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Chiral separations of basic drugs and quantitation of bupivacaine enantiomers in serum by capillary electrophoresis with modified cyclodextrin buffers

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ABSTRACT

Modified cyclodextrin derivatives were evaluated as the buffer additives in capillary electrophoresis of several racemic pharmaceutical bases. Uncoated and polyacrylamide-modified silica capillaries were compared for their effectiveness in the enantiomeric resolution and migration reproducibility of model solutes. Using cationic detergents in the mixed-micellar mode, optimized separations of the racemic drug bupivacaine are demonstrated in a spiked serum sample at the therapeutic level. Precision, linearity and sensitivity of the method appear adequate for reliable quantitation required in pharmacokinetic and clinical studies.

INTRODUCTION

In pharmaceutical research and quality control, separation of optical isomers is important throughout the development and manufacturing of numerous therapeutics. In the course of checking the composition of bulk drug substances as well as in the more involved pharmacokinetic and clinical studies, the ability to measure separately quantities of optical isomers has become essential. Many pharmaceutical preparations are now administered as a 1:1 racemic mixture of (*R*)- and (*S*)-enantiomers. In the body, various enantiomers behave as two different entities; stereoselectively controlled processes such as drug absorption, distribution, metabolism and elimination may differ substantially between the enantiomers [1]. The pharmacokinetic studies in body fluids impose challenging demands for the separation systems in use. These include, in addition to the enantioselective separations, the need for resolution between a number of endogenous biological molecules and relatively small pharmaceutical molecules present in the same sample. In addition, the performance of the analytical system must remain constant during the long analysis periods despite the presence of interfering matrix compounds.

The separation principle of capillary electrophoresis (CE) offers considerably greater separation efficiency, within a short analysis time, than that of high-performance liquid chromatography or gas chromatography [2,3]. The possibility of varying the selectivity of separation through the use of dif-

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ferent modifiers in buffer solutions is yet another valuable property of the general method. For example, micellar electrokinetic chromatography [4] has shown an excellent capability to solubilize disturbing proteins and other endogeneous materials during the analysis of pharmaceuticals after a direct injection of blood serum [5–7]. Short of sample preconcentration, however, this approach is adequate only at high concentrations of pharmaceuticals.

Recently, cyclodextrins (CDs) and their derivatives have been employed in CE of various optical and geometrical isomers as well as for other small molecules of pharmaceutical interest [8–11]. The uses of CDs in CE have been reviewed by Snopek *et al.* [12]. At this date, analyses of biological materials following this approach remain unexplored.

In the recent applications of CD-modified electrokinetic chromatography, a combination of anionic surfactants and CDs has been utilized in the buffer systems to aid resolution of hydrophobic polyaromatic compounds [13] and closely related corticosteroids [14]. Structurally similar peptides, both in their native forms and as fluorescent derivatives, were successfully separated using both anionic and cationic detergents above their critical micelle concentration, together with CDs [15]. Under such conditions, the analytes are believed to incorporate themselves either into the micelles or the CD cavity [13], thus enhancing the selectivity potential of the separation system. Interactions of CD molecules with cationic and anionic surfactants under the micellar conditions have been studied by careful conductometric measurements [16,17]. According to these measurements, CDs induce an increase in the apparent critical micellar concentration of a surfactant, since the available monomers are partly associated in a complex with CDs. Furthermore, interactions of surfactants with the outer hydrophilic shells of CDs were suggested [16].

In the present study, we have investigated the properties of β-CD solutions modified by certain cationic surfactants in the separations of optical isomers of basic pharmaceuticals. Uncoated fused-silica and polyacrylamide-coated silica capillaries were used in the study. Effective separations of optical isomers for model pharmaceuticals —verapamil, fluoxetine, bupivacaine, mepivacaine, carvedilol and pindolol— are described. Chemical structures of these compounds are shown in Fig. 1. A variety of additives in the CD–buffer systems were tested for both enhancing resolution between the enantiomeric pairs and improving analytical reproducibility. The effect of the cationic detergents hexadecyltrimethylammonium bromide and cetylpyridinium chloride, in combination with the β-CD trimethylderivative, on the resolution of the enantiomeric pairs was investigated for fluoxetine and verapamil. Finally, an optimized separation system with the β-CD dimethylderivative was demonstrated for the quantitation of bupivacaine enantiomers in spiked serum samples at therapeutic levels.

