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Evaluation of quail egg white riboflavin binding protein as a chiral selector in capillary electrophoresis by applying a modified partial filling technique

A preliminary evaluation of the enantioselective properties of quail egg yolk riboflavin binding protein (qRfBP) was carried out in capillary electrophoresis by using the complete filling technique. The most promising results obtained by this screening of nineteen chiral drugs were singled out with the aim of optimizing enantiomer separations by applying the partial filling technique, which allows operating at much higher protein concentrations without detection problems. The building of the separation zone in the partial filling technique has been modified in order to enable on-line monitoring, before each run, of the actual protein plug application velocity and, consequently, the building of a plug of the desired length. The electrophoretic conditions chosen gave opposite migration directions for the chiral selector and the analytes, with qRfBP migrating away from the detector. A polyvinyl alcohol-coated capillary was first totally filled with protein and the optimal plug length was obtained by further applying negative pressure together with positive voltage for the time needed. Separations of basic drugs were optimized by using protein concentrations ranging from 200 μM up to 900 μM and different plug lengths, while the running buffer pH (6.0), temperature (25°C) and operating voltage (+20 kV) were kept constant. The enantioresolution of all solutes was affected by both the chiral selector concentration and protein plug length. Baseline separations were obtained for oxprenolol, prilocaine and bupivacaine.

Keywords: Enantioseparation / Quail egg riboflavin binding protein / Capillary electrophoresis / Partial filling technique
EL 3561

1 Introduction

In the early '90s proteins began to be used as chiral selectors in capillary electrophoresis [1, 2]. Purified proteins are expensive and furthermore, in some cases, not even commercially available; the CE approach is thus of particular advantage over HPLC because only a small amount of material is required and immobilization on a solid support is not needed. Most initial papers [3–7] took the simple approach of completely filling the capillary with the background electrolyte (BGE) in which the protein is dissolved (complete filling technique, CFT).

Over the past few years in our laboratory we have undertaken the evaluation of riboflavin binding proteins (RfBPs) as chiral selectors, both as HPLC columns and as BGE additives in free solution CE (FSCE) with the complete filling technique. From the first successful results obtained with an HPLC column based on chicken egg white RfBP [8] also described by other authors [9, 10], we moved to quail egg white RfBP (qRfBP) [11] in order to investigate whether the subtle structural differences described for the same protein of different species [12] could reflect different stereoselective properties. The FSCE experiments carried out with this RfBP were planned on the basis of findings and ideas originated in HPLC in order to evaluate whether CE could be used as a rapid scouting technique for screening the enantioselectivity of novel proteins. This approach made it possible to evaluate the extent to which the protein structure is affected by the HPLC immobilization, as data obtained with the two techniques were found to be comparable, even in terms of k' and α values. Despite these interesting findings, the performance of the CFT with RfBP as BGE additive in terms of peak efficiency and sensitivity was disappointing; furthermore, it was impossible to enhance resolution by using RfBP concentrations higher than 30 μM, as sensitivity deterioration systematically occurred. It is well known that these disadvantages are due to the presence of the protein in the detection region and that an elegant response to the problem is represented by the partial filling technique.

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Abbreviations: CFT, complete filling technique; **DMA**, dimethylacrylamide; **PVA**, polyvinyl alcohol; **qRfBP**, quail riboflavin binding protein

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This approach was first described by Valtcheva *et al.* [13] and later redefined by Tanaka and Terabe [14]. According to the classical "partial separation zone technique", the capillary is partially filled with the protein solution by applying a given pressure for a given time so as to obtain a separation zone that does not reach the detection window. This system may also be set up with other selectors that elicit a strong detector response, such as macrocyclic antibiotics [15–17], cyclodextrins [18], and sodium dodecyl sulfate [19]; however, it was first developed for protein-based separations since proteins cause the worst problems. The attractive features of the technique are mainly the ability to use much higher selector concentrations than in the CFT [20–24], higher efficiencies, low consumption of material, and compatibility with the mass spectrometer interface [19, 25, 26].

