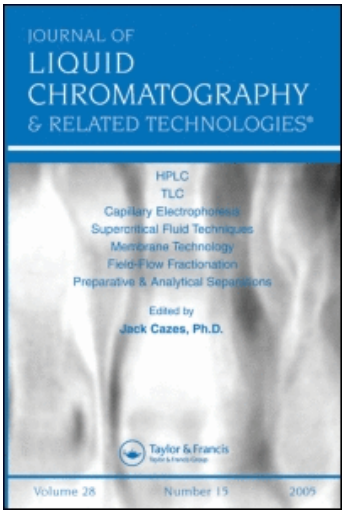

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Helén Ljungberg[a]; Staffan Nilsson[a]
[a] Department of Technical Analytical Chemistry, Chemical Center, University of Lund, Lund, Sweden

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PROTEIN-BASED CAPILLARY AFFINITY GEL ELECTROPHORESIS FOR CHIRAL SEPARATION OF β-ADRENERGIC BLOCKERS

HELÉN LJUNGBERG AND STAFFAN NILSSON*

Department of Technical Analytical Chemistry
Chemical Center
University of Lund
P.O. Box 124
S-221 00 Lund, Sweden

ABSTRACT

The proteins cellulase and bovine serum albumin (BSA) have in this study been cross-linked with glutaraldehyde to form a gel which has been used in capillary affinity gel electrophoresis (CAGE) to resolve enantiomeric pairs of ß-adrenergic blockers. Both proteins have earlier been used as chiral selectors, especially in HPLC. We have utilized the major quantitatively cellulase, cellobiohydrolase I (CBH I) produced by the fungus *Trichoderma reesei*, in CAGE to separate enantiomers. Since it was difficult to obtain a stable gel with cellulase alone, we copolymerized it with BSA. With this cellulase/BSA gel we could resolve the optical isomers of the ß-blocking drugs, *rac*-propranolol, *rac*-metoprolol, *rac*-pindolol and partially *rac*-atenolol. The used capillaries have an inner diameter of 75 µm, a total length of 23,5 cm and an effective length of 15,5 cm filled with gel to the detection window. The buffer used for separation was, 50 mM potassium phosphate buffer at pH 6.8 with 1% 2-propanol added as organic modifier. Samples were electrokinetically introduced and separated at a voltage of 3-3,5 kV. With

this study we want to propose a new model based on copolymerization of proteins, as chiral selectors, to create a gel which has the potential to resolve different types of chiral compounds in capillary affinity gel electrophoresis.

INTRODUCTION

During recent years, the enantiomeric composition of biologically active compounds have become increasingly important due to the different physiological activities/effects that can exist for drug enantiomers. A large interest for different analytical methods within the area of enantiomeric separation techniques has developed. For quite some time researchers have used proteins as chiral selectors for separating optical isomers within the field of chiral chromatography (1, 2). New techniques, such as capillary electrophoresis, have developed into a powerful tool for chiral separations since extremely high efficiency, short separation times, less material and chemicals are consumed. This can be of value in terms of cost and/or when a compound is available in limited amounts (3-7). Another substantial advantage is that only minute amounts of chemical waste is produced.

Two different proteins have been used in this study, cellulase and bovine serum albumin (BSA). Cellobiohydrolase I (CBH I) is the major quantitatively cellulase produced by the fungus *Trichoderma reesei*. The three-dimensional (3D) structure has recently been determined and refined to 1,8 Å resolution by X-ray crystallography. The enzyme contains a 40 Å long tunnel which is thought to be part of the active site (8) were the enantioselection also are thought to take place. Both CBH I, an acidic glycoprotein, and BSA, a globular plasmaprotein, or parts of them, are well known to discriminate between enantiomers. These proteins have earlier been used as chiral stationary phases by bonding (9-11) or adsorbing BSA (12) to silica. In a recent work, CBH I has been used in liquid chromatography (13) and in free solution as an

enantioselective protein in high performance capillary electrophoresis, providing chiral selectivity for some ß-blocking drugs (14).

In this study we have used BSA to create a gel which can copolymerize or possibly entrap or hold another protein, CBH I, in a gel-network formed with glutaraldehyde as a crosslinker. BSA has been used earlier, as a model protein for capillary affinity gel electrophoresis (15). However, here we propose a new approach to copolymerize CBH I with another protein, BSA, within a closed network, in order to resolve the enantiomers of the ß-adrenergic blockers. The advantages with this mild crosslinking is that the 3D-structure of the selector is kept intact, which is favourable for chiral recognition and separations. Conformational changes seem to be minimized and the chiral selector is more available and stable for interactions with the sample molecules.

MATERIALS AND METHODS

Apparatus.

The experiments were performed with the capillary electrophoretic system from Beckman Instruments Inc., P/ACE 2050 Series, controlled by P/ACE Version 3.0 software and an on-capillary UV-absorbance detector set at 214 nm (Palo Alto, CA, USA). To fill the capillary with the gelation mixture we used a Minipuls peristaltic pump from Gilson (Gilson Medical Electronics, Villiers le Bel, France) with a FloRated, PVC pump tubing 0.015 cc/min Lot. No.:K 1652 6 from Interlab (Westboro, MA, USA).

Material and reagents.

Fused-silica capillary tubing with an internal diameter of 75 µm and an outer diameter of 375 µm was from Polymicro Technologies

(Phoenix, AZ, USA). For the separations we used capillaries with a total length of 23,5 cm and 16,5 cm to the detection window. Bovine serum albumin (BSA), cellulase from *Trichoderma reesei* , (R)-, (S)-, *rac*-propranolol and *rac*-pindolol were obtained from Sigma (St. Louis, MO, USA). *rac*-atenolol (Batch 900232), *rac*-metoprolol (Batch 76029001) from Leiras (Åbo, Finland) and (S)-metoprolol (H 150/65) Batch No H 18 was a kind gift from Astra Hässle (Mölndal, Sweden). 2-propanol, 85% phosphoric acid, sodium citrate and tri-potassium-phosphate-7-hydrate were purchased from Merck (Darmstadt, Germany). Glutaraldehyde, 50%, was bought from Fluka (Buchs, Switzerland). Buffer and sample solutions were prepared from MilliQ water. All solutions were degassed (with helium or ultrasonication) and filtered (0,2 µm DynaGuard ME, Microgon Inc., Laguna Hills, California, USA) before use.

Procedure.

Preparation of cellulase/BSA gel filled capillaries. The on-capillary detection window was prepared 7 cm from outlet end of the capillary, by quickly flaming approximately a 1 cm segment of the polyimide coating, which normally protects the fused silica capillary. The capillary is filled with gelation mixture following the procedure described below (summarized in figure 1). It was first washed with 0,1 ml each of water, 1 M NaOH, water and finally 50 mM sodium-citrate buffer pH 5.0 by flushing it with a syringe. The capillary was then connected to the peristaltic pump. To estimate the capillaries filling time, with the gel mixture up to the the previously made detection window, the capillary was emptied by air injection. Refilling was timed by following the liquid air interface movement. Gelation mixture was prepared of 5 parts 20% (w/v) BSA in water, 21 parts 20 % (w/v) cellulase in 50 mM sodium-citrate buffer pH 5.0 and mixed with 4 parts 50 % glutaraldehyde, in an Eppendorf vial and then the buffer containing capillary was immediately

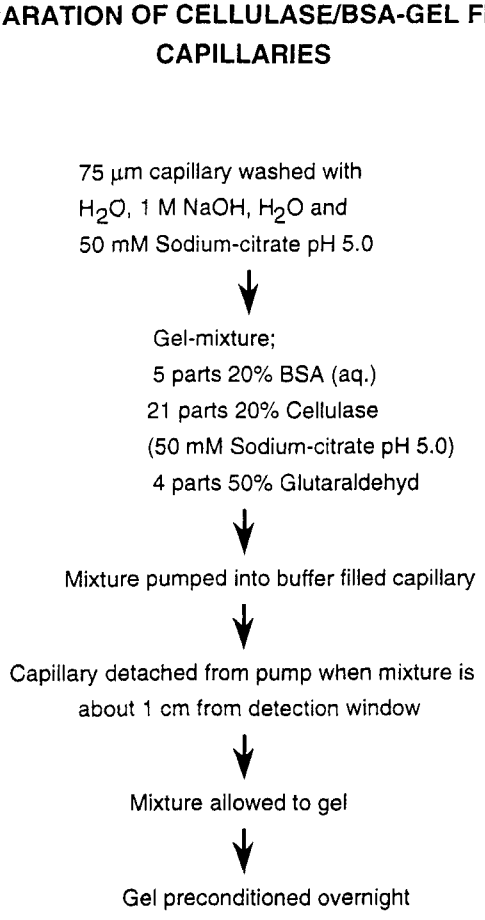

Figure 1. Flow diagram for preparation of gel filled capillaries.

filled. When the mixture had reached approximately 1 cm up to the detection window the filled capillary was disconnected, the pumping tube was snipped with a pair of scissors. Thus, the detection window region of the capillary contained phosphate buffer and not protein gel. After this, the gel filled capillary was placed flat on the bench for gelation. The ends were sealed with (children's) clay preventing the gel to dry out. Before

the capillary was mounted into a Beckman cartridge and preconditioned, it was carefully inspected under a microscope to make absolutely sure that no air bubbles were trapped in it.

Electrophoretic procedure. The gel filled capillary was pre-conditioned overnight by applying a potential of 2 kV, current limitation was 20 μA, with the cathode placed at the injection side (reversed polarity). During this time, the buffer was changed to 50 mM phosphate pH 6.8 with 1 % (v/v) 2-propanol. Electrophoresis of the samples were then performed with the anode at the injection side (normal polarity). Samples were electrokinetically introduced, 2 kV, 3 seconds, containing a buffer with half the ionic concentration of the running buffer. The conditions to perform the electrophoresis were, the voltage were varied from 2-7 kV, the gel length was normally 15,5 cm and the total capillary length 23,5 cm, running buffer used was 50 mM potassium phosphate pH 6.8 supplemented with different concentrations of 2-propanol.

RESULTS AND DISCUSSION

Protein-gel preparation.

Various experiments were made to select the proper conditions for making the protein-gel. Initially we simply started by mixing a few drops protein solutions with different concentrations, buffers and glutaraldehyde on open glass ware. With BSA we were able to obtain gelation under several different conditions. It was on the other hand rather difficult to find the proper conditions to obtain a stable gel with cellulase. Instead, a mixture of cellulase and BSA was used and a stable gel could easily be prepared. Conditions were selected to minimize the amount of BSA, in combination with an excess of cellulase. It was found that a stable opaque gel could be formed with 3,3 % (w/v) BSA and 14 % (w/v)

cellulase as final protein concentrations. Gelation was then performed in Eppendorf vials and a stable gel formed in about 3 minutes which gave enough time for filling the capillaries. The capillaries were prepared and filled as described earlier.

Electrophoresis.

Preconditioning. Before use the capillaries were conditioned with reversed polarity. This procedure was established for eliminating dissolved air which often are formed during the capillary filling and/or gelation, if the reversed polarity is used the air bubbles will not be trapped, in the interface between the gel and the buffer. Another reason for reversed polarity, is that non-bound cellulase can be detected as it passes towards the anode during equilibration of the CAGE-capillary, since it carries a net negative charge at pH 6,8. When the UV absorbance was stable the unbound protein has been removed and stable conditions for separation were obtained.

Sample injection. Electrokinetic injection technique was used (2 kV, 3 sec) to introduce the samples. The samples were dissolved in the running buffer, diluted once with water to develop a sharp sample enrichment zone to enhance efficiency (15).

Separation of the enantiomers of ß-adrenergic blockers in cellulase/BSA-gel with capillary affinity gel electrophoresis. At pH 6,8 the basic ß-adrenergic blockers used in this study all carries a positive charge (pKa above 9) and migrates towards the cathode. We were able to obtain a chiral separation of *rac*-propranolol with CAGE based on cellulase/BSA stationary phase. The electropherogram in figure 2a represent the resolution of 0,5 mM *rac*-propranolol, R- and S-forms were used for verification, (fig 2b,c), although they were not 100 % enantiopure (personal communication with Lars I. Andersson at Astra Pain Control, Sweden). A constant voltage of 3,5 kV was applied giving a stable

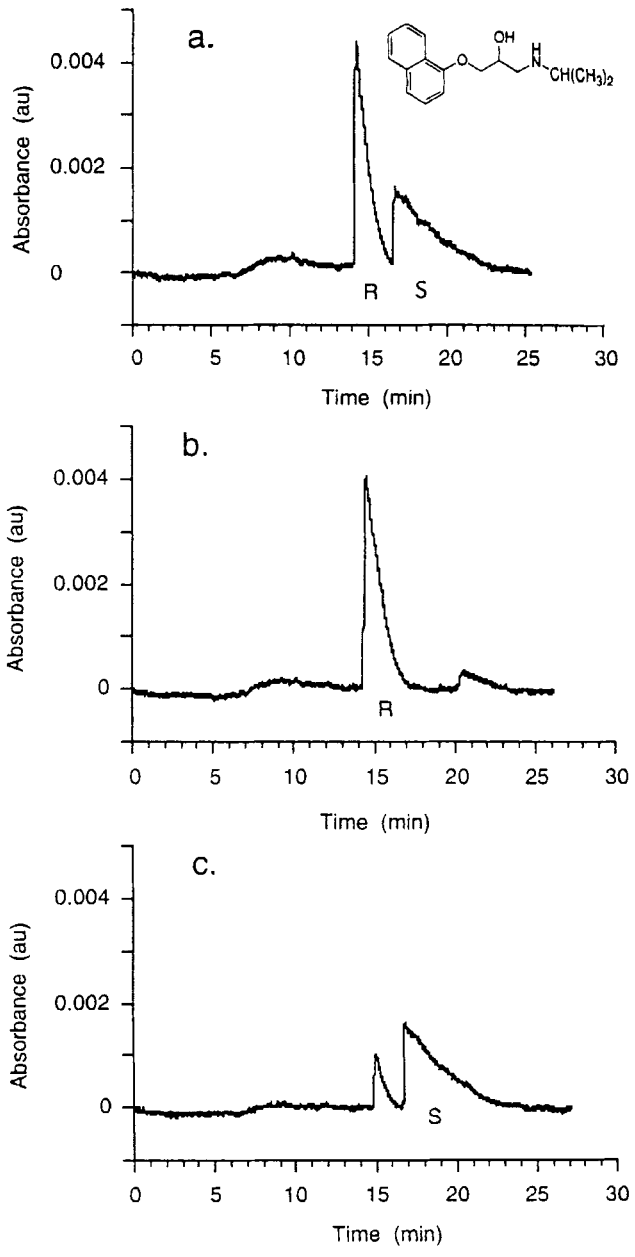

Figure 2. Capillary affinity gel electrophoretic separation of *rac*-propranolol with cellulase/BSA gel. **a**. 1 mM *rac*-propranolol. **b**. 0,5 mM R-propranolol. **c**. 0,5 mM S-propranolol. Conditions: constant applied electric field 3,5 kV (~150 V/cm), 45 μA; gel length=16.5 cm, total length=23.5 cm; buffer 50 mM potassium phosphate pH 6.8 with 1 %(v/v) 2-propanol; sample injection 2 kV, 3 s.

current of 45 μA and as a running buffer 50 mM phosphate pH 6.8 with addition of 1% 2-propanol was used. Higher theoretical plate number and some improvement of the separation was achieved if the 2-propanol concentration was raised to 25 % and the constant voltage applied was increased to 7 kV (data not shown). However, under these separation conditions the protein gels were not stable. Therefore, it is preferable if the electrophoresis can be performed under milder conditions. Still the separation is performed within reasonable time. To investigate if it was possible to resolve other ß-blocking agents under these milder conditions (3,5 kV), separation of metoprolol, pindolol and atenolol were tested.

