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Chiral separation of β-blockers by high-performance capillary electrophoresis based on non-immobilized cellulase as enantioselective protein

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ABSTRACT

Optical isomers of some basic parmaceutical drugs (β-blockers) were separated by means of high-performance capillary electrophoresis in a carrier-free solution, using the chiral recognition properties of a cellulase (cellobiohydrolase I). High resolution of the isomers and peaks with satisfactory symmetry were obtained only when the enzyme was dissolved at a high concentration (40 mg/ml; total 10 μg) in a buffer of high ionic strength (0.4 *M* sodium phosphate) supplemented with 2-propanol. Surprisingly, the isomer selectivity was lost when the electrophoresis was carried out in the buffers used for chromatographic separation of the isomers on a bed derivatized with cellulase. At pH 5.1 (the experimental pH), the enantiomers are positively charged and the enzyme is negatively charged. With the cathode at the detection end of the capillary the enzyme accordingly migrated away from the detection point and the enantiomers toward it. Disturbances in the UV detection of the enantiomers otherwise caused by the presence of the enzyme were thus avoided. As the runs are performed in the absence of a supporting medium the analyses can be automated easily, which also facilitates the screening of different proteins for their chiral recognition properties and studies to establish the optimum experimental conditions.

A large number of pharmaceutical drugs contain one or more chiral atoms and thus exist in two or more isomeric forms. In most instances only one of the forms is highly active therapeutically. The other one can be either much less active, inactive or sometimes even toxic. Only the highly active isomers are used in the production of medicines. The requirements of the US Food and Drug Administration for the purity of substances used in the pharmaceutical industry are very strict, and the presence of non-highly active forms is not allowed. For efficient purity control, rapid and sensitive analytical methods are therefore needed.

Most chiral separation methods are based on chromatography. The enantioselective ligands can be synthetic, low-molecular-mass compounds, *e.g.*, β-cyclodextrin and Pirkle phases [1], or natural polymers, such as albumin [2,3], α_1-acidic glycoprotein [4,5], conalbumin [6] and α-chymotrypsin [7].

Only low-molecular-mass compounds, such as cyclodextrins, have been used as enantioselective agents for the separations of *R*- and *S*-forms by high-performance capillary electrophoresis (HPCE) [8–11]. In this paper we show the usefulness of a high-molecular-mass enantioselector (a protein) for this purpose.

Cellobiohydrolase I (CBH I), a cellulase produced by the fungus *Trichoderma reesei*, was recently used successfully for chiral recognition chromatography following immobilization on silica [12,13] and piperazine diacrylamide–methacrylamide [14] beds. It was therefore of interest to investigate whether this enzyme could be used also for electrophoretic chiral separations. For this purpose we used HPCE, as this

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method gives high resolution and short run times and requires only small amounts of sample. The last requirement was essential, as we wanted to develop a method based on simple addition of the enantioselective enzyme to the buffer.

The electrophoresis experiments could therefore be performed in free solution without time-consuming immobilization of the enzyme on a solid support. Such a method is attractive as it permits rapid screening of many substances for their enantioselective properties under different experimental conditions. The method is also easy to automate, as the absence of a solid phase makes it easy to suck washing liquid and fresh enzyme and buffer solutions through the electrophoresis tube for each run.

EXPERIMENTAL

Apparatus

The experiments were performed with a laboratory-designed HPCE system consisting of a Linear 206 PHD variable-wavelength UV detector, (a kind gift from Mr. K. Weinberger, Linear Instruments, Reno, NV, USA, and Mr. L. Lidman, Metric, Malmö, Sweden), a 10-kV power supply (constructed by Mr. Per-Axel Lidström of this Department) and a W + W 1100 recorder (LKB, Bromma, Sweden). The part of the detector that holds the capillary tube was modified slightly in order to accomodate tubes with small diameters.

Materials and reagents

Fused-silica tubing (75 μm I.D., 150 μm O.D.) was bought from Polymicro Technologies (Phoenix, AZ, USA).

The sodium hydrogenphosphate, disodium hydrogenphosphate and 2-propanol used for the preparation of the electrophoresis buffers were of analytical-reagent grade (Merck, Darmstadt, Germany). The agarose gel plug at one end of the capillary was prepared from agarose zero-m_r (Bio-Rad, Richmond, CA, USA).