In the partial filling technique, conditions are usually chosen so that the protein has a net negative charge and migrates toward the anode, whereas the analytes migrate toward the detector at the cathodic end. Amini *et al.* [27] defined an equation for the calculation of the effective separation zone length, taking into account that, as the protein zone migrates toward the anode, the effective length is necessarily shorter than the applied one. On this basis, Amini and Westerlund [28] carried out affinity capillary electrophoresis experiments and applied the partial filling technique to the determination of association constants between drug enantiomers and human α_1-acid glycoprotein. A modification of the classical format, defined as "partial defilling", has recently been reported (Quaglia *et al.*, submitted). The capillary is totally filled with protein and when voltage is applied, the protein selector moves toward the anode, thus clearing the detection window during the run.

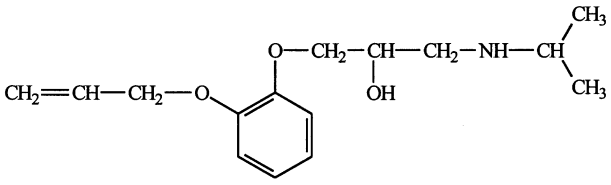
In the present study the separation of some basic drug racemates is obtained by using the partial filling technique and quail RfBP as selector. A modification of the plug building procedure allows run-by-run monitoring of the actual protein plug velocity; accordingly, the building of a more reliable plug of the desired length is achieved.

2 Materials and methods

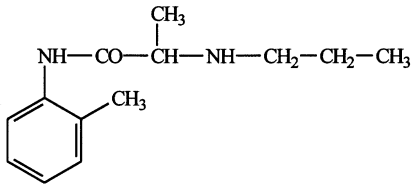
2.1 Chemicals

Oxprenolol, prilocaine, bupivacaine and bepridil were purchased from Sigma (St. Louis, MO, USA), amlodipine was donated by Pfizer (Sandwich, UK). Chemical structures of all analytes are reported in Table 1. Sample preparation was carried out by dissolving known amounts of chiral drugs in bidistilled water and each stock solution was diluted with bidistilled water to a concentration of

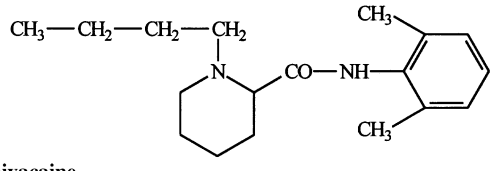
Table 1. Chemical structures of tested analytes



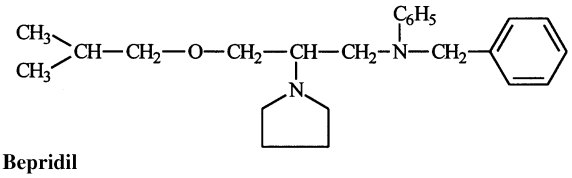
Oxprenolol



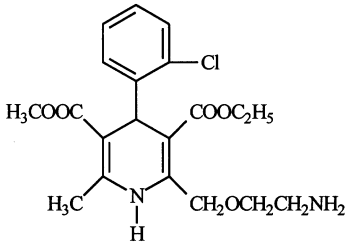
Prilocaine



Bupivacaine



Bepridil



Amlodipine

200 μM. Amlodipine stock solution was instead prepared in methanol/water (1:99 v/v) and then diluted with bidistilled water. NaH_2PO_4, Na_2HPO_4, H_3PO_4 (85%) were purchased from Merck (Darmstadt, Germany). All buffer solutions were prepared fresh daily using bidistilled water. Prior to use, all solutions were filtered through a 0.45 μm membrane filter and degassed by sonication. 50 mM and 100 mM phosphate buffer solutions were prepared by mixing analytical-grade dibasic sodium hydrogen phosphate and sodium dihydrogen phosphate solutions to give a pH of 6.0.

