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Chiral separations by complexation with proteins in capillary zone electrophoresis

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ABSTRACT

The direct separation of enantiomeric drugs by capillary zone electrophoresis using proteins as chiral complexing agents in the background electrolyte is described. The influence of protein type and organic modifiers added to the background electrolyte on the resolution of enantiomers was studied. Separations of the enantiomers of tryptophan, benzoin, pindolol, promethaxine and warfarin are shown.

INTRODUCTION

Stereochemical effects can be significant for the biological activity of a drug. Racemic drugs exhibit pharmacological activities and/or side-effects different from those of the optically pure drugs [1]. It is therefore important to develop methods for the separation of enantiomers.

A variety of chromatographic approaches, particularly those using high-performance liquid chromatography (HPLC), have been developed [2]. Much work has been reported on the direct resolution of enantiomers by chiral stationary sorbents and a wide variety of these sorbents are now commercially available. These chiral sorbents include the Pirkle type [3], those based on inclusion complex formation with, e.g., cyclodextrin [4] and cellulosic materials [5], ligand exchange [6] and immobilized protein supports [7]. In liquid chromatography, protein-based stationary sorbents have become popular for the direct separation of drug enantiomers because of their broad applicability and the use of aqueous buffered mobile phases that are compatible with many biological samples. The initial immobilized protein HPLC columns for chiral recognition are prepared from bovine serum albumin [8] and α_1-acid glycoprotein (orosomucoid) [9]. Recently, columns based on immobilized human serum albumin [10], conalbumin [11] and some glycoproteins, fungal cellulase [12] and ovomucoid [13], have been used to resolve enantiomers.

Capillary zone electrophoresis (CZE) has been demonstrated to be a highly efficient separation technique suitable for the separation of ionic compounds even with very small differences in migration. However, for stereomers the difference in migration is usually too small to resolve the enantiomers unless specific stereoselective agents are introduced in the running buffer. At present several various chiral complexing agents are available that can be added to the background electrolyte to induce the separation of enantiomers. Enantiomers of drugs could be resolved successfully by the addition of several kinds of cyclodextrins to the buffer system [14–16]. An interesting alternative was demonstrated by Terabe and co-workers [17,18], who separated racemic amino acids using micellar electrokinetic chromatography with chiral surfactants for the formation of micelles. Kuhn et al.

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[19] demonstrated the successful separation of racemic compounds using a chiral crown ether as a buffer constituent. Although the above-mentioned enantioselective electromigration methods are suitable for many compounds, we have focused our attention on protein-based separations, primarily because of the general stereoselectivity of enzymes and cell membrane receptors observed *in vivo*. Distinctive features of the various proteins used in this study are shown in Table I. Orosomucoid is an acidic protein with negatively charged groups in the aspartic acid residues and in the terminal serine group, whereas positively charged groups are present in the arginine, lysine and histidine residues. Uncharged, chiral hydrogen bonding sites are present in the peptide chain and in the carbohydrate units that constitute 45% of the molecular mass [23]. The major difference between the ovomucoid and the orosomucoid proteins, apart from the molecular mass, is in their isoelectric points. This is a direct consequence of the higher sialic acid residue content of orosomucoid. Further, the larger number of disulphide bridges contributes to a more rigid structure, which may account for the exceptional stability of the ovomucoid protein [21]. Fungal cellulase is stabilized by twelve disulphide bridges. Fungal cellulase has a low carbohydrate content (6%) in comparison with the other two glycoproteins, ovomucoid (30%) and orosomucoid (45%).

In this paper, we report the preliminary results of an explorative study using the aforementioned types of proteins in the running buffer as chiral selectors for the separation of enantiomers of some test compounds.

EXPERIMENTAL

Instrumentation

Electrophoresis was performed at 25°C in an untreated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) (50.7 cm x 50 μm I.D.). The length from injection to the detection point was 29.8 cm. The UV absorption at different wavelengths was measured with an adapted UV detector (Model 757, Applied Biosystems, Foster City, CA, USA). As a regulated high-voltage power supply, an FUG HCN 35-35000 (FUG Elektronic, Rosenheim, Germany) was used. The total laboratory-built set-up was placed in a Plexiglas box; opening the door automatically shut off the high voltage. The current in the system was measured over a 1-kΩ resistance in the return circuit of the power supply. Samples were injected electrokinetically at constant voltage (10 kV) at the positive side for a fixed period of time (2-5 s). All experiments were carried out with an applied voltage of 10 kV. This voltage was selected to avoid too large a heat production in the capillary with the applied relatively high buffer concentrations. Electropherograms were recorded with a Type BD41 chart recorder (Kipp and Zonen, Delft, Netherlands) at a chart speed of 0.1 mm/s.

Material

The proteins bovine serum albumin, ovomucoid, orosomucoid fungal cellulase and analytical-reagent grade hydrochloric acid, sodium hydroxide, D,L-tryptophan and warfarin were purchased from Sigma (St. Louis, MO, USA), analytical-reagent grade disodium hydrogenphos-

TABLE I

CHARACTERISTICS OF THE PROTEINS USED AS CHIRAL COMPLEXING AGENTS

Property	Orosomucoid [20]	Ovomucoid [21]	Fungal cellulase [12]	Bovine serum albumin [22]
Molecular mass	41 ooo	28800	60 ouo-70 ooo	66 000
Isoelectric point	2.7	3.9-4.5	3.9	4.7
Sialic acid residues	14	0.3		
Disulphide bridges	2	8	12	17
Carbohydrate (%)	45	30	6	

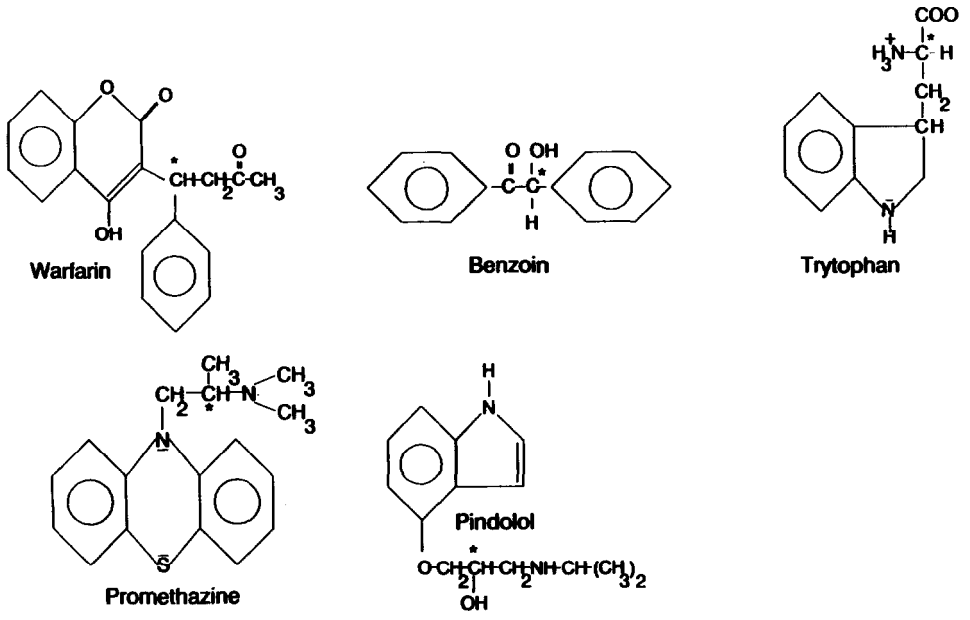

Fig. 1. Structures of the test solutes.

phate, sodium dihydrogenphosphate, ethanol, 1-propanol and benzoin from Merck (Darmstadt, Germany) and N ,N-dimethyloctylamine from Aldrich (Milwaukee, WI, USA). The drugs pindolol, promethazine and disopyramide were kindly provided by a local pharmacist. Solute structures are shown in Fig. 1. Distilled water was used to prepare the buffer solutions.

Procedures

The phosphate buffers were 67 mmol/l (pH 7.4), or as given otherwise in the text or figure captions. The buffer was prepared from the two 67 mmol/l solutions in such proportions that the desired pH was obtained. The protein solution was prepared by dissolving the proteins at a given concentration in the phosphate buffer. The two solutions in the two electrode reservoirs were changed after a certain period to avoid changes in pH due to electrolysis of the water. Stock solutions of cu. 0.2 mg/ml of all test compounds in phosphate buffer were prepared. Before analysis these stock solutions were diluted in phosphate buffer to give 50 and 100 μmol/l solutions. Tryptophan was available as single enantiomers in this study. Chiral separations were performed using solutions of the racemic amino acid prepared by mixing equal volumes of each enantiomer. The capillary was flushed daily and after a buffer change for 5 min with 1 *M* NaOH and subsequently for 5 min with buffer.

The capacity factor, $\tilde{k}'$, is defined in a similar way to that by Terabe *et al.* [24] for MECC, *i.e.*, using the symbol $\tilde{k}'$ instead of widely accepted k' in order to emphasize the difference in the relationship of the capacity factor to t_R from the well known equation $t_R = t_0(1 + k')$:

$$\tilde{k}' = \frac{t_R - t_0}{t_0\left(1 - \frac{t_R}{t_{Prot}}\right)} \quad (1)$$

For the calculation of the capacity factors the migration time of the drugs in the same buffer without the protein was used as t_0, that of the drug in the buffer system with the protein as t_R and that of the protein in the buffer system without the protein as t_{Prot}. Electroosmotic mobilities in both background electrolytes, with and without the protein, were the same to within 5%. The selectivity factor, a, is equal to $\tilde{k}_2'/\tilde{k}_1'$. The resolution was measured from the electropherograms.

RESULTS AND DISCUSSION

The migration behaviour of the racemic test compounds was investigated with four types of proteins: bovine serum albumin, ovomucoid, orosomucoid and fungal cellulase protein. These four proteins were selected because previous studies, with these proteins immobilized on HPLC sorbents, suggested a broad range of applicability. The separations were performed with electrolyte solutions consisting of only phosphate buffers and organic modifier. The effect of pH, ionic strength and addition of organic modifier (e.g., 1-propanol or dimethyloctylamine) to the running buffer on the resolution of the enantiomers was investigated.

BSA

On the basis of the results of HPLC experiments with native BSA by Sebille and Thaud [25], a 50 μmol/l BSA solution in phosphate buffer was selected. Tryptophan, benzoin and warfarin were taken as test solutes. Under these standard conditions, enantiomeric resolution was observed for tryptophan and benzoin. By addition of a small amount of 1-propanol to the running buffer, resolution of warfarin could also be achieved. The effect of the addition of 1-propanol on $\tilde{k}'$, α, the resolution R_s of the enantiomers and the peak shapes are given in Table II and Fig. 2a–d.

The migration behaviour of tryptophan can be seen in Fig. 2a and Table II. Under standard conditions (1-propanol absent) the enantiomers are well resolved but the L-form shows significant peak tailing. This behaviour might indicate a slow mass transfer between the L-form and the protein or the effect is due to an interaction of the solute with BSA absorbed on the surface of the capillary. However, the latter reason is unlikely in our experiments because the electroosmotic flow with the buffer and buffer + protein is the same. From Fig. 2a it can be seen that the addition of 1-propanol increases significantly the peak shape of the L-enantiomer but the resolution between the two enantiomers decreases. The presence of 1-propanol will decrease the protein binding of the solutes and/or induce a conformational change of the protein.

TABLE II

CAPACITY FACTORS ($\tilde{k}'$), SEPARATION FACTORS (α) AND RESOLUTION (R_s) FOR TRYPTOPHAN, BENZOIN AND WARFARIN

Conditions for tryptophan, benzoin and warfarin as in Fig. 2a, b and c, respectively.

Solute	1-Propanol (%)	$\tilde{k}_1'$ [a]	$\tilde{k}_2'$ [a]	a	R_s
Tryptophan	0	0.03	0.54	14.7	9.1
	3	0.04	0.25	5.7	4.0
	6	0.18	0.28	1.6	3.3
	9	0.29	0.35	1.2	3.1
	12	0.72	0.76	1.1	4.0
Benzoin	0	0.24	0.39	1.6	3.8
	3	0.28	0.54	1.9	4.7
	6	0.18	0.35	2.0	5.6
	9	0.44	0.59	1.3	4.4
	12	0.65	0.73	1.1	3.3
Warfarin	0	7.44	7.44	1.0	0.0
	3	0.32	0.21	1.6	0.7
	6	0.23	0.06	3.5	0.8
	9	0.05	0.05	1.0	0.0
	12	0.02	0.02	1.0	0.0

[a] **The $\tilde{k}'$ values given are approximate.**

The favourable effect of 1-propanol on the peak shapes is also found with protein-modified packings in HPLC. For tryptophan, 6% 1-propanol seems to be a good choice for resolving the enantiomers.

The results for benzoin are reflected in Fig. 2b and Table II. Under standard conditions the enantiomers of benzoin are completely resolved. With increasing addition of 1-propanol the resolution first increases and then decreases again. The behaviour of the capacity factor with the organic modifier is strange and is not understood. From Fig. 2b it can be seen that 3-6% of 1-propanol is a good composition for resolving the enantiomers of benzoin.

The enantiomers of warfarin could not be resolved under standard conditions. Partial resolution of the enantiomers could be achieved by addition of 3-6% of 1-propanol, as can be seen from Fig. 2c and Table 11. At higher modifier concentrations the resolution was lost again. Apart from the organic modifier, the pH and ionic strength of the buffer also might influence the resolution. Lowering of the ionic strength of

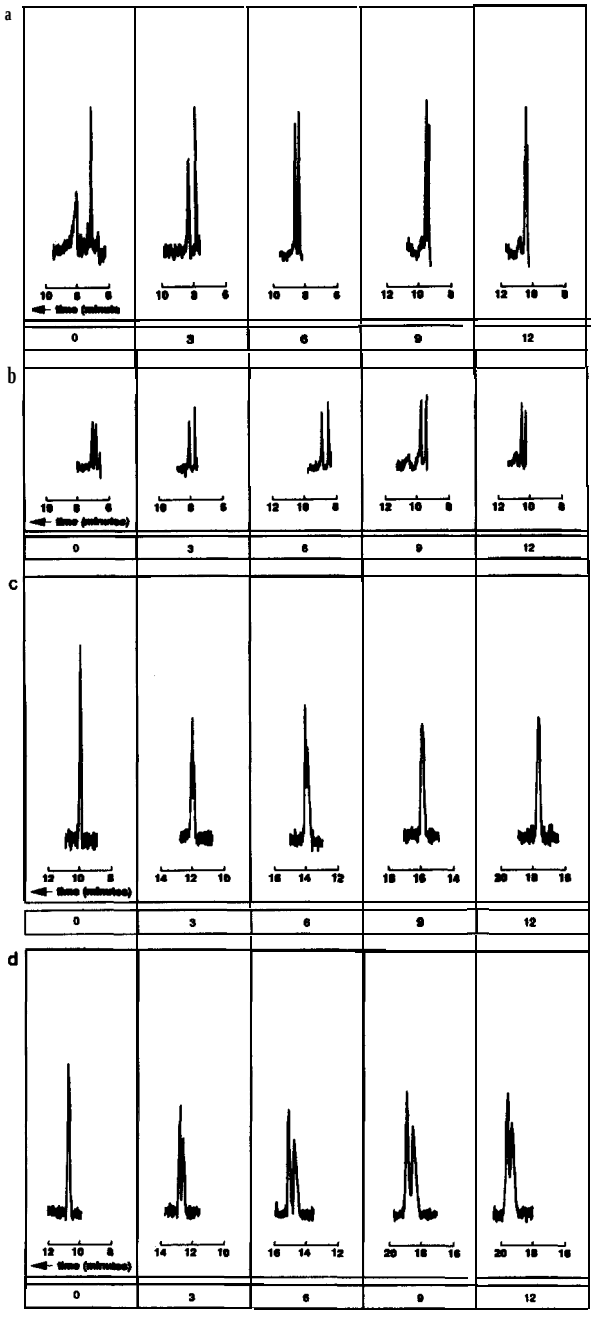

Fig. 2. Electropherograms showing the influence of 1-propanol (%1-propanol indicated on bottom line of each panel) on chiral separations. (a) D,L-Tryptophan. Conditions: bovine serum albumin solution, 50 μmol/l in phosphate buffer; detection wavelength, 280 nm. (b) (*R*,*S*)-Benzoin. Detection wavelength, 260 nm. Other conditions as in (a). (c) (R,S)-warfarin. Detection wavelength, 315 nm. Other conditions as in (a). (d) (*R*,*S*)-warfarin. Conditions: bovine serum albumin solution, 50 μmol/l in 50 mmol/l phosphate buffer (pH 6.8); detection wavelength, 315 nm.

the buffer but keeping the **pH** the same did not improve the resolution. However, a significant improvement in the resolution was gained by lowering the **pH** of the buffer, as can be seen from Fig. 2c and d. The greatest resolution was again obtained with 6% of 1-propanol.

The separation system with BSA as additive in the running buffer behaves similarly, as expected on the basis of HPLC measurements with immobilized BSA packings. From these studies it became apparent that BSA is suitable for the separation of acidic racemates **[21]**.

Ovomucoid

An interesting protein to be tested was the glycoprotein ovomucoid because we expected from the results obtained with HPLC that the ovomucoid protein might permit the separation of both acidic and basic enantiomers **[21]**. Several experiments with this protein were carried out by varying the protein concentration in the buffer (5, 15, 50 and 174 **μmol/l),** the **pH** (6, 7 and 8) the type and concentration of the modifier (N,N-dimethyloctylamine, methanol, **acetonitrile**, 1-propanol). However, no separation of the enantiomers of the test components (tryptophan, benzoin, warfarin, propanolol, pindolol and atenolol) could be achieved. A few test solutes showed some interaction with the protein, indicated as a change in migration time and a very broad peak, but changing the **pH** and modifier did not result in any separation of the **enantiomers**. In view of the results obtained, the achievements obtained with the ovomucoid protein immobilized on an HPLC sorbent is puzzling. From the results one might conclude that immobilization brings about a change in the (tertiary) structure of the ovomucoid protein that results in an excellent chiral stationary phase for HPLC experiments.

Orosomucoid (AGP)

This glycoprotein is often used for chiral separations in HPLC. This glycoprotein has been shown to be applicable to different types of chiral solutes such as **amines,** acids and **non**proteolytes, which indicates that many different binding groups are present on the protein. It has been demonstrated that the retention and

stereoselectivity on orosomucoid can be varied over a wide range in HPLC by changing the pH of the buffer and the content of charged and uncharged organic compounds [26]. The effects of these changes are highly dependent on the structure of the solute and bear no simple relationship to general properties such as charge or hydrophobicity. In a chiral AGP column, AGP is immobilized on silica particles by a covalent linkage and cross-linking of adjacent protein molecules. Some of the binding groups which are free for interaction in native AGP have been demonstrated to be utilized in the immobilization and cross-linking procedures [23].

The interaction between the solute and the protein was studied by monitoring the effects of 1-propanol and N,N-dimethyloctylamine (DMOA), a cationic additive. The ionic modifiers will influence the coulombic interactions between the protein and the drug. Also, the charged modifier might affect the conformation of the protein and it can compete with the enantiomers for the binding groups on the protein. It has been shown that enantioselectivity can reversibly be induced by organic modifiers [23]. It was suggested that the enantiomers compete with the uncharged modifier for binding to the same site(s) on the protein and that the hydrophobicity and hydrogen-bonding properties are of great importance for the effects on the enantioselectivity. The drugs chosen as test analytes were benzoin, warfarin, pindolol, promethazine and disopyramide. First a 20 μmol/l AGP solution in phosphate buffer was used. Under these standard conditions, resolution was only achieved for promethazine. The electropherogram is shown in Fig. 3. The bad peak shape can be accounted for, at least partly, by the kinetics of the drug-protein interaction. The enantiomers of warfarin are not resolved but elute as a very broad peak. This behaviour indicates that interaction with the protein occurs. In order to improve the separation, different organic modifiers (DMOA and 1-propanol) were added to the running buffer.

Addition of 4.2 mmol/l of DMOA to the running buffer improves the peak shape of promethazine significantly (see Fig. 3) but has no

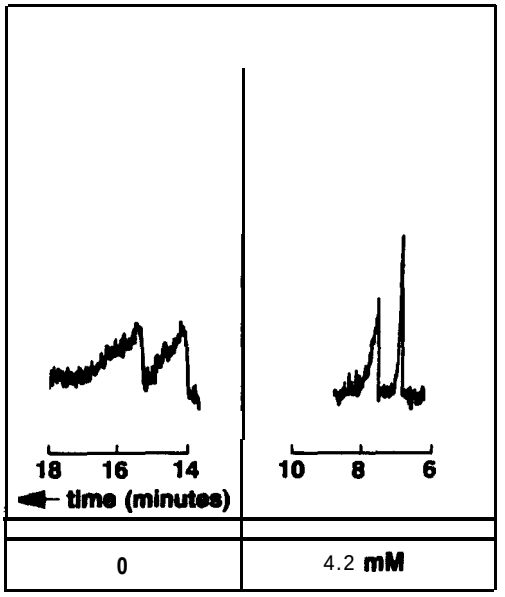

Fig. 3. Electropherograms of (*R*,*S*)-promethazine showing the influence of DMOA (concentration indicated on bottom line) on the chiral separation. Conditions: orosomucoid solution, 21 μmol/l in phosphate buffer; detection wavelength, 260 nm.

effect on the migration behaviour of the other test solutes. This again shows that solutes interact differently with specific sites on the protein.

Addition of 3% of 1-propanol has a negligible effect on the migration behaviour of the test solutes except for warfarin, which elutes as a sharp peak. The improvement of the peak shape of warfarin with 3% of 1-propanol compared with the shape under the standard conditions indicates that the enantiomers can probably be resolved by fine tuning of the organic modifier content or type of modifier. Although the effect of 1-propanol on the resolution of enantiomers with orosomucoid is small, for other types of proteins the effect of 1-propanol can be significant, as is demonstrated for BSA and promethazine in Fig. 4.

Fungal cellulase

Fungal cellulase is a glycoprotein isolated from a culture filtrate of the fungus *Aspergillus niger*. It has been demonstrated that with this (immobilized) protein as a stationary sorbent for chiral HPLC, the enantiomers of acidic and basic

drugs could be resolved [12]. With this glycoprotein as a chiral complexing agent in the running buffer, some explorative experiments were carried out with the same test compounds as used with orosomucoid. Under these standard conditions only the enantiomers of pindolol could be resolved and the separation is shown in Fig. 5.

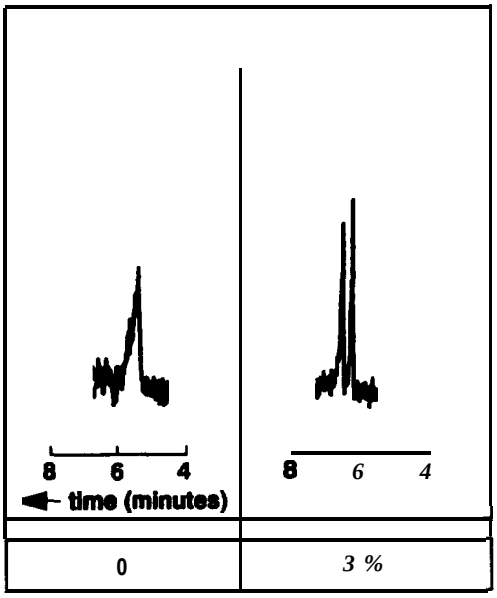

Fig. 4. Electropherograms of (*R*,*S*)-promethazine showing the influence of 1-propanol (% 1-propanol indicated on bottom line). Conditions: bovine serum albumin solution, 50 μmol/l in phosphate buffer; 3% (v/v) 1-propanol; detection wavelength, 280 nm.

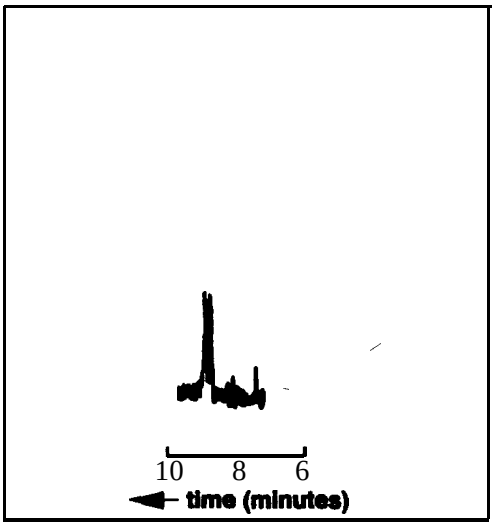

Fig. 5. Electropherograms of (*R*,*S*)-pindolol. Conditions: fungal cellulase solution, 20 μmol/l in phosphate buffer; detection wavelength, 260 nm.

CONCLUSIONS

From the preliminary experiments it can be concluded that in principle proteins dissolved in the running buffer can be used as chiral complexing agents in capillary zone electrophoresis to induce chiral separations. It is also clear from the above experiments that the enantioselectivity differs with the type of protein, as is known from protein-based packings for HPLC systems. The simplicity of using all types of proteins is a substantial advantage of the CZE technique compared with HPLC, where the immobilization of proteins is laborious. Addition of organic modifiers to the running buffer is sometimes essential to obtain chiral separations. In that respect, addition of compounds that bind specific sites on the protein, e.g., concanavalin A to sugar residues, will provide valuable information about the chiral recognition.

The results also show clearly that CZE is a very useful one-phase separation technique for studying interactions between (bio)molecules. It can also shed light on possible conformational changes induced in proteins by immobilization procedures.

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