Culture filtrate from *Trichoderma reesei*, strain QM 9414, was kindly provided by the Biotechnical Laboratory of the Technical Research Centre of Finland (VTT) (Espoo, Finland).

Cellobiohydrolase I (CBH I), a glycoprotein with a molecular mass of 64 000 and a p*I* of 3.9 [15] was purified as described [16]. The enzyme was concentrated by ultrafiltration (cell UH 100/10 with M_r cut-off *ca.* 10 000; Schleicher & Schüll, Dassel, Germany).

The samples, some drugs of the groups of the β-blockers, vasodilatators and antiadrenergics, were a gift from Dr. Bengt-Arne Persson (Astra Hässle, Mölndal, Sweden). A list of the samples and their structures are given in Table I.

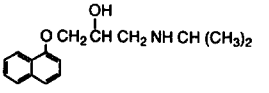
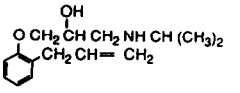
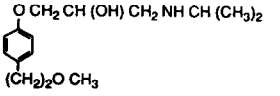
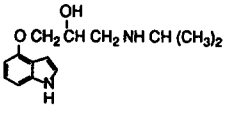
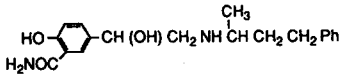
Electrophoretic procedure

The capillary tubes were 11.5 cm long (8.5 cm to the detector) × 75 μm I.D. The polyamide coating was removed from a 2–3-mm zone of the silica wall at the detection point by burning it on a hot Kantal wire [17]. The inner surface of the capillary was coated with non-cross-linked polyacrylamide in order to suppress electroendosmotic flow and interaction between the solutes and the silica wall [18].

Agarose (20 mg) was mixed with 1 ml of electrophoresis buffer. The mixture was first boiled to dissolve the agarose and then cooled to allow gelation. One end of the coated buffer-

TABLE 1

STRUCTURE OF THE DRUGS

No.	Name	pK_a	Structure
1	Propranolol	9.5	
2	Alprenolol	9.5	
3	Metoprolol	9.7	
4	Pindolol	9.7	
5	Labetolol	7.4	

filled electrophoresis tube was pressed into the agarose gel. The gel plug thus obtained had a length of a few millimetres.

The enzyme solution was dialysed against the electrophoresis buffer and concentrated by ultrafiltration to a final concentration of 40 mg/ml. An additional dialysis is preferable, as some concentration of buffer ions takes place during the ultrafiltration step.

The electrophoresis buffer used was 0.4 *M* sodium phosphate buffer (pH 5.1) containing different concentrations of 2-propanol (up to 30%). The experiments were carried out as follows. A 7-cm long buffer zone was drawn up into an 11.5-cm coated fused-silica tube from the end where the sample was to be applied. The same end of the tube was then dipped into a vial containing CBH I solution (40 mg/ml in the electrophoresis buffer) to fill, by capillary force, the remainder of the tube with enzyme solution (see Fig. 1). A 2–3-mm long agarose plug was then introduced at the application end as described above to prevent any hydrodynamic flow through the tube.

The sample, 1 mg/ml of a racemate dissolved in electrophoresis buffer diluted tenfold, was applied electrophoretically at 1000 V for 20 s. As the solutes and the enzyme have opposite charges at pH 5.1 they migrate in opposite directions. The basic sample molecules migrate towards the cathode, passing through the CBH I zone, which moves considerably more slowly towards the anode (Fig. 1). This approach ensures that protein molecules do not pass through the detection point and do not disturb the detection of the sample zones. UV detection was performed at 220 nm.

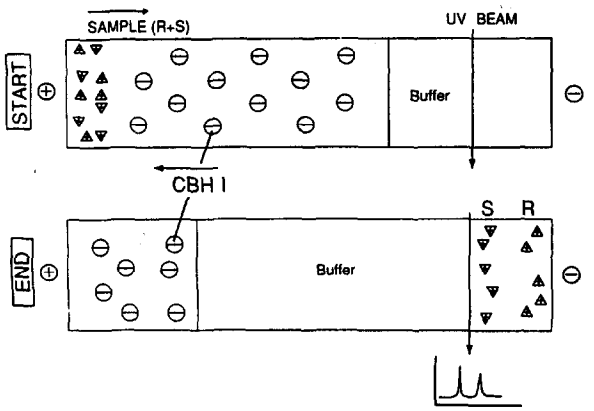


Fig. 1. Principle of the separation of enantiomers by capillary electrophoresis in free solution. The electrophoresis was conducted at a pH such that the enantioselective agent (in this instance cellulase) migrates in a direction opposite to that of the enantiomers. The UV-absorbing cellulase will thus not disturb the UV detection of the enantiomers.

RESULTS AND DISCUSSION

Importance of pH, ionic strength and buffer additive for chiral separation

The electropherograms in Fig. 2 represent the separation of optical isomers of different drugs. Propranolol (a), pindolol (b) and metoprolol (c) possess one asymmetric atom and therefore appear in two isoforms. The first peak in electropherogram (a) corresponds to the *R*-form of propranolol and the second to the *S*-form. Unfortunately, we could not find standards for the *R*- and *S*-forms of the other drugs. In Fig. 2d and

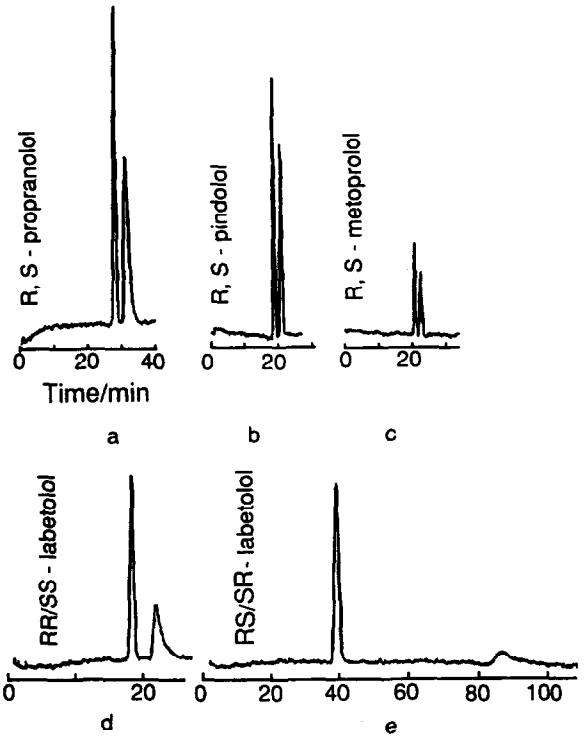


Fig. 2. Separation of (a) (*R*,*S*)-propranolol, (b) (*R*,*S*)-pindolol, (c) (*R*,*S*)-metoprolol, (d) (*RR*/*SS*)-labetolol and (e) (*RS*/*SR*)-labetolol. Buffer, 0.4 *M* sodium phosphate (pH 5.1) supplemented with (a, b, c) 25% and (d, e) 30% 2-propanol.

e a separation of the isomers of labetolol is presented. Labetolol is a diastereomer and thus appears in four isoforms: *RR*, *SS*, *RS* and *SR*. In all runs the isomers were baseline resolved and the peaks showed satisfactory symmetry. The asymmetry factor for the first peaks varied from 1.08 to 1.17 and for the second peaks from 1.6 to 2.7. (For the calculation of the symmetry factor, see ref. 19.)

To resolve the enantiomers we had to perform the experiments under conditions that are unusual for HPCE: a high ionic strength (0.4 *M* sodium phosphate buffer), a high concentration of organic solvent (up to 30% 2-propanol) and a relatively low voltage (the voltage was kept at 1000 V to avoid high temperatures in the electrophoresis tube and thereby the risks of precipitation of CBH I and bubble formation). Without 2-propanol in the buffer the enantiomers separated, but the second peak was extremely broad and asymmetrical, probably owing to a strong hydrophobic interaction with the non-polar patches on the surface of the CBH I molecule. Even at a propanol concentration as high as 10–15%, the tailing was very pronounced. We had to increase the concentration above 20% to obtain a satisfactory peak shape. The asymmetry factor for the second alprenolol peak in the presence of 25% 2-propanol was 2.0. The influence of 2-propanol on the appearance of the peaks is illustrated by the separation of (*R*, *S*)-alprenolol. (Fig. 3). Different samples required different concentrations of 2-propanol to suppress tailing of the second peak (see the legend to Fig. 2), which suggests differences in their hydrophobic interaction with the enzyme. Attempts to suppress the hydrophobic interaction by using ethylene glycol instead of 2-propanol were not successful. Ethylene glycol at high concentrations increases the viscosity of the buffer, thereby extending the run times and increasing the peak broadening.

As we have not studied systematically the influence of each parameter (pH, ionic strength, 2-propanol concentration) there are certainly other experimental conditions that can be used with advantage. The difficulty in finding the optimum conditions for the separation of enantiomers originates from the nature of the chiral

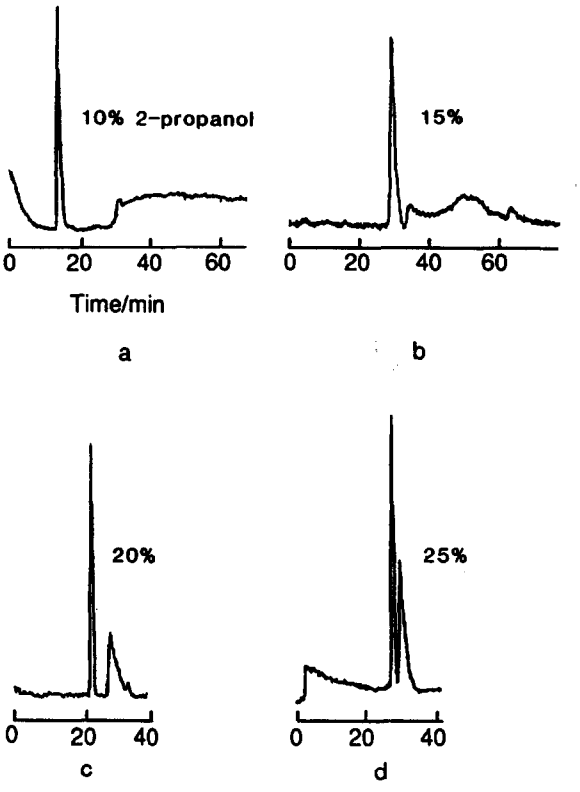


Fig. 3. Influence of the concentration of 2-propanol on the chiral separation of (*R*,*S*)-alprenolol. Buffer, 0.4 *M* sodium phosphate (pH 5.1) supplemented with (a) 10%, (b) 15%, (c) 20% and (d) 25% 2-propanol.

recognition: a complicated multiple (three)-point interaction [20,21], including electrostatic, hydrophobic, $\pi-\pi$, Van der Waals and hydrogen bonds. A strict balance among the interactions is needed to obtain optimum resolution. As the contributions of the different kinds of interactions are a function of, for instance, ionic strength, pH, temperature and the presence of buffer additives, the selectivity is dependent on fine tuning of the separation conditions.

Comparison of HPLC and HPCE separations of enantiomers interacting with CBH I

We also studied the separation of enantiomers of the same drugs by chromatography on a continuous polymer bed (synthesized from piperazine diacrylamide and methacrylamide) to which CBH I had been immobilized [14]. A comparison can therefore be made. The enan-

tioselectivity was lost both for the free enzyme and the enzyme immobilized on the continuous bed when the buffers used for chromatography (0.005–0.1 *M* potassium phosphate, pH 7.5) were employed in the HPCE runs. We have no satisfactory explanation for this unexpected finding. In addition, *RR/SS* and *RS/SR* isomers of labetolol (Fig. 2d and e) could be baseline separated by HPCE but not by chromatography. Obviously, the selectivity of the free (in HPCE) and the immobilized enzyme (in HPLC) is not the same, probably owing to some structural changes in the protein molecules on immobilization. Some non-specific interactions with the supporting material may also explain the differences in selectivity.

Separation mechanism

CBH I consists of one cellulose-binding domain, the three-dimensional structure of which has been solved [22], and a catalytically active core. Structural studies on this core, which constitutes the main part of the enzyme, are in progress. As cellobiose, an inhibitor of the enzyme, strongly impairs the enantioselectivity (14,23) it is evident that the active site is involved in the chiral recognition. Interestingly, propranolol inhibits the enzymatic activity [23].

CONCLUSIONS

We have shown that CBH I, and therefore probably also other proteins enantioselective in HPLC, can be used for the separation of enantiomers by HPCE utilizing the approach described. It is stressed, however, that it is not necessary that the protein and the enantiomers migate in different directions to avoid disturbances in the UV detection; it may be sufficient if they have different migration velocities. The advantage of HPCE over HPLC is that CBH I need not be immobilized. As the enzyme is included in the buffer, it is easy to automate the analyses and to use this method for screening many proteins for stereoselective properties.

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