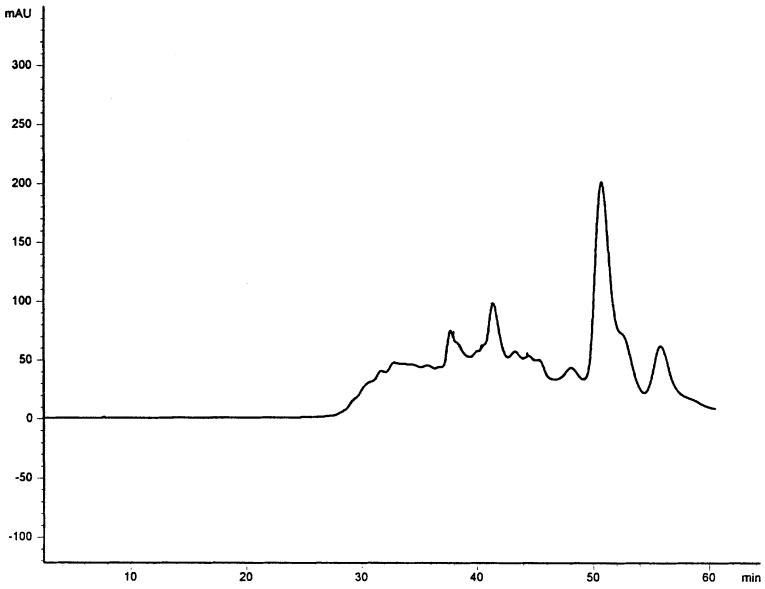


Figure 1. Electropherogram of purified quail egg white RfBP. Background electrolyte, 100 mM phosphate buffer, pH 6.0. Voltage, +20 kV; detection, UV at 200 nm; protein concentration, 30 μM.

Quail egg white RfBP was purified in our laboratory [11] by a suitable modification of a method that is widely used for chicken RfBP [29]. Holo RfBP was kept in the freezer (–70°C) until one week before use and then extensively dialyzed at pH 3.0 in order to eliminate the riboflavin bound to the protein. Apo RfBP was further dialyzed at pH 6.0 against 50 mM or 100 mM phosphate buffer and appropriately diluted with the same buffer to obtain concentrations in the range of 200–900 μM. The purity of quail egg white RfBP was investigated at different purification stages by capillary electrophoresis. By means of preparative isoelectric focusing, purified material revealed the presence of several isoforms which are believed to differ in their carbohydrate and/or phosphate content. This was confirmed by the electropherogram of purified quail egg white RfBP, shown in Fig. 1. Some preliminary experiments were performed with a dynamic coating of dimethylacrylamide (DMA), which was kindly donated by Dr. Marcella Chiari (CNR, Milan, Italy). DMA was dissolved in background electrolyte in order to obtain a concentration of 0.005%; a fused-silica capillary (MicroQuartz, München, Germany) was then rinsed with this solution.

2.2 Apparatus

A Hewlett Packard 3D capillary electrophoresis system (Waldbronn, Germany) with built-in diode-array detector was employed for the partial filling technique. The CE instrument control was performed with an HP Vectra XA/166 computer utilizing Chemstation A.0501 software. A 64.5 cm (56 cm effective length) × 50 μm ID polyvinyl alcohol (PVA)-coated capillary (Hewlett Packard) was used for the partial filling technique. The capillary was thermostated at 25°C. A 61.5 cm (46.5 cm effective length) × 50 μm ID fused-silica capillary (MicroQuartz) was used for the preliminary experiments carried out with the CFT.

2.3 Procedure

At the beginning of each day a PVA-coated capillary was conditioned for 20 min with phosphate buffer (50–100 mM, pH 6.0); at the end of each day the capillary was washed with water for 10 min and dried by flushing with air. Between runs the capillary was rinsed with 10 mM H_3PO_4, H_2O, and running buffer for 1, 2, and 3 min, respectively. All washes and rinses were performed using a pressure of 1000 mbar applied to the capillary inlet. The capillary was then totally filled for 4 min with the separation solution by applying a pressure of 1000 mbar. Strong background UV absorption was observed because of the presence of RfBP in the detection cell. The ends of the capillary were then dipped in the running buffer and a negative pressure (–50 mbar) or negative pressure together with voltage (–50 mbar and +20 kV or +30 kV) were applied. As the isoelectric point of qRfBP is about 4.0, in these conditions the separation zone moves back toward the anode. When the end of the separation zone reaches the detection cell from the cathodic end, UV absorption starts to decrease. The "return velocity" (v_r) of the chiral selector solution was determined by measuring the time required to clear the detection window from the outlet end, visualized by a change in the background signal, which is calculated at half-height point. The length of the applied separation plug (PL_{app}) was determined experimentally by the following formula:

$$PL_{app} = L_{tot} - v_r t \quad (1)$$

where L_{tot} is the total length of the capillary and t is the necessary application time of negative pressure (or negative pressure with positive voltage) to obtain PL_{app}. The sample solution was then introduced into the capillary by dynamic injection with a pressure of 30 mbar for a time of 25 s. The ends of the capillary were then dipped into the running buffer and a voltage of 20 kV was applied. Electropherograms were monitored both at the maximum wavelength for each analyte (225 nm for oxprenolol, prilocaine, and bupivacaine, 240 nm for amlodipine, and 257 nm for bepridil) and at 200 nm, in order to detect potential migration of protein plug together with analyte at the detection window. A dynamic coating (using a 0.005% solution of DMA) was considered at the beginning of the study [30], but it was found inadequate for the partial filling application, as capillary blockage systematically occurred, probably due to interactions between qRfBP and DMA.