EXPERIMENTAL

Materials

Verapamil hydrochloride, or α-[3-{[2-(3,4-dimethoxyphenyl)ethyl]-methylamino}propyl]-3,4-dimethoxy-α-(1-methylethyl)benzene-acetonitrile hydrochloride; pindolol, or 1-(1H-indol-4-yloxy)-3-[(1-methyl-ethyl)amino]-2-propanol; carvedilol, or 1-(9H-carbazol-4-yloxy)-3-{[2-(2-methoxyphenoxy)ethyl]amino}-2-propanol; and bupivacaine hydrochloride, or 1-butyl-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide hydrochloride, were a gift from Orion Pharmaceutica, Espoo, Finland. Pure (*R*)- and (*S*)-bupivacaine hydrochloride were provided by Astra (Södertälje, Sweden). Mepivacaine, or N-(2,6-dimethylphenyl)-1-methyl-2-piperidinecarboxamide, was a USP reference standard (Twinbrook Parkway, Rockville, MD, USA). Fluoxetine hydrochloride, or (±)-N-methyl-3-phenyl-[(α,α,α-trifluoro-*p*-tolyl)-oxyl]-propylamine hydrochloride was a gift from Lilly Research Laboratories (Indianapolis, IN, USA). Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD); heptakis(2,6,di-O-methyl)-β-cyclodextrin (DM-β-CD); hexadecyltrimethylammonium bromide (HTAB); cetylpyridium chloride (CTPC); 1-heptanesulfonic acid; and finally, Trizma base (Tris), were obtained from Sigma (St. Louis, MO, USA). Methylhydroxyethylcellulose 1000 and 4000 (MHEC) were purchased from Serva (Heidelberg, Germany). Ethylene glycol, hexane (HPLC grade), sodium hydroxide (1 *M* solution) and phosphoric acid (HPLC grade, 85%) were from Fisher Scientific (Fair Lawn, NJ, USA). Diethyl ether and ethylacetate (ChromAR) were purchased from Mallinckrodt (Paris, KY, USA).

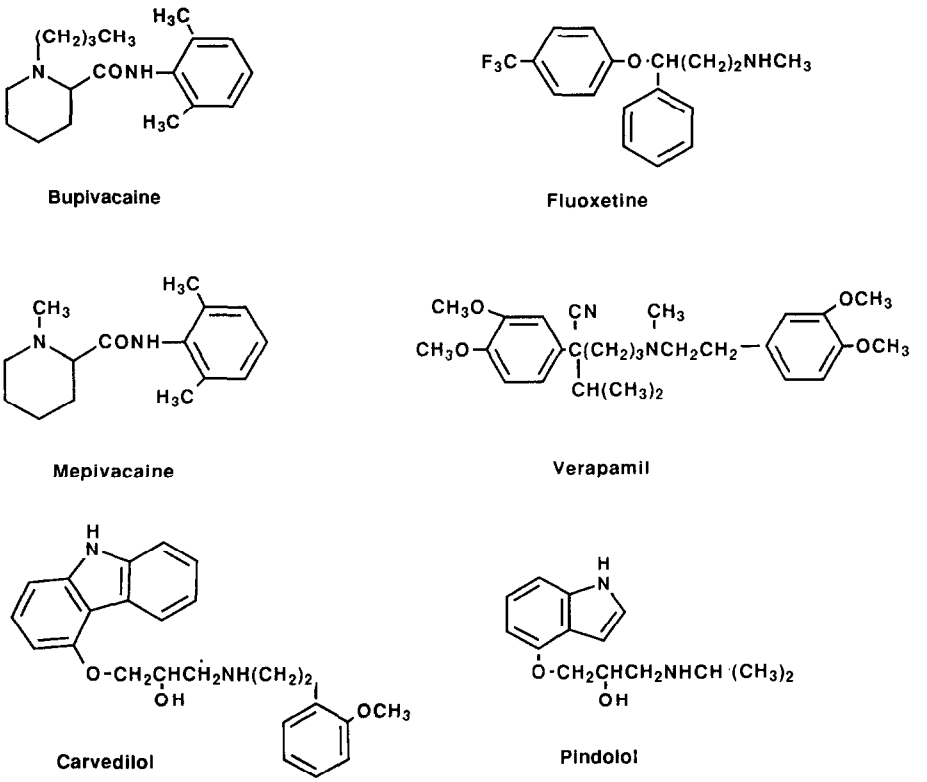

Fig. 1. Chemical structures of the basic racemic pharmaceuticals used in this study.

Polyacrylamide coating of the capillary was carried out according to the procedure described by Hjertén [18]. All buffers were filtered with 0.2-μm Nylaflo filters (Gelman Sciences, Ann Arbor, MI, USA). Water was distilled before use.

Equipment

A laboratory-made CE apparatus and Beckman (Palo Alto, CA, USA) P/ACE System 2100 were used in all measurements. Capillaries (50 cm × 150 μm O.D., 30 μm I.D. and 64 cm × 185 μm O.D., 50 μm I.D.) used in the laboratory-made instrument were from Polymicro Technologies (Phoenix, AZ, USA). A high-voltage power supply (0–60 kV) was a product of Spellman High Voltage Electronics (Plainview, NY, USA). Voltages 18–20 kV were used (negative ground). The detector was a Jasco (Tokyo, Japan) UVIDEC-100-IV adjusted to 220 nm (0.01 AUFS), where all model compounds exhibited relatively strong UV absorption. The polyimide coating of the capillary was removed in a small area (16 cm from the capillary end) to form an on-column flow-cell for UV-absorbance detection. The Beckman instrument used a capillary of 57 cm (50 cm effective length) × 367 μm O.D., 75 μm I.D. Buffers contained tri- and dimethylcyclodextrin derivatives (10 m*M* TM-β-CD or DM-β-CD), Tris (18 m*M*, pH adjusted to 2.8—3.0 with phosphoric acid) and methylhydroxyethylcellulose 1000 or 4000 (0.1%, w/w). Modifiers were added in the described buffer systems. Under the above conditions, the analytes possessed positive charge and migrated towards the negative electrode (detector end).

Sample preparation

Stock solutions of racemic drugs were prepared in methanol. Further dilutions were made in the same solvent. In the ethylacetate extraction of verapamil from serum, 0.25 ml of serum spiked with about 10 μg of verapamil was alkalinized with 30 μl of 1 *M* sodium hydroxide and shaken vigorously for

2 min with 0.5 ml ethylacetate. After the organic layer (0.4 ml) was evaporated to dryness under the stream of nitrogen, the residue was dissolved in 10 μl methanol.

For the spiked bupivacaine samples, blank serum (1.0 ml) was transferred to a 5-ml test tube and the racemic bupivacaine (0.19–3.8 μg in 15–50 μl methanol) and mepivacaine (160 μg/30 μl methanol) were added. After thorough mixing, the samples were alkalinized with 200 μl of 1 *M* sodium hydroxide by mixing for 20 s. The samples were extracted with 1.5 ml hexane–diethyl ether (1:1) by shaking the test tube gently for 5 min in the horizontal position to avoid emulsion formation. After standing in a refrigerator 5 min to cool, the 1.3 ml organic layer was evaporated to dryness in a polyethylene conical tube by a stream of nitrogen. The residue was redissolved into 15 μl of methanol. Sample introduction was carried out hydrodynamically by lifting the sample 13 cm above the buffer level and dipping the capillary end into the sample for 15-s time intervals.

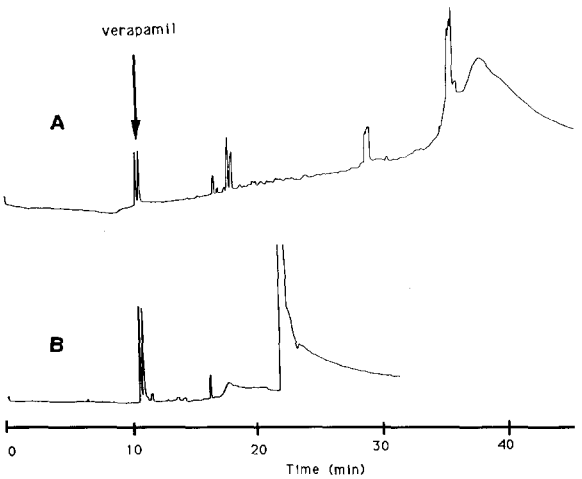

Fig. 2. Ethyl acetate extract of an alkalinized serum sample containing verapamil. (A) Buffer: 10 m*M* TM-β-CD–20 m*M* Tris, pH 2.7–0.1% MHEC 1000, capillary 64 cm × 50 μm, uncoated silica; 20 kV voltage; 215 nm UV-absorbance detection, 0.01 AUFS. (B) Buffer as in (A), but with 0.05 m*M* HTAB added (pH 3.0).

