Investigation of Enantioselective Ofloxacin–Albumin Binding and Displacement Interactions using Capillary Affinity Zone Electrophoresis

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The direct chiral separation of ofloxacin by capillary affinity zone electrophoresis using serum albumins from different animal sources as chiral selector in the supporting electrolyte is described. In addition, the effects of displacers on the mobility and enantioselectivity of ofloxacin were studied. Firstly, the separation behaviour of the enantiomers of the ofloxacin (OFLX) and tryptophan (Trp) was compared. The influence of albumin types, including chemically modified bovine serum albumins (BSAs), and buffer types on the migration behaviour of enantiomers was investigated. The results showed that stereoselectivity of Trp is independent of the type of albumin used. However, chiral separation of OFLX depends on the biological species of albumin. Use of chemically modified BSA led to poorer resolution of enantiomers. Only with acetylated BSA could chiral separation of Trp be achieved. Using Good's buffer solutions (DIPSO and HEPES) as a supporting electrolyte affected the migration times of OFLX enantiomers. Finally, a variety of displacers were added to the buffer along with the protein, and the effects on separation behaviour were observed. The displacers included warfarin, ketoproten, diazepam, propranolol, benzoinphenylbutazone, digitoxin and octanoic acid. From the results obtained, it is concluded that capillary affinity zone electrophoresis using albumin as a chiral selector may allow screening of OFLX–displacer interactions.

INTRODUCTION

It is well known that the chirality of pharmaceutical compounds can have significant effects on their biological activities. Racemic drugs exhibit pharmacological activities different from those of the optically pure drugs (Ariens and Schweiz, 1990). For example, ofloxacin [OFLX; *R*,*S*-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-pyrido(1,2,3-*de*)-1,4-benzoxazine-6-carboxylic acid] is an excellent new quinolone antibacterial agent in clinical use. Its optically active form, levofloxacin, is 4–160 times more active than OFLX in *in vitro* trials (Hayakawa *et al.*, 1986). In order to study the pharmacokinetics and protein binding of enantiomers and to control the quality of the pharmaceuticals, it is important to develop methods of chiral separation. Practically, a variety of approaches have been attempted so far. Chromatographic approaches, particularly those using high-performance liquid chromatography (HPLC) (Krstulovic, 1989, Hermansson, 1983), gas chromatography and capillary zone electrophoresis (CZE) (Dobashi *et al.*, 1989; Terabe *et al.*, 1989; Otsuka *et al.*, 1991), have progressed remarkably during the past 10 years.

One factor contributing to enantioselective drug action and disposition is differences in the serum protein binding of the enantiomers of some chiral drugs. This phenomenon has been exploited in protein-based packings for HPLC systems (Allenmark and Bromgren, 1983; Miwa *et al.*, 1987). These stationary phases have become popular for the direct resolution of drug enantiomers because of their broad applicability and the use of aqueous buffered mobile phases. CZE, which has been developed recently, represents an attractive alternative method to HPLC for chiral separation because of its high efficiency and selectivity. At present, various chiral complexing agents are available that can be added to the supporting electrolyte to induce the separation of enantiomers. Enantiomers of drugs can be resolved successfully by the addition of several kinds of cyclodextrins to the buffer system (Otsuka *et al.*, 1991).

OFLX has interesting physico- and biochemical properties in terms of chirality. Chiral separations of ofloxacin have been achieved by ligand-exchange HPLC or on a BSA immobilized HPLC column. Recently, we reported the use of BSA as a running buffer additive to incorporate enantiomeric selectivity into the separation system. Excellent separation of OFLX enantiomers was achieved by this method (Arai *et al.*, 1994). However, using human serum albumin (HSA) as chiral selector, enantioselective separation of ofloxacin was not achieved. Okazaki *et al.* (1991) reported the metabolism and disposition of OFLX enantiomers in humans and some animals (e.g. rat, dog, and monkey). Differences in the pharmacokinetics of OFLX enantiomers were observed in the animals but not in humans. Thus we became interested in the ability of serum albumin from different animals to separate OFLX enantiomers (Arai *et al.*, 1994).

In this paper, we report the observed results of an explorative study using serum albumin from different animal sources in the supporting electrolyte as chiral

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Table 1. List of examined reagents

Reagents	Lot. no.	Grade
Bovine serum albumin (BSA)	A-6003	Essential fatty acid free
Palmitic-BSA	A-7922 (92H9307)	Palmitic acid content: 16 mol/mol
Glucosamide-BSA	A-6158 (27F8240)	28 mol glucosamide/mol (BSA)
Acetyl-BSA	B-2518 (121H67871)	Lyophilized
Rabbit albumin	A7065 (19F9301)	99% pure
Egg albumin	A-3154 (51H0245)	Hydrolysate
Horse albumin	A-9888 (37F9326)	Chon fraction V (Edsall, 1947)
Guinea pig albumin	A-2639 (129F9300)	Chon fraction V
Dog albumin	A-9236 (108F9327)	Chon fraction V
Goat albumin	A-2514 (22H9324)	Chon fraction V

selectors for the separation of OFLX enantiomers. The results are compared with those of Trp, the enantiomers of which are well known to be separated by BSA. The effects of buffer solutions, especially Good's buffers, on the retention of BSA and enantioselectivities are also discussed. And recently, Lloyd *et al.*, 1994), using other ligands as co-additives in the separation buffer, reported that considerable information could be obtained on the interactions between the additive and analyte as they bind to the protein. The effects of a variety of competitive ligands on OFLX enantioselective behaviour are also described in this report. These include compounds known to bind to HSA site I (e.g. warfarin), to HSA site II (e.g. diazepam) and to HSA site III (e.g. digitoxin).

EXPERIMENTAL

Apparatus. Electrophoresis was performed on a Jasco (Tokyo, Japan) model CE-800 capillary electrophoresis system at 23 °C in an untreated fused-silica capillary (50 mm i.d. × 50 cm or 30 cm). The distance from injection to the detection point was 20 cm. The ultraviolet absorption detector was connected to a C-R7A integrator (Shimadzu, Kyoto, Japan). Samples were injected electrokinetically at constant voltage at the positive side for a fixed period of time. Capillaries were stored overnight filled with water. Each day operation was started by purging with 0.5 M sodium hydroxide solution followed by water.

Chemicals. (*S*, *R*)-OFLX was obtained from Daiichi Pharmaceuticals (Tokyo, Japan). (D, L)-Trp and all kinds of albumin, including chemically modified BSA and different biological species of albumin, were purchased from Sigma (St Louis, MO, USA). The names of the albumins, lot nos. and grade are listed in Table 1. Good's buffer reagents (DIPSO and HEPES) were purchased from Wako (Osaka, Japan). The structures of the examined analytes (OFLX and Trp) and of Good's buffer reagents are shown in Fig. 1. Other chemicals used were reagent grade.

Procedures. Phosphate buffer and Good's buffers were used at a concentration 20 mmol/L (pH 7.5). The desired pH values of buffers were achieved by adding phosphoric acid or hydrochloric acid respectively as necessary. The carrier solutions were prepared by dissolving the proteins at an indicated concentration in the buffers. The two solutions in the two electrode reservoirs were changed after a certain period to avoid changes in pH owing to electrolysis of water. Sample

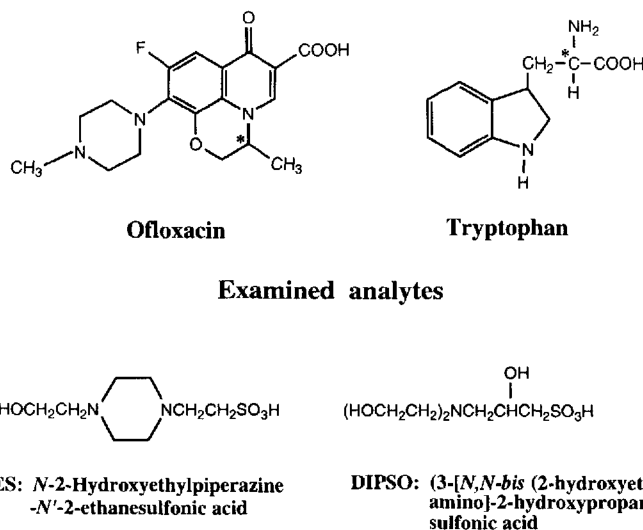

Figure 1. Structures of analytes examined and reagents for Good's buffer solutions.

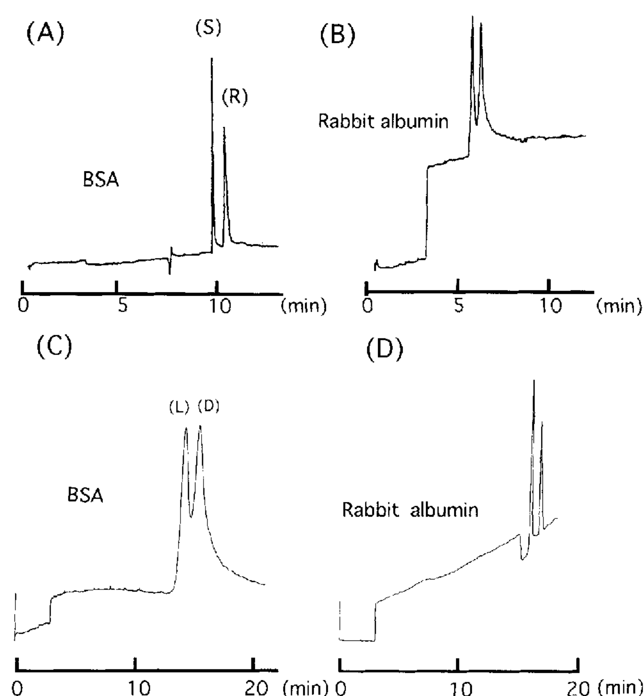

Figure 2. Electropherograms of (*R*, *S*)-OFLX and (D, L)-Trp obtained using different biological species of albumin as a chiral selector. (A) (*R*, *S*)-OFLX, 20 mM phosphate buffer (pH 7.5) with 0.4% (w/v) BSA. (B) (*R*, *S*)-OFLX, 20 mM phosphate buffer (pH 7.5) with 0.4% (w/v) rabbit albumin. (C) (D, L)-Trp, 20 mM phosphate buffer (pH 7.5) with 0.4% (w/v) BSA. (D) (D, L)-Trp, 20 mM phosphate buffer (pH 7.5) with 0.4% (w/v) rabbit albumin. Conditions: (A and B) applied voltage 30 kV (driving current 28 μA), detection wavelength 300 nm; (C and D) applied voltage 10 kV (driving current 10 μA). Other conditions: uncoated capillary with a 50 cm effective length; temperature 23 °C. Sample solutions (0.2 mg/mL) were electrically injected: 15 kV × 3 s.

solutions of ca 0.2 mg/mL OFLX and Trp were prepared; by dissolving in water and 0.5 M hydrochloric acid solution respectively. The detailed analytical conditions are shown in the figures and tables.

RESULTS AND DISCUSSION

The migration behaviour of racemic OFLX and Trp was investigated with three types of chemically modified BSA (glucosamide-BSA, acetylated BSA and palmitic acid-BSA) and seven types of serum albumin from different animals. These combinations of analytes and albumin were selected because previous studies have suggested good applicability of albumin-containing supporting electrolytes in capillary zone electrophoresis (Bush *et al.*, 1993; Sun *et al.*, 1993; Arai *et al.*, 1994). The effects of albumin types and Good's buffers on the resolution of the OFLX and Trp enantiomers were investigated. In addition, it is shown that this capillary affinity zone electrophoresis (CAZE) method can be used as a procedure to screen for competitive interactions between drugs which bind to albumin and OFLX.

Chiral separation with serum albumin from different animals. Okazaki *et al.* (1991) have demonstrated stereoselective disposition of OFLX in some animals. However, there is no difference in protein binding in humans between OFLX enantiomers. In fact, the concentration profiles of *S*-(−)-OFLX and *R*-(+)-OFLX in serum after oral administration of racemic OFLX in humans are almost identical. In this study, we attempted to use capillary electrophoresis as a tool to screen for enantioselective interactions between OFLX and albumin from different animals.

Typical electropherograms of OFLX and Trp enantiomers obtained by CAZE using BSA and rabbit serum albumin as the chiral selector in the supporting electrolyte are shown in Fig. 2. Some of the parameters of electrophoretic behaviour of OFLX and Trp with different types of biological albumin are shown in Table 2. The capacity factor $\tilde{k}'$, is defined in a similar way to that described by Terabe *et al.* (1985) for micelle electrokinetic chromatography using the symbol $\tilde{k}'$ instead of widely accepted k'.

$$\tilde{k}' = (t_R - t_0)/([t_0(1 - t_R/t_p)]$$

Table 2. Separation parameters of (*S*, *R*)-ofloxacin (OFLX) and (D, L)-tryptophan (Trp) by CAZE using serum albumins from different animals (analytical conditions are the same as those in Fig. 2)

Separation parameters	BSA	Rabbit	Egg	Horse	Guinea pig	Dog	Goat
OFLX							
(*S*): t_1 (min)	9.4	5.6	7.5	9.8	7.7	5.3	9.9
(*R*): t_2 (min)	10.1	6.1	7.5	9.8	7.8	5.3	10.5
t_2/t_1	1.07	1.09	1.00	1.00	1.01	1.00	1.06
t_P (min)	10.9	10.8	8.2	9.1	8.6	10.6	9.7
t_0 (min)	5.4	5.4	5.4	5.4	5.4	5.4	5.4
$\tilde{k}'_1$	5.5	0.1	5.0	−10.9	3.7	−0.0	−35.6
$\tilde{k}'_2$	11.0	0.3	5.0	−10.9	4.4	−0.0	−11.7
α	2.02	4.51	1.00	1.00	1.17	1.00	0.33
Trp							
(*S*): t_1 (min)	16.2	14.2	23.0	32.0	26.1	21.5	31.7
(*R*): t_2 (min)	16.9	15.4	24.2	32.6	27.6	22.2	31.7
t_2/t_1	1.04	1.08	1.05	1.02	1.06	1.04	1.00
t_P (min)	33.8	32.5	24.6	27.4	25.9	31.7	29.1
t_0 (min)	14.9	14.9	14.9	14.9	14.9	14.9	14.9
$\tilde{k}'_1$	0.2	−0.1	8.7	−6.9	−175.5	1.4	−12.5
$\tilde{k}'_2$	0.3	0.1	44.0	−6.3	−13.6	1.7	−12.5
α	1.62	−0.77	5.04	0.92	0.08	1.21	1.00

Table 3. Separation parameters of (*S*, *R*)-ofloxacin (OFLX) and (D, L)-tryptophan (Trp) by CAZE using chemically modified albumin (analytical conditions are the same as those in Fig. 2)

Separation parameters	BSA	Palmitic-BSA	Glucosamide-BSA	Acetyl-BSA
OFLX				
(*S*): t_1 (min)	9.4	8.7	>60	9.0
(*R*): t_2 (min)	10.1	8.7	>60	9.0
t_2/t_1	1.07	1.00	—	9.0
t_P (min)	10.9	13.3	>60	24.4
t_0 (min)	5.4	5.4	5.4	5.4
$\tilde{k}'_1$	5.5	1.8	—	1.1
$\tilde{k}'_2$	11.0	1.8	—	1.1
α	2.02	1.00	—	1.00
TRP				
(*S*): t_1 (min)	16.2	14.4	>60	11.9
(*R*): t_2 (min)	16.9	14.4	>60	12.2
t_2/t_1	1.04	1.00	–	1.03
t_P (min)	33.7	33.4	>60	77.4
t_0 (min)	14.9	14.9	14.9	14.9
$\tilde{k}'_1$	0.16	−0.1	–	−0.2
$\tilde{k}'_2$	0.27	−0.1	–	−0.2
α	1.62	1.00	–	0.90

For calculation of the capacity factors, the migration time of the analytes in the same buffer without the protein was used as t_0, that of the analytes in the buffer system with the protein as t_R and that of the protein in the buffer system without the protein as t_p. The selectivity factor, α, is equal to $\tilde{k}_2/\tilde{k}_1$. In addition tr_2/t_1 values were calculated for reference. The resolution was measured from the electropherograms.

Under our analytical conditions, Trp enantiomers were more widely distinguished than OFLX enantiomers by serum albumin from different animals. From the results, one might conclude that natural substances such as amino acids are more easily discriminated than artificial compounds such as OFLX. The affinity, represented by $\tilde{k}'$ of Trp is rather poorer than that of OFLX. However, CAZE might be applicable to the investigation of drug–protein interactions.

Since Cohn fraction V albumins contain some impurities, for example dimeric albumin, α-globulin, fatty acids and enzymes, we could not draw any conclusions about the mechanism of separation from these results. However, as far as chiral separation methods are con-

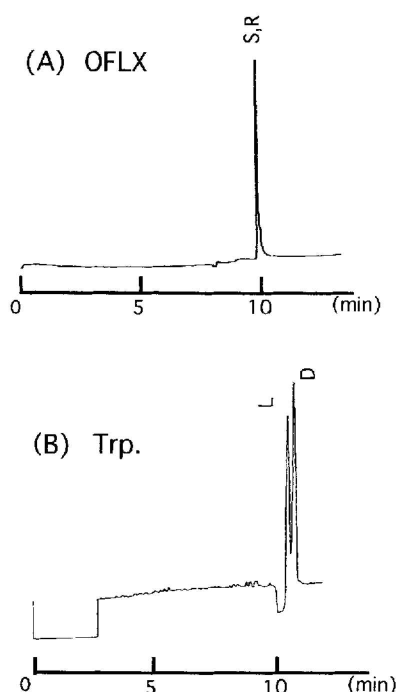

Figure 3. Electropherograms of (*R*, *S*)-OFLX and (D, L)-Trp using chemically modified BSA as a chiral selector. (A) (*R*, *S*)-OFLX; (B) (D, L)-Trp. Conditions: running buffer, 20 mM phosphate buffer with 0.4% (w/v) acetylated BSA. Other conditions were as described in Fig. 2.

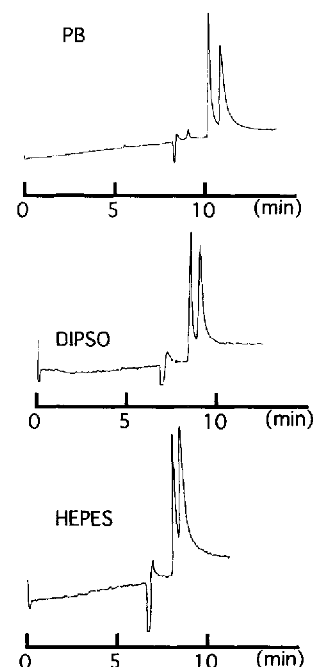

Figure 4. Electropherograms of (*R*, *S*)-OFLX showing the influence of Good's buffer. (A) 20 mM phosphate buffer (pH 7.5), (B) 20 mM DIPSO buffer (pH 7.5), (C) 20 mM HEPES buffer (pH 7.5). Conditions: 0.4% (w/v) BSA in the buffer solution, applied voltage 15 kV, uncoated capillary with a 30 cm effective length. Other conditions are as in Fig. 2.

Table 4. Effects of Good's buffers on separation parameters of (*S*, *R*)-{OFLX and (D, L)-Trp by CAZE (analytical conditions are the same as those in Fig. 4)

Separation parameters	Phosphate Buffer	HEPES	DIPSO
OFLX			
(*S*): t_1 (min)	10.3	7.7	8.0
(*R*): t_2 (min)	11.0	8.1	8.5
t_2/t_1	1.06	1.05	1.06
t_P (min)	16.9	7.1	8.3
t_0 (min)	7.9	4.9	5.4
$\bar{k}'_1$	0.8	−7.8	−18.3
$\bar{k}'_2$	1.10	−5.1	−18.3
α	1.42	0.65	−1.1
Trp			
(*S*): t_1 (min)	10.8	10.2	14.7
(*R*): t_2 (min)	11.2	10.7	15.2
t_2/t_1	1.04	1.04	1.03
t_P (min)	29.1	18.5	14.2
t_0 (min)	13.5	17.3	13.6
$\bar{k}'_1$	−0.3	−0.9	−2.3
$\bar{k}'_2$	−0.3	−0.9	−1.7
α	0.85	0.99	0.74

cerned, this procedure should be widely applicable to the development of chiral separation systems using serum albumin from different animals.

Albumins act as a pseudo-phase in this separation system, and $\bar{k}'$ is an indicator of the affinity of analytes for proteins. However, correlation between $\bar{k}'$ and separation efficiency was not observed.

Chiral separation with chemically modified BSA. Some parameters of the electrophoretic behaviour of OFLX and Trp enantiomers with chemically modified BSA are shown in Table 3. Typical electropherograms of OFLX and Trp enantiomers with acetylated BSA are shown in Fig. 3.

In all cases using chemically modified albumin, the separation efficiency was decreased. Ony when using acetylated BSA was chiral separation achieved. The results suggest that chemical modification causes alteration of the parts of the protein required for chiral discrimination and that the migration behaviour of albumin, which acted as a pseudo-mobile phase in electrokinetic chromatography, was also changed.

Palmitic acid-BSA has a higher negative charge, which is due to the carboxylic acid group of palmitic acid, than non-modified BSA and it slowly moved toward the negative electrode side as pseudo-phase; thus t_p and $\bar{k}'$ were increased. Regarding chiral discrimination, the effect of acetylation of BSA might be rather minor compared with other chemical modifications.

Effect of using Good's buffers. We have previously reported that OFLX forms chelates with borate when it is present in the supporting electrolyte (Morin *et al.*, 1993a, b; Arai *et al.*, 1994) and the enantiomeric resolution of OFLX is poor using BSA in CAZE In this study, the effect of using Good's buffers on CAZE was observed. Good's buffers are often used in biochemistry. The characteristics of Good's buffers are: (1) their structures are based on aminoethane sulphonic acid derivatives or aminopropane sulphonic acid derivatives; (2) they form zwitterions; (3) their solubility in water is good, and their degree of acid dissociation is stable; (4) their ability to form chelates is low; (5) their UV absorption is low. Considering these advantages, we attempted chiral separation by CAZE using Good's buffers.

Electropherograms of OFLX enantiomers obtained using phosphate buffer and Good's buffer are shown for comparison in Fig. 4, and their separation parameters are shown in Table 4. In all cases, the separation efficiency was almost identical. However, in spite of using the same mole concentration of buffer solution and applied voltage, migration time and capacity factor were decreased. The reason for this phenomenon is not well understood, but it might be presumed that Good's buffers act as ion pairing reagents and their complexes may affect the migration behaviour of analytes and pseudo-phase (protein) (see Fig. 5).

Simulation of OFLX–displacer interactions on albumiin. The effect of the addition of various concentrations of other drugs to the albumin-containing carrier solution was studied. This method should reveal and simulate interactions between the drugs by causing changes in analyte mobility and selectivity. The drug–drug replacement interactions on protein may be the result of either competition, at either primary or secondary sites, or allosteric effects. The simple model of three drug binding sites on HSA (site I, e.g. warfarin; site II; e.g. benzodiazepines; and site III, e.g. digitoxin) can now be seen to be a rather gross approximation, but it is nevertheless still a useful starting point to try and explain observations of interactions between drugs binding to albumin. Lloyd *et al.* (1994) attempted to use drugs as co-additives with HSA, including compounds known to bind to HSA sites I and II. In this study, in order to investigate the site of binding of OFLX to

Table 5. Summary of the effect of various co-additives on the mobility and enantioselectivity of OFLX

Coadditive	Warfarin	Benzoin	Phenylbutazone	Propranolol	Kynurenin	Diazepam	Ketoprofen	Octanoic acid	Tryptophan	Digitoxin
Mobility	(−)	(−)	(−)	(−)	(+)	(−)	(−)	(−)	(−)	(−)
Enantioselectivity	(−)	(−)	(+)	(−)	(+)	(++)	(++)	(+)	(−)	(−)
Binding site to HSA	I	I	I	?	?	II	II	II	II	III

Separation conditions as in Fig. 2 with 60 μM BSA and applied voltage 20 kV. + indicates a slight change, while ++ indicates obvious change between enantiomers. Little or no change is indicated by −.

Coadditive concentrations were 300–900 μM. Carrier solutions contaiining digitoxin, phenylbutazone and ketoprofen were prepared by addition of their methanol solutions (containing about 3% methanol). The results were compared with the control (blank) electropherogram. The definition of HSA binding site followed the report of Fehske *et al.* (1981). Site I; warfarin site; site II, benzodiazepine site; and site III, digitoxin site.

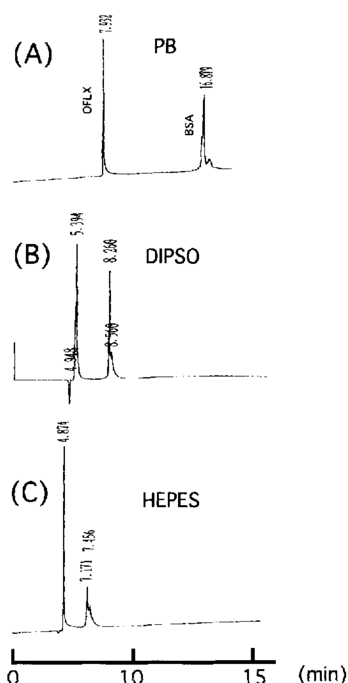

Figure 5. Electropherograms of BSA and OFLX using protein-free supporting electrolyte (determination of t_p, t_0 and effects of Good's buffer on migration times) (A) 20 mM phosphate buffer (pHH 7.5),m (B) 20 mM DIPSO buffer (pH 7.5), (C) 20 mM HEPES buffer (pH 7.5). Other conditions in Fig. 4.

BSA, drugs used as co-additives with BSA included compounds known to bind to HSA sites I (warfarin, propranolol, etc.), II (ketoprofen, diazepam, etc.) and III (digitoxin). The OFLX enantiomers were separated using a constant concentration of BSA (60 μM), with a variety of concentrations of co-additive.

Figures 6 and 7 show the typical effects of co-additives on the electropherograms of the OFLX enantiomers. The measurements made with these and other co-additives are summarized in Table 5. Site I (e.g. warfarin) and site III drugs (digitoxin) had no effect on the electrophoretic behaviour of OFLX enantiomers. However, addition of ketoprofen (Fig. 6) or diazepam (Fig. 7) caused a slight reduction in stereoselectivity and/or peak shape for OFLX, but only at concentrations >300 μM. We can conclude that the stereoselective component of OFLX binding to BSA occurs at site II (ketoprofen, diazepam), but that the major, achiral binding occurs elsewhere. In any case, there were no specific interactions between OFLX and examined co-additives, and it may be concluded that the enantioselective binding of OFLX to BSA occurs at neither the warfarin nor the propranolol binding site.

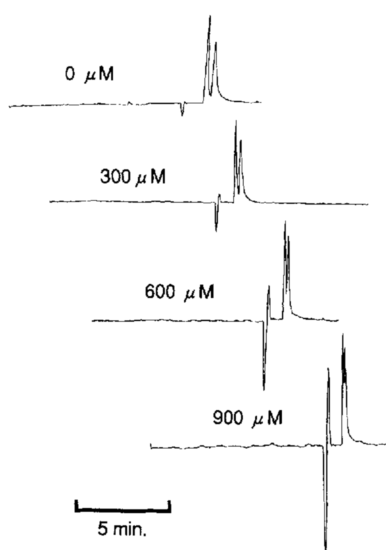

Figure 6. Effect of the co-additive ketoprofen on the electropherograms of OFLX enantiomers obtained by CAZE using BSA. BSA concentration, 60 μM; applied voltage, 20 kV. The carrier buffer solution contained 3% methanol in order to dissolve ketoprofen in the solution. Other conditions as in Fig. 4.

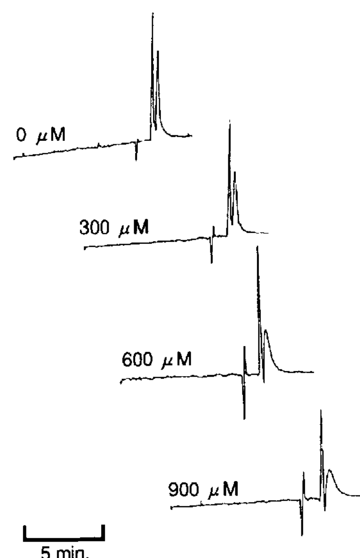

Figure 7. Effect of the co-additive diazepam on the electropherograms of OFLX enantiomers obtained by CAZE using BSA. BSA concentration, 60 μM; applied voltage, 20 kV. Other conditions as in Fig. 4.

CONCLUSION

We investigated the chiral separation, binding and interactions of OFLX–displacer with albumins using CAZE. Serum albumin from different animals showed varying capacity to discriminate between chiral enantiomers using CAZE. Chemical modification of albumin affected the separation efficiency and migration behaviour of enantiomers. Using Good's buffers as the supporting electrolyte did not affect the separation efficiency, but decreased the migration time. Using other drugs as co-additives in the separation buffer, considerable information can be obtained on the interactions between the additive and OFLX as they bind to albumin.

This procedure is applicable to the study of drug–protein binding affinity or drug–displacer interactions instead of as an alternative to protein immobilization procedures. In this respect, it will provide valuable information about not only chiral recognition but also interactions between (bio)molecules.

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