3 Results and discussion

In this study a modified partial filling technique was used to improve and optimize the performance obtained by quail egg white RfBP as chiral selector when using the complete filling technique. RfBPs have already demonstrated an excellent performance as HPLC-chiral stationary phase (CSP) [8–11]; furthermore, the study of this protein family as chiral selector is of particular interest because the three-dimensional structure has recently been determined by X-ray diffraction [31].

3.1 Characterization of the chiral selector

The RfBPs are acidic proteins with an important role in maintaining the supply of vitamins to the developing embryo. The first protein of this family to be isolated and studied was chicken egg white RfBP [31]. The family of the chicken RfBPs includes three well-known proteins that are purified from egg white, egg yolk, and from the plasma of laying hens. They are the product of the same gene, but have undergone different post-translational modifications, *i.e.*, chicken egg white and yolk RfBP share the same amino acid sequence, but differ in respect to their carbohydrate chains; furthermore, the latter lacks the last 11–13 amino acids which are proteolytically cleaved. Peculiar characteristics of the three chicken RfBPs are the presence of a highly phosphorylated region and the presence of nine disulfide bridges that play an important structural role in terms of stability. The molecular weight is 36 000 and the carbohydrate part constitutes approximately 14%; its isoelectric point is about 4.0. There are some small, but significant differences between the riboflavin binding proteins from quail and domestic fowl: although the overall secondary structures of the two proteins are similar, there are differences in the environments of aromatic amino acids side chains as revealed by the near UV circular dichroism and fluorescence properties [12]. These differences in tertiary structures must reflect differences in amino acid sequence. Further characterization of the riboflavin binding site of qRfBP has recently been investigated in our laboratory by means of HPLC frontal analysis and zonal elution experiments (Massolini *et al.*, submitted).

3.2 Complete filling technique preliminary data

A preliminary systematic study was carried out on nineteen chiral drugs with different chemical structures including, among others, calcium antagonists, β-blockers and local anaesthetics, by filling completely a fused-silica capillary with a 30 μM qRfBP solution. In order to optimize the enantioseparations, pH (6.0, 7.0, and 8.0), temperature (18, 25, and 30°C), and analyte concentrations (50, 100, and 200 μM) were varied.

The outcome of this screening revealed that higher temperature values resulted in better efficiency but lower resolution, and therefore 25°C was considered to be a good compromise. By decreasing the analyte concentration the α values increased, but it was prohibitive to further reduce this parameter because of sensitivity problems. During these experiments, adhesion of qRfBP to the capillary wall was sometimes encountered, with subsequent building up and capillary blockage phenomena. Despite a suitable between-run rinsing procedure with sodium dodecyl sulfate [32] that was successfully employed throughout all experiments, the problem reappeared from time to time. In general, the low k' and poor α values observed, revealed that higher protein concentrations could be envisaged to reach the necessary number of theoretical plates and achieve better separations [11]. For this reason some analytes were singled out to perform enantioseparations using the partial filling technique, so to arrive at protein concentrations higher than 30 μM and overcome low k' values without detection problems. The racemates chosen were oxprenolol (α = 1.25, $k'1$ = 0.17, $k'2$ = 0.21) and prilocaine (α = 1.23, $k'1$ = 0.082, $k'2$ = 0.10), which showed a hint of separation; amlodipine, which showed good separation (α = 1.65) but low retention ($k'1$ = 0.27; $k'2$ = 0.45); and bepridil, which gave a good separation (α = 2.43), but high retention ($k'1$ = 1.35, $k'2$ = 3.28) and very low efficiency. The retention factor is calculated according to [33]. Bupivacaine, structurally related to prilocaine, was added to the list, despite the fact that for this compound no enantioresolution was observed. The results obtained by employing the CFT for the above-mentioned compounds are reported in Fig. 2.

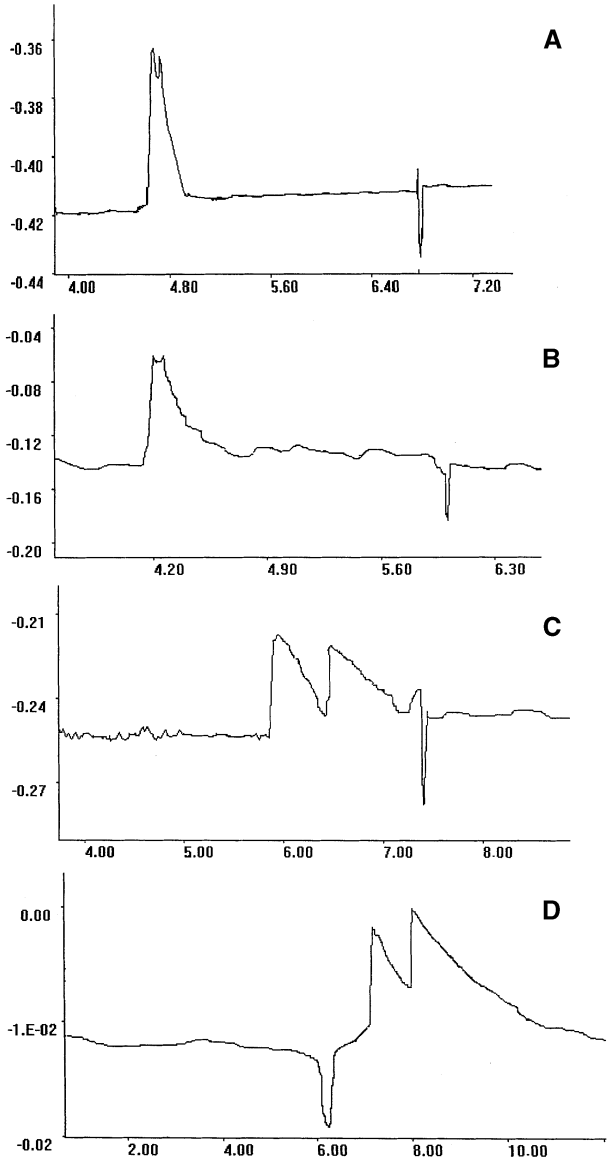


Figure 2. Complete filling technique. Electropherograms of (A) oxprenolol, (B) prilocaine, (C) amlodipine, and (D) bepridil. Background electrolyte, 30 μM qRfBP in 50 mM phosphate buffer, pH 6.0; temperature, 25°C; voltage, +20 kV; detection, UV at 225 nm (A and B) and at 247 nm (C and D).

3.3 Partial filling technique

The operating pH was chosen to be 6.0 where the protein has a net negative charge and moves toward the anode, whereas the solutes, bearing a pK_a between 8.0 and 9.0, give a migration direction toward the cathode. To date, in all the papers describing the partial filling technique with protein selector [13, 14, 20, 21, 23–25, 27, 28], no attention is paid to the reproducibility of the plug application velocity. It is nevertheless reported that this parameter depends on the protein concentration [14, 25, 27, 28], as this, in turn, depends on the different viscosity of the protein. Following the traditional method we initially filled the capillary with a 300 μM solution of qRfBP by applying a constant pressure and calculated the RSD% values of the application velocity on eight consecutive determinations by measuring the time required for the protein solution to reach the detection window. As shown in Table 2, the precision is disappointing, probably due to both instrument and capillary performance. The RSD% values become even higher when measured on an interday basis for the same capillary. Bearing in mind that the applied plug length (PL_{app}) controls the enantioresolution [27], it was decided to modify the technique in order to achieve an "on-line" monitoring of the plug building, run by run. As described in Section 2.3 it therefore became necessary to first fill the whole capillary with protein selector and then to apply negative pressure, so as to measure the actual PL_{app} while building it before the beginning of each analytical run (Eq. 1).

Table 2. Precision of application velocity

	v_{app} [a)] (cm/min)	RSD%
Interday $n = 16$ [b)]	3.75	3.4
Intraday $n = 8$	3.84	1.9
Protein concentration, 300 μM		

a) Application velocity (filling pressure, +50 mbar)
b) Four replicates on four consecutive days

The use of positive voltage instead of negative pressure was found inadequate as the change in the background signal took place gradually, probably because the RfBP, as shown in Fig. 1, