Parameter		Acetonitrile–TEA		SDS–propanol
Retention	A	−0.555	P	−0.322
	T	−0.081	S	−0.477
	AT	0.006	PS	−0.008
Efficiency	A	−1140	P	675
	T	263	S	−1280
	AT	−208	PS	−450
Asymmetry	A	−0.017	P	0.083
	T	−0.041	S	0.081
	AT	−0.053	PS	0.072

Table IV. Efficiencies and Asymmetry Factors in Aqueous–Organic and Micellar Mobile Phases*

Compound[†]	Pure aqueous–organic		Hybrid aqueous–organic		Pure micellar		Hybrid micellar	
	N	B/A	N	B/A	N	B/A	N	B/A
1	255 ± 80	3.1 ± 0.06	800 ± 350	1.6 ± 0.07	795 ± 130	2.2 ± 0.1	2030 ± 560	1.3 ± 0.2
2	325 ± 60	2.7 ± 0.5	870 ± 340	1.8 ± 0.5			1900 ± 540	1.3 ± 0.2
3	490 ± 90	2.5 ± 0.3	1110 ± 300	1.5 ± 0.3			2230 ± 540	1.3 ± 0.2
4	360 ± 40	2.6 ± 0.3	1670 ± 290	1.6 ± 0.2	340 ± 110	2.8 ± 0.4	2180 ± 500	1.2 ± 0.1
5	405 ± 70	2.5 ± 0.3	1680 ± 400	1.5 ± 0.2			1770 ± 1100	1.6 ± 0.6
6	550 ± 140	2.6 ± 0.3	2680 ± 480	1.5 ± 0.2			2780 ± 610	1.2 ± 0.1
7	285 ± 120	2.9 ± 0.3	2590 ± 370	1.5 ± 0.1	80 ± 15	4.6 ± 0.7	2550 ± 520	1.2 ± 0.1
8	175 ± 100	3.0 ± 0.3	2540 ± 500	1.7 ± 0.1	60 ± 9	5.2 ± 0.3	2540 ± 590	1.1 ± 0.1
9	505 ± 120	2.6 ± 0.2	4000 ± 490	1.5 ± 0.1			3190 ± 510	1.1 ± 0.1
10	295 ± 130	2.8 ± 0.3	2940 ± 480	1.6 ± 0.2	150 ± 30	3.5 ± 0.3	3530 ± 600	1.1 ± 0.1
11	425 ± 200	2.3 ± 0.3	2630 ± 480	1.5 ± 0.2	240 ± 115	2.7 ± 1.0	3640 ± 670	1.0 ± 0.0
12	400 ± 90	2.6 ± 0.3	4120 ± 410	1.5 ± 0.1			3340 ± 630	1.1 ± 0.1
13	830 ± 390	2.7 ± 0.3	2160 ± 380	1.8 ± 0.1			3660 ± 640	1.1 ± 0.0
14	315 ± 100	2.6 ± 0.3	3760 ± 490	1.5 ± 0.1	215 ± 131	2.6 ± 0.6	2130 ± 910	1.1 ± 0.0
15	335 ± 100	2.8 ± 0.5	3890 ± 420	1.6 ± 0.1	165 ± 33	1.6 ± 0.1	2620 ± 990	1.0 ± 0.0
16	655 ± 520	2.4 ± 1.0	4040 ± 360	1.7 ± 0.1			3100 ± 910	1.0 ± 0.1

* Mean values of the data in all experimental mobile phases.
† Codes 1–16 correspond to the compound numbers given in the Experimental section.

system increased appreciably by the addition of TEA (Table II). However, the effect of the amine was rather weak between 0.05% and 0.15% TEA. In this range, the retention decay was smaller at the greater amine concentration for the least retained solutes, but larger for those most retained. Also, the elution strength of TEA was scarcely affected by the acetonitrile content. Similarly to aqueous–organic RP-LC, in the micellar system the greatest change in retention took place in the transition from pure micellar to hybrid eluents. The change was usually small between the addition of 5% and 15% propanol, or between 0.075 and 0.15M SDS. On the other hand, the elution strength was 0.5–2 units greater for the surfactant than for the alcohol, which means that the changes in retention were larger when the concentration of surfactant was varied. This can be explained by the strong association of the protonated β-blockers with the anionic SDS micelles.
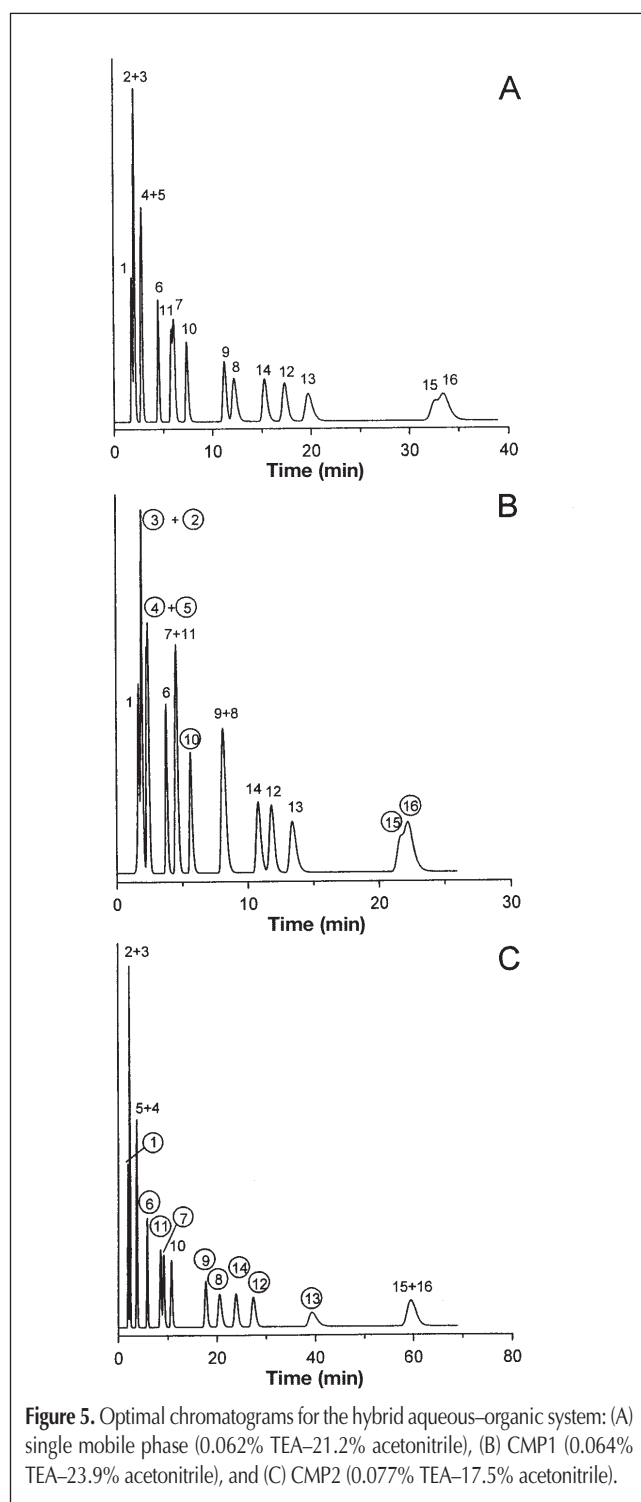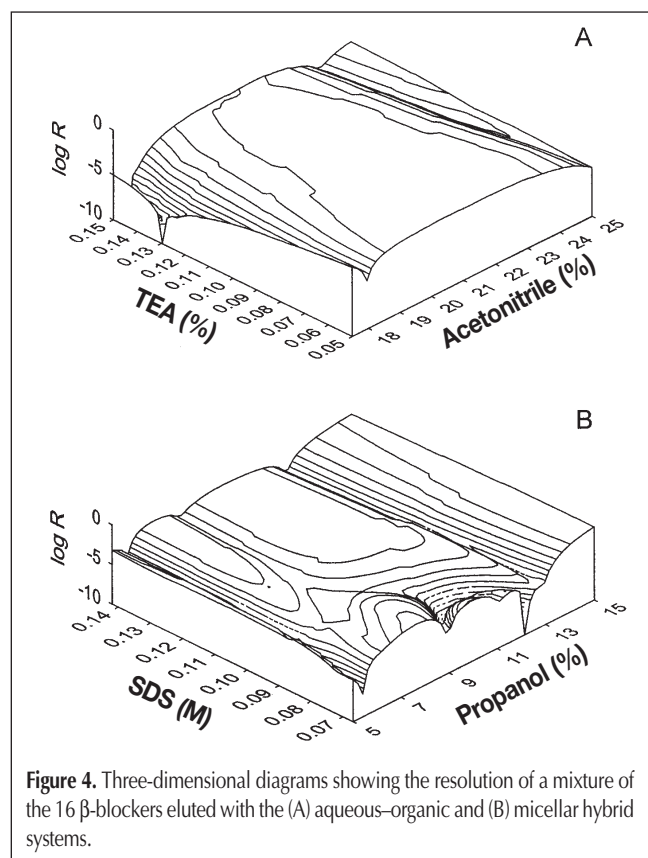
Table III shows the mean main effects and first-order interactions in both hybrid RP-LC systems. Acetonitrile was the factor having the strongest effect on retention, followed by SDS and propanol. The effect of TEA was almost negligible. This conclusion can also be reached from the comparison of the elution strengths in Table II. Although the effect of SDS was greater than that of propanol, the range of retention factors in the experimental domain was similar for both modifiers, which was because the ratio between the extreme concentrations was larger for propanol.

Finally, in the aqueous–organic system with or without an amine, there was a remarkable variation in elution strength along the series of β-blockers, which increased with compound hydrophobicity (Table II). This behavior permitted the use of gra-
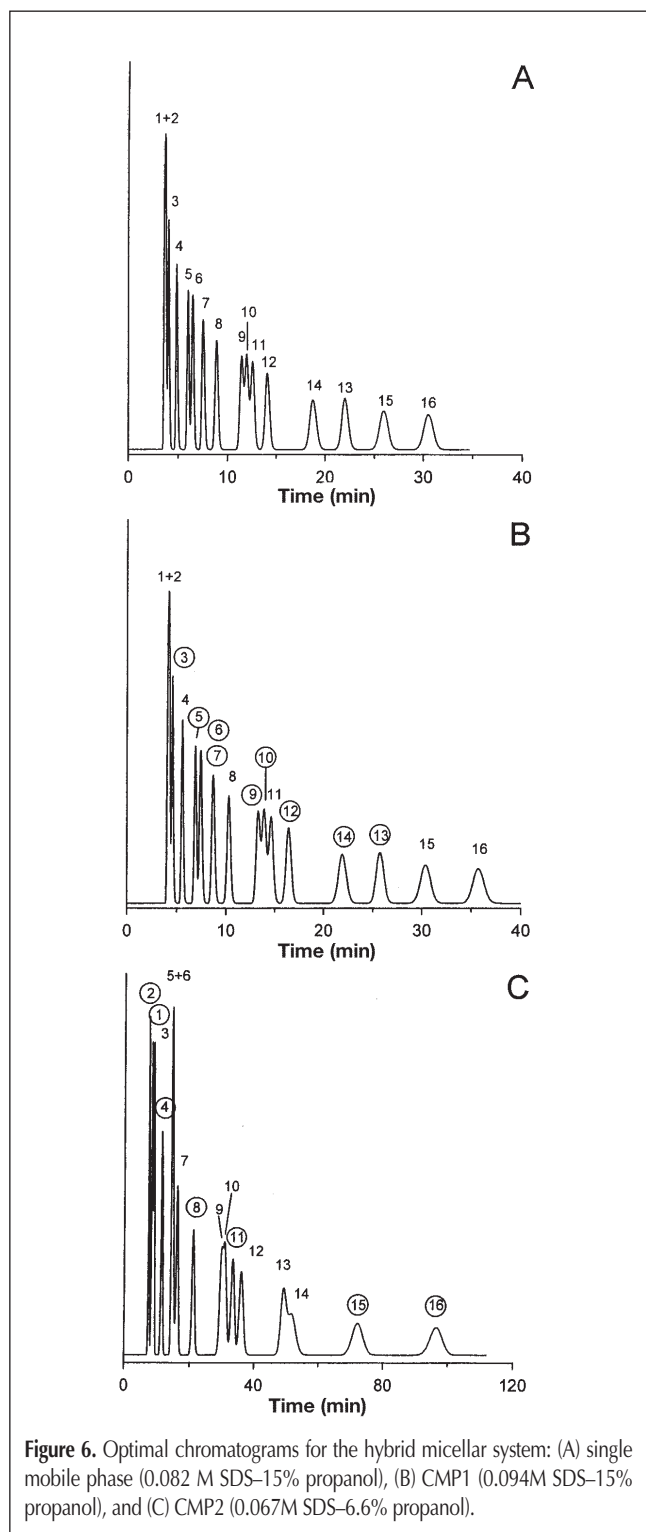
dient elution to achieve practical analysis times for the determination of a mixture that included compounds of extreme polarities. The elution strength range was much smaller for TEA, SDS, and propanol.

Peak efficiencies and asymmetries

Peak efficiencies, expressed as theoretical plates (N), were estimated at 10% of peak height according to Foley and Dorsey (16). Asymmetry factors were calculated as the ratio (B/A) of the distances between the peak maximum and the tailing edge (B), and

Figure 5. Optimal chromatograms for the hybrid aqueous–organic system: (A) single mobile phase (0.062% TEA–21.2% acetonitrile), (B) CMP1 (0.064% TEA–23.9% acetonitrile), and (C) CMP2 (0.077% TEA–17.5% acetonitrile).

Figure 4. Three-dimensional diagrams showing the resolution of a mixture of the 16 β-blockers eluted with the (A) aqueous–organic and (B) micellar hybrid systems.

the peak maximum and the leading edge (A), measured also at 10% of peak height. An analysis of the mean main effects (Table III) in the hybrid systems shows that the improvement in efficiency was larger for propanol than for TEA, whereas this deteriorated similarly for SDS and acetonitrile. This result can be also observed in Figure 3, in which the mean values of N for the 16 β-blockers, chromatographed in all experimental mobile phases with a given concentration level of TEA or propanol, are plotted. The data for the pure systems are also included. The plot that shows the dependence of the efficiency with TEA is remarkably similar to that for propanol, and the same can be said for acetonitrile and SDS. A steep transition was observed with the first additions of TEA or propanol to the pure mobile phases. Further additions of these modifiers yielded constant efficiencies, especially in the case of TEA. Concentrations of propanol of greater than 15% were not examined in order to avoid the disruption of micellar aggregates.

The mean values of N for each β-blocker eluted with all of the experimental mobile phases are given in Table IV. The efficiencies were larger for the amine-modified acetonitrile eluents than for the pure eluents, with enhancement factors of 2.3–3.1 for atenolol, practolol, and sotalol, to more than 10 for some of the most hydrophobic compounds. In the micellar eluents, a similar trend was observed, with even higher enhancement factors. The efficiencies obtained by the addition of TEA and propanol to the mobile phases of acetonitrile–water and SDS, respectively, were usually similar for the compounds of intermediate polarity, but larger values were obtained for the β-blockers of lowest polarity in TEA–acetonitrile and those of the highest polarity in SDS–propanol.

The peak symmetries experienced an abrupt improvement upon the addition of TEA or propanol to the pure systems (Figure 3 and Table IV). However, in the studied experimental domain of both amine- and propanol-modified systems, peak symmetry did not change appreciably. Note that, although the initial B/A values obtained in pure SDS eluents were larger than in acetonitrile–water for some compounds, the improvement for the former was greater. In fact, peak tailing was almost suppressed in SDS–propanol (B/A = 1.0–1.3, except for nadolol), while some tailing remained in TEA–acetonitrile.

Resolution

Individual resolution was measured as peak purity (i.e., peak area free of overlap), r, according to a previously reported methodology (17). The resolution values were reduced to a single measurement (the product of peak purities, R) to describe the global separation for all of the peaks in the chromatogram. Peak purities varied between 0 (full overlap) and 1 (full resolution). Because the magnitude of R depended on the number of multiplied individual resolutions, the number of compounds must have been considered in its evaluation. Therefore, if all compounds in a complex mixture exhibit small overlap, R can be small even when the resolution is fully satisfactory.

Full resolution is usually accomplished by searching one optimal mobile phase. However, the separation degree may not be satisfactory, especially if the mixture is too complex . In order to explore the possibilities of the chromatographic system, the examination of the limiting individual resolutions, r_{lim} (i.e., peak purity that can be achieved when a given compound is resolved maximally from the others) is useful. In case the individual resolutions do not reach the limiting ones, an alternative approach is to search two or more complementary mobile phases (CMPs) (17). Each CMP resolves optimally some compounds in the mixture, and the other compounds can overlap among them.

Figure 4 shows the three-dimensional resolution diagrams for the separation of the 16 β-blockers with the two hybrid systems.

Figure 6. Optimal chromatograms for the hybrid micellar system: (A) single mobile phase (0.082 M SDS–15% propanol), (B) CMP1 (0.094M SDS–15% propanol), and (C) CMP2 (0.067M SDS–6.6% propanol).

Both TEA and SDS scarcely affect the resolution capability. In both cases, a plateau existed at intermediate concentrations of the organic solvents, which was more extensive for acetonitrile although with smaller global resolution values. The steep valleys at the plateau extremes indicated peak crossings. In the aqueous–organic system, only peaks 8 and 9 reverse their elution order at an acetonitrile concentration of greater than 23%, whereas in the micellar system, multiple peak crossings (i.e., peaks 1/2, 5/6, 10/11/12, and 13/14) took place in different regions of the experimental domain. In SDS–propanol, maximum resolution occured at the highest organic solvent concentration (0.082M SDS–15% propanol), whereas in TEA–acetonitrile, this was found at an intermediate value of acetonitrile (0.062% TEA–21.2% acetonitrile).

Figures 5A and 6A depict the chromatograms for the optimal single mobile phases in the hybrid aqueous–organic and micellar systems, respectively. The analysis times were 35 and 32 min, respectively. The elution order of intermediate polarity compounds (compounds 8–12) differs in both systems. Also, the efficiencies are remarkably larger in the optimal micellar mobile phase. Thus, for example, for carteolol, celiprolol, and labetalol, N = 1590, 2070, and 2190 with B/A = 1.8 in 0.062% TEA–21.2% acetonitrile, whereas N = 2600, 3270, and 4490 with B/A = 1.1–1.2 in 0.082M SDS–15% propanol.

Peak resolutions for the optimal compositions are given in Table V, in which they are compared with the limiting values. Peaks 2/3, 4/5, 7/11, and 15/16 remain unresolved with the optimal single aqueous–organic mobile phase, and peaks 1/2 and 9/10/11 coelute partially with the micellar mobile phase. Limiting peak purities indicated that satisfactory resolution was feasible for peaks 7 and 11 in the former system (i.e., individual resolutions can be improved from r = 0.776 to 0.979). When two optimal CMPs were selected, these solutes almost reached the maximal expected resolution (r = 0.970), but six solutes still remained poorly resolved (Figures 5B and 5C).

For SDS–propanol, limiting resolutions were noteworthy and larger than in the previous case, which denoted the greater capability of the micellar system to resolve the mixture of β-blockers. This was the final consequence of the higher efficiency and asymmetry enhancements, together with a larger variation in selectivity. Thus, peak purities of solutes 1, 2, and 11 can be largely improved (from r = 0.604, 0.565, and 0.884 to r = 0.949, 0.998, and 0.960, respectively). Interestingly, these solutes reached almost their maximum expected resolution with two optimal CMPs (r = 0.911, 0.996, and 0.960, respectively). This means that only solutes 9 and 10 were still partially overlapped (Figures 6B and 6C). Note that one of the CMPs had a composition similar to the optimal single mobile phase (0.094M SDS–15% propanol), whereas the other, with a smaller elution strength (0.067M SDS–6.6% propanol), succeeded in the separation of some of the most problematic solutes (1, 2, and 11). Some solutes (peaks 4, 8, 15, and 16) were well resolved in both CMPs.

In previous work (7), high efficiencies were obtained for most of the studied β-blockers (N = 3000–8000) with a base-deactivated XTerra MS C_{18} column (Waters, Milford, MA), using acetonitrile–water mobile phases in the absence of amine. Asymmetry factors (B/A = 1.2–1.5) were not quite as low as in the micellar system. Also, the resolution was poorer because of the incapability of the column to differentiate the elution of several of the least retained compounds. The difference in retention between polar and low polar compounds was also too large to permit the elution by isocratic RP-LC.

Table V. Limiting (r_{lim}) and Elementary Resolutions (r) for the Optimal Single and Complementary Mobile Phases

Compound*	Acetonitrile–TEA				SDS–propanol			
	Limiting	Single	CMP1[†]	CMP2[†]	Limiting	Single	CMP1[†]	CMP2[†]
1	0.991	0.931	0.942	**0.991**	0.949	0.604	0.584	**0.911**
2	0.423	0.296	**0.423**	0.205	0.998	0.565	0.546	**0.996**
3	0.457	0.344	**0.444**	0.233	0.990	0.951	**0.954**	0.915
4	0.784	0.667	**0.763**	0.576	1.000	1.000	**1.000**	**1.000**
5	0.774	0.670	**0.760**	0.579	0.980	0.973	**0.967**	0.096
6	1.000	1.000	**1.000**	**1.000**	0.980	0.973	**0.967**	0.174
7	0.979	0.776	0.432	**0.970**	1.000	1.000	**1.000**	0.952
8	1.000	0.990	0.459	**1.000**	1.000	1.000	**1.000**	**1.000**
9	1.000	0.990	0.466	**1.000**	0.902	0.851	**0.873**	0.730
10	1.000	1.000	**1.000**	**1.000**	0.756	0.736	**0.756**	0.705
11	0.979	0.776	0.420	**0.970**	0.960	0.884	0.882	**0.960**
12	1.000	1.000	0.990	**1.000**	1.000	0.999	**0.999**	0.985
13	1.000	1.000	**1.000**	**1.000**	1.000	1.000	**1.000**	0.785
14	1.000	1.000	0.991	**1.000**	1.000	1.000	**1.000**	0.785
15	0.769	0.695	**0.666**	0.238	1.000	1.000	**1.000**	**1.000**
16	0.766	0.691	**0.661**	0.235	1.000	1.000	**1.000**	**1.000**
R	0.066	0.012	0.045	**0.589**	0.170	0.512		

* Codes 1–16 correspond to the compound numbers given in the Experimental section.
[†] Bold values indicate the CMP.

Conclusion

In conclusion, micellar LC seems to be superior to classical RP-LC in the separation of basic β-blockers. The combination of improved peak shapes (i.e., peak sharpness and less tailing), larger selectivity, and smaller range in retention among compounds of extreme polarity leads to the logical observation that a greater number of solutes can be resolved in one run using isocratic elution. Micellar mobile phases enable the direct injection of physiological samples into the chromatographic system. The following limits of detection were obtained in our laboratory for β-blockers administered in our country by use of fluorimetric detection: acebutolol (30 ng/mL), atenolol (19 ng/mL), celiprolol (200 ng/mL), labetalol (20 ng/mL), metoprolol (16 ng/mL), nadolol (8 ng/mL), and propranolol (3 ng/mL). This permitted the quantitation of the drugs at least up to 24–48 h after administration (18).

Acknowledgments

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