The electropherograms in figure 3a represent the resolution of *rac*-metoprolol. The enantiomers has been completely resolved and verified with the (S)-metoprolol (fig 3b). Shorter retention times were achieved, than for *rac*-propranolol, likely according to less hydrophobic interaction with the chiral stationary phase.

In figure 4 *rac*-pindolol has been separated. The loading capacity of the protein-gel capillary was studied by resolving different concentrations of *rac*-pindolol, using UV-detection at 214 nm, 75 μm inner diameter and 23.5 cm long capillary. The limit of detection (LOD) was estimated to 10 μM defined as a peak height larger than twice the noise. The upper concentration limit was 1 mM accepting a resolution of, R=1 where $R=\Delta V_{RS}/(W_R+W_S)$ and ΔV_{RS} = difference in elution volume of the R- and S-forms, W_R and W_S equals peak width at the inflection points. Thus, the range of sample concentration separated and analyzed was 0.01-1 mM which is a considerable improvement compared to the previous report (15) although seven times higher concentration of the chiral selector was used in this study.

A very promising split in the electropherogram was obtained for *rac*-atenolol as shown in figure 5. The same separation conditions as described earlier, was used. However, no further attempts were tried to resolve this enantiomeric pair, since that was beyond the intention of this study.

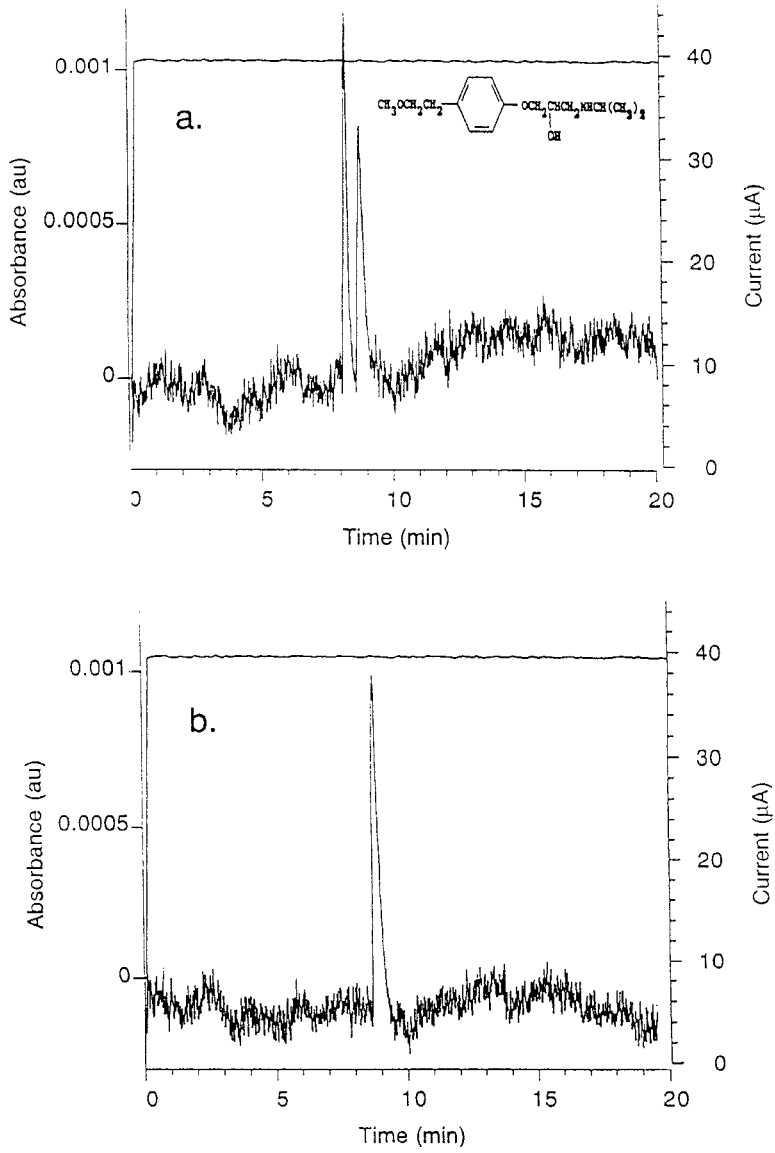

Figure 3 Capillary affinity gel electrophoretic separation of *rac*-metoprolol with cellulase/BSA gel. **a.** 0,15 mM *rac*-metoprolol **b.** 0,075 mM S-metoprolol (Identification of racemate.). Conditions: constant applied electric field 3 kV (~130 V/cm), 40 μA; gel length=16.5 cm, total length=23.5 cm; buffer 50 mM potassium phosphate pH 6.8 with 1 %(v/v) 2-propanol; sample injection 2 kV, 3 s.

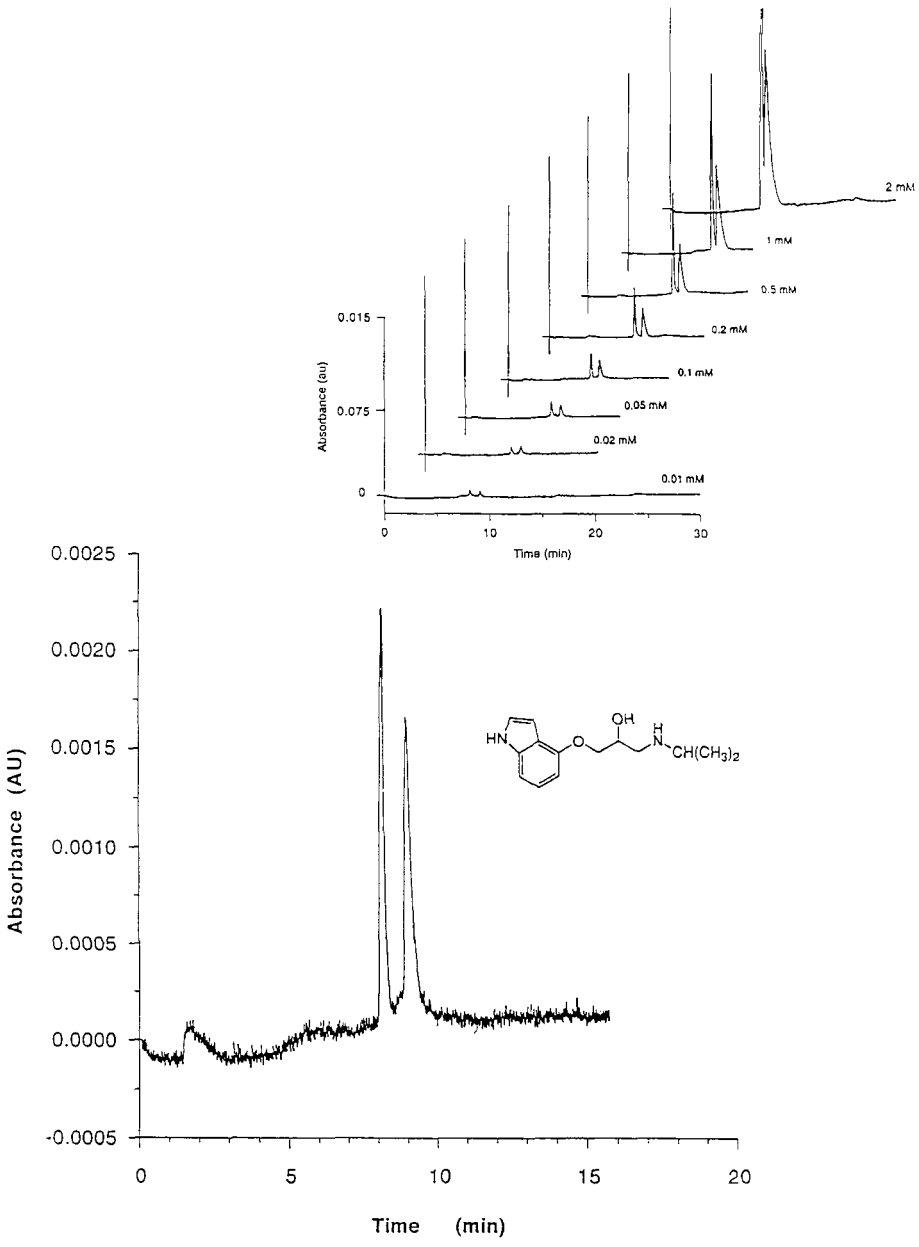

Figure 4. Capillary affinity gel electrophoretic separation of 0,1 mM *rac*-pindolol with cellulase/BSA gel. Insert: Sample loading capacity 0.01-2 mM. Conditions: constant applied electric field 3.5 kV (~150 V/cm), 45 μA; gel length=16.5 cm, total length=23.5 cm; buffer 50 mM potassium phosphate pH 6.8 with 1 %(v/v) 2-propanol; sample injection 2 kV, 3 s.

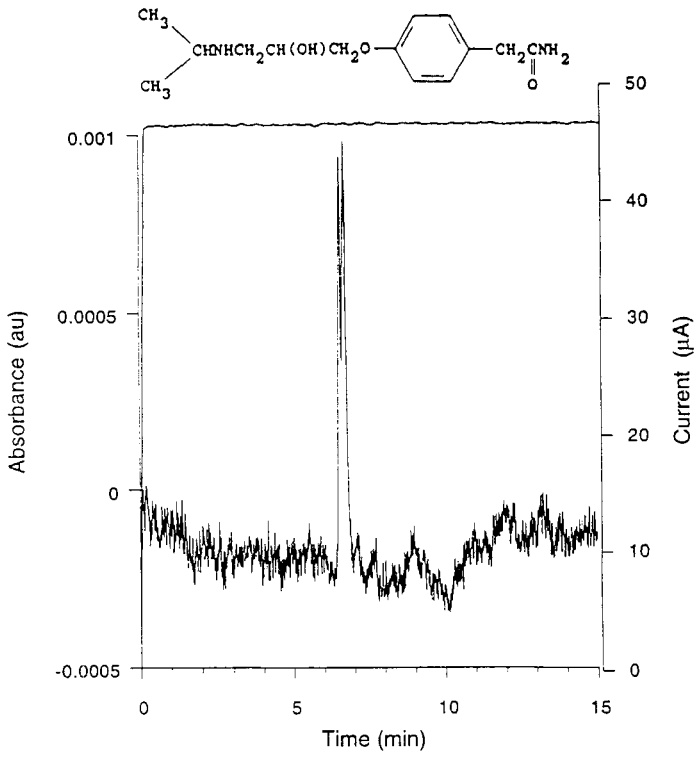

Figure 5. Capillary affinity gel electrophoretic separation of 0,05 mM *rac*-atenolol with cellulase/BSA gel. Conditions: constant applied electric field 3.5 kV (~150 V/cm), 47 μA; gel length=16.5 cm, total length=23.5 cm; buffer 50 mM potassium phosphate pH 6.8 with 1 %(v/v) 2-propanol; sample injection 2 kV, 3 s.

Further improvement of the separations of *rac*-propranolol and *rac*-atenolol as well as resolving other enantiomeric pairs of ß-blocking drugs, will be attempted with this selector in a more comprehensive study. Developing studies will also include the addition of other proteins into the gels and by doing so possibly create other chiral phases which can be used for enantiomeric separations of many different kinds of compounds, in the same capillary.

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