RESULTS

In a previous qualitative study [11] we used heptakis (2,6-di-O-methyl)-β- and γ-cyclodextrins in a buffer (pH between 2 and 3), with methylhydroxyethylcellulose addition. The buffer system showed a promising ability to separate the optical isomers of basic pharmaceuticals using uncoated capillaries. In this present study, buffers with hydroxycellulose derivative (pH 2.7–3.0) and heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin or heptakis(2,6-di-O-methyl)-β-cyclodextrin were modified. The aim of these experiments was to achieve separation conditions adequate for quantitative analysis of pharmaceuticals in serum samples containing a biological matrix.

Uncoated capillaries

A dramatic effect of HTAB (0.05 m*M*) in suppressing the slow-migrating serum proteins from about 40 min to 20 min, in a spiked serum extract sample (levels of verapamil around 40 μg/ml), is shown in Fig. 2. Apparently, the HTAB molecules cover the negatively charged silica wall of a capillary, forming a positive net charge. Consequently, adsorption of proteins on the wall is minimized, while the positively charged analytes are repelled.

Under the above acidic conditions (in the presence of cyclodextrins and cellulose), the electromigration of positively charged species towards the negative electrode was the main force in effect. Electroosmosis became negligible. Therefore, the reversed electroosmosis which had been observed in the cationic detergent buffer systems earlier under neutral and basic conditions [19,20] did not have a significant effect in our buffer system.

To enhance the enantiomer resolution and sampling precision for quantitation purposes, we explored the addition of methanol, ethylene glycol, and 1-heptanesulfonic acid to the original buffer-plus-additive system (TM-β-CD–0.1% MHEC–0.05 m*M* HTAB). The addition of methanol and 1-heptanesulfonic acid improved the peak shapes of fluoxetine enantiomers, while their effect on verapamil separation appeared negative. With methanol, the verapamil enantiomers co-migrated, and broad and tailing peaks were experienced due to the use of 1-heptanesulfonic acid. The effects of selected additives are demonstrated in Fig. 3.

The merits of using cationic detergents, HTAB and CTPC, in conjunction with the modified CDs and untreated silica capillary, were examined. The literature values [21] of the critical micelle concentration are 0.028 m*M* and 0.9 m*M* for HTAB and

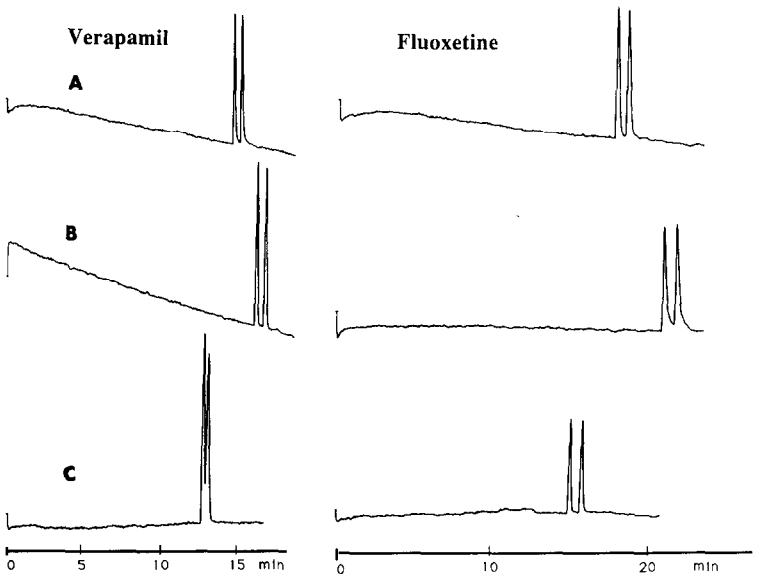

Fig. 3. Comparison of the effects of buffer modifiers on verapamil and fluoxetine enantiomer separation. Capillary: 50 cm × 30 μm, uncoated silica, detection at 220 nm, 0.01 AUFS, 25 kV voltage. (A) Buffer: 12 m*M* TM-β-CD–20 m*M* TRIS, pH 2.7–0.1% MHEC 1000–0.05 m*M* HTAB. (B) Buffer: ethylene glycol–buffer (A) (2:98). (C) Buffer: 10 m*M* 1-heptanesulfonic acid–buffer (A) (47:53) (6.4 m*M* TM-β-CD–4.7 m*M* 1-heptanesulfonic acid–0.05% MHEC 1000–0.027 m*M* HTAB).

CTPC, respectively. Hansen *et al.* [22] reported that HTAB adsorptivity on porous silica is much stronger than the values for shorter-chain cationic detergents. It is thus expected that small changes in HTAB concentration may be effective in regulating the analytical conditions of our experiments. In addition, its minor addition to the buffer media does not significantly alter the current conditions in comparative experiments.

The enantiomeric resolution and migration reproducibility for verapamil and fluoxetine were measured here as a function of detergent concentration. Resolution (R_s) was determined according to the usual formula, $R_s = 2(t_2 - t_1)/w_1 + w_2)$, where t_1 and t_2 are the respective migration times and $(w_1 + w_2)/2$ is the average peak width at the peak base. Appropriate amounts of HTAB or CTPC were added to the buffer of standard composition [10 m*M* TM-β-CD–18 m*M* Tris-phosphate (pH = 2.9)–0.1% MHEC 1000]. The resolution *vs.* detergent concentration plots are shown in Figs. 4 and 5, while Tables I and II demonstrate migration reproducibility for the first isomer of fluoxetine and verapamil at different detergent concentrations.

The results indicate that there are optimum values for both migration reproducibility and the resolution of enantiomers. For HTAB, this optimum concentration is equal to or slightly higher than the critical micelle concentration (cmc); it is somewhat lower for CTPC. The optimized separations are shown in Fig. 6 A–C.

The optical isomers of mepivacaine, bupivacaine, pindolol and carvedilol were subsequently separated using dimethyl-β-cyclodextrin buffer with the es-

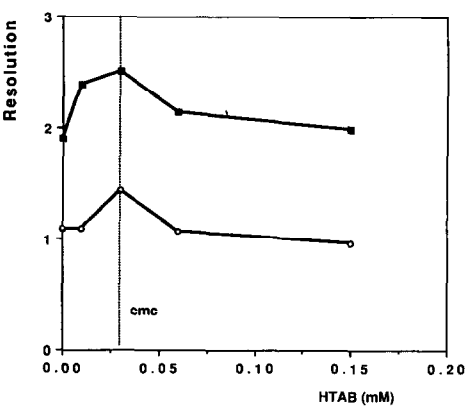

Fig. 4. Resolution of the enantiomeric pairs of verapamil (○) and fluoxetine (■) *vs.* HTAB concentration.

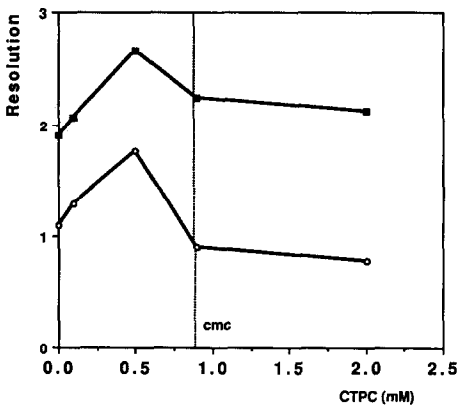

Fig. 5. Resolution of the enantiomeric pairs of verapamil (○) and fluoxetine (■) *vs.* CTCP concentration.

tablished optimum concentration of HTAB. Buffer composition was DM-β-CD (10 m*M*)–Tris (18 m*M*, pH 2.9 with phosphoric acid)–0.1% MHEC 4000–0.03 m*M* HTAB.

Separations of additional analytes are seen in Figs. 7A and B and 8. For pindolol, only partial separation was achieved. The migration order of bupivacaine enantiomers was verified with pure (*R*)- and (*S*)-isomers. (*R*)-Bupivacaine eluted before (*S*)-bupivacaine, as seen in Fig. 9, where an excess of (*R*)-isomer was added to the racemic mixture.

Bupivacaine in serum

Bupivacaine hydrochloride is an amide-type local, long-lasting anaesthetic agent. Following the epidural analgesia with administration of 150 mg of the drug before orthopaedic surgery, therapeutic levels of bupivacaine were reported to remain in serum typically at the level of 1.0–1.2 μg/ml up to 3 h after treatment [23]. Therapeutic levels of the race-

TABLE I

MIGRATION TIME VARIATION OF VERAPAMIL AND FLUOXETINE (RELATIVE STANDARD DEVIATION, R.S.D., n = 4) AS FUNCTION OF HTAB CONCENTRATION

HTAB (m*M*)	R.S.D. (%)	
	Verapamil	Fluoxetine
0.00	2.8	6.1
0.01	0.4	0.9
0.03	0.7	0.4
0.06	0.6	0.5
0.15	3.5	3.7

TABLE II

MIGRATION TIME VARIATION OF VERAPAMIL AND FLUOXETINE (R.S.D., n = 4) AS FUNCTION OF CTPC CONCENTRATION

CTPC (m*M*)	R.S.D. (%)	
	Verapamil	Fluoxetine
0.0	2.8	6.1
0.1	1.4	1.7
0.5	1.7	1.0
0.9	2.4	2.8
2.0	2.8	2.9

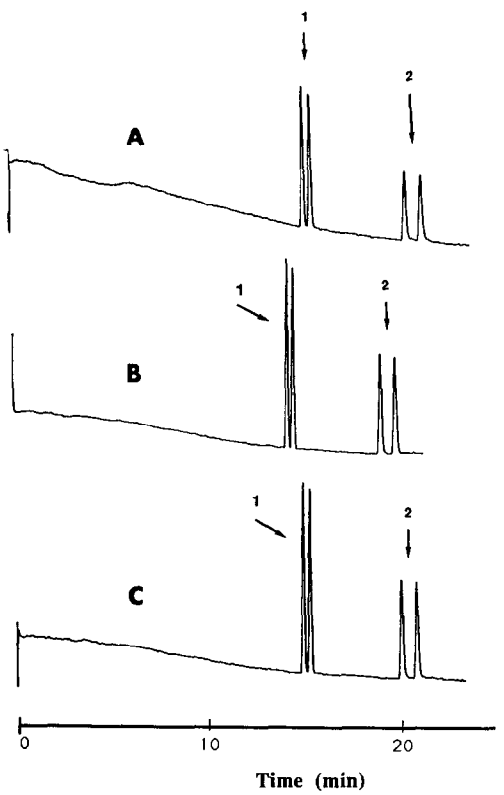

Fig. 6. Electropherograms of the enantiomers of verapamil (1) and fluoxetine (2). (A) Buffer: 10 m*M* TM-β-CD–30 m*M* Tris, pH 2.8–0.1% MHEC 1000. (B) Buffer as in (A) with 0.03 m*M* HTAB. (C) Buffer as in (A) with 0.15 m*M* HTAB. Capillary: 64 cm × 50 μm uncoated silica; voltage 20 kV; detection at 220 nm, 0.01 AUFS.

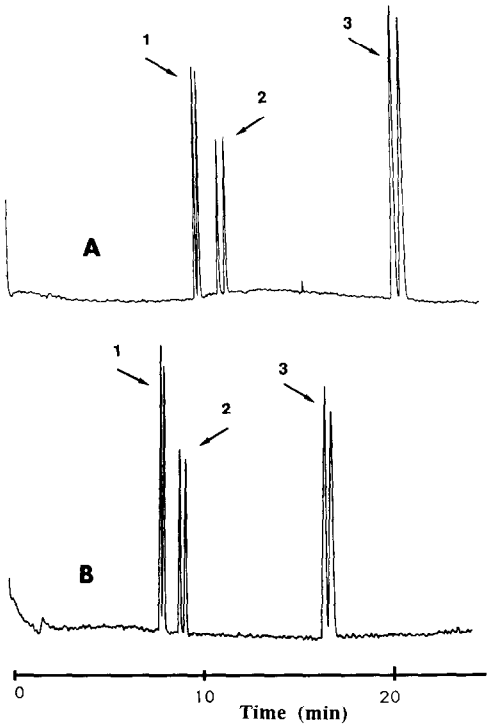

Fig. 7. Separations of the enantiomers of mepivacaine (1), bupivacaine (2) and carvedilol (3). Dimethyl-β-cyclodextrin buffer composition is described in the text (*Uncoated capillaries*). Detection at 220 nm, 0.01 AUFS. (A) Uncoated 64 cm × 50 μm capillary; 24 kV voltage. (B) Coated 64 cm × 50 μm capillary; no HTAB added to the buffer; 24 kV voltage.

mic bupivacaine after administration of 187.5–250 mg doses can be as high as 1.5–3.5 μg/ml using different regional anaesthesia techniques [24].

Using the DM-β-CD buffer, as described above, calibration was performed when the serum samples

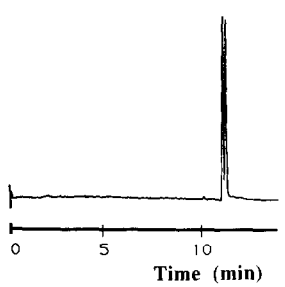

Fig. 8. Electropherogram of pindolol enantiomers (R_s 0.89) with 10 m*M* DM-β-CD–18 m*M* Tris, pH 3.0–0.1% MHEC 4000–0.03 m*M* HTAB buffer. Capillary: 64 cm × 50 μm uncoated silica; voltage 24 kV; detection at 220 nm, 0.01 AUFS.

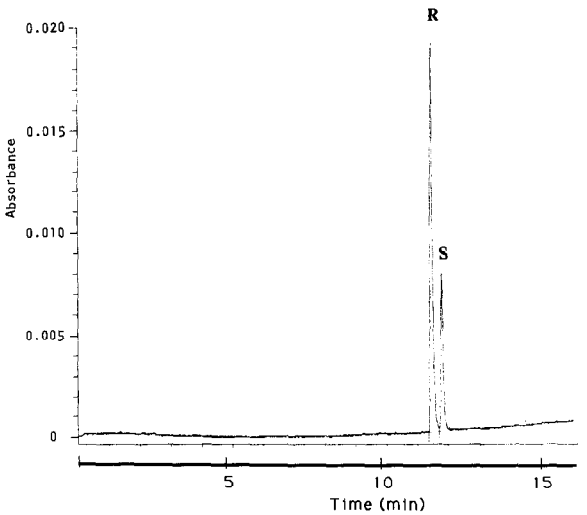

Fig. 9. Migration order of bupivacaine enantiomers: (*R*)-bupivacaine (99.9 μg/ml; water–methanol), (*S*)-bupivacaine (35.7 μg/ml; water–methanol). Detection at 200 nm, 0.02 AUFS; capillary: 50 cm × 75 μm I.D. uncoated silica; other conditions as described in the text.

were spiked with the racemic bupivacaine in the concentration range of 0.19–3.8 μg/ml. Mepivacaine was used as an internal standard. (*R*)- and (*S*)-enantiomers were quantitated separately by constructing linear regression calibration curves from the peak heights of the two bupivacaine enantiomers, which were compared with those of the mepivacaine enantiomers. Our previous experience has demonstrated that using a close analog of the substance analyzed, which migrates just before the analyte of interest, will yield excellent precision of peak-height ratio measurements [25]. The peak height ratios of (*R*)- and (*S*)-bupivacaine (RB and SB) and mepivacaine (1M and 2M) were measured for the sake of calibration and precision of sampling. Each peak height is considered here to represent 50% of the total amount of the racemic drug. For comparison, the first appearing enantiomer of mepivacaine (1M) was used as an internal standard for both enantiomers of bupivacaine.

Because a non-polar solvent at an alkaline pH was used to extract the bupivacaine, the serum background did not interfere with the CE measurements (Fig. 10). Specificity of determination was further explored by injecting the drugs cimetidine, warfarin, and diltiazem, which all migrated before

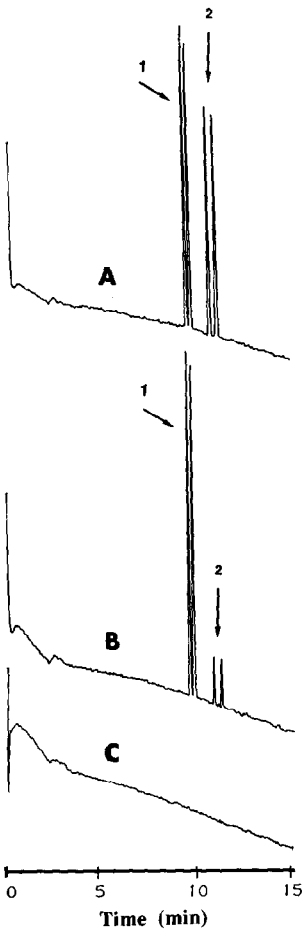

Fig. 10. Electropherograms of the racemic bupivacaine samples extracted from serum. Internal standard as: 1 = mepivacaine; 2 = bupivacaine. (A) 3.8 μg/ml; (B) 0.57 μg/ml; (C) serum blank. Run conditions: buffer, 10 m*M* DM-β-CD–18 m*M* Tris, pH 2.9–0.1% MHEC 4000–0.03 m*M* HTAB; voltage 24 kV; capillary: 64 cm × 50 μm uncoated silica, detection at 220 nm, 0.01 AUFS.

mepivacaine and bupivacaine. The acidic drugs indomethacin and ibuprofen were not detected under the analytical conditions employed. In general, acidic drugs are unlikely to interfere under the conditions we employed.

Table III demonstrates the linear regression measurements ($y = a + bx$) for different methods of peak-height comparison. The calibration curves were measured for the (*R*)- and (*S*)-bupivacaine enantiomers in the concentration range of 0.095–1.9 μg/ml. During repeated injections of serum sample with 1.15 μg/ml (for racemic bupivacaine), the injection precision (R.S.D.) was 1–2% for both bupivacaine enantiomers ($n = 5$) using peak-height comparisons with the first eluted isomer of mepivacaine (1M).

TABLE III

LINEAR REGRESSION CALIBRATION DATA FOR (*R*)- AND (*S*)-BUPIVACAINE (RB AND SB)

Mepivacaine enantiomers 1M and 2M were used as internal standards

Peak height ratio	*r*	*a*	*b*	*n*
RB/1M	0.9995	0.002	0.205	5
SB/2M	0.9990	0.008	0.210	5
SB/1M	0.9992	0.005	0.201	5

Polyacrylamide-coated capillaries

Resolutions for mepivacaine, bupivacaine, and carvedilol were slightly decreased as shown in Fig. 7B on a coated capillary. Using a DM-β-CD-HTAB buffer medium similar to the one described above for uncoated capillaries, the performance of coated capillaries was also evaluated. The remainder from the bupivacaine serum samples was injected in the multiple doses into the coated capillary and the peak-height ratios were measured.

We used the coated capillaries primarily to explore whether the presence of HTAB in the buffer would make any difference to the cyclodextrins' effectiveness (given that in a coated capillary there would be no free silanol moieties to cover). Our results suggest that the benefit of a cationic detergent is mainly due to the interaction of the detergent with the silanol groups in uncoated capillaries. Measurement precision for the coated capillaries (containing no silanol moieties) actually improved when the buffer did not contain HTAB. Table IV lists the precision (R.S.D.) of the measured ratios of the racemic bupivacaine sample (1.15 μg/ml serum). The interaction of HTAB with CDs, then, is evidently not as powerful as that of the detergent with silanol moieties.

DISCUSSION

Interest in the use of high-performance electromigration techniques for resolution of optical iso-

TABLE IV

PEAK HEIGHT RATIOS OF (*R*)- AND (*S*)-BUPIVACAINE (RB AND SB) TO MEPIVACAINE ENANTIOMERS (1M and 2M)

	R.S.D. (%) (n = 5)	
	With HTAB	Without HTAB
RB/1M	4.4	1.3
SB/2M	5.7	3.8
SB/1M	3.8	1.9

mers has been on a rapid rise in spite of a short induction period. Cyclodextrin derivatives [8–11] have been particularly singled out as attractive buffer additives in such separations. In general, chiral separations following this approach appear much easier to develop and optimize than the methodologies based on gas chromatography or high-performance liquid chromatography.

While the number of successful demonstrations of capillary electrokinetic chromatography in pharmaceutical applications increases, most (if not all) reports have been confined to the use of standard substances. Determination of chiral substances in biological matrices represents a set of special problems. One of the purposes of this study has been to evaluate detergents for overcoming the interference from endogeneous substances in serum. Cationic micellar systems have proven effective in this regard. In addition, HTAB used at levels around its critical micelle concentration stabilized the migration times and peak shapes of various basic enantiomers with uncoated capillaries, suggesting it has a role in compensating for the negative surface charge and in decreasing the amount of irreversible adsorption.

The micellar agents can also be used to modify the selectivity of cyclodextrins towards various enantiomers, as shown with several chiral therapeutics investigated in this work. This may lessen the need to develop additional chemically modified cyclodextrins for the analysis of chiral drugs and other isomer separations.

The surface chemistry of the capillary wall is yet another variable in optimizing separations in capillary electrokinetic chromatography. In the work reported here, the use of HTAB with surface-coated capillaries was found counterproductive. Yet a surface modification proved beneficial in resolving ketotifen derivatives and chloramphenicol in a different study [26]. Optimization strategies in chiral separations may need to take into account the effects of subtle changes in the buffer systems.

When biological interferences from serum samples are suppressed, it will be feasible to quantify certain chiral drugs at their therapeutic levels by CE and UV detection with adequate precision. This has been indicated by the example of bupivacaine in this study. However, reaching lower levels of detection will necessitate methodologies based on a more sensitive detection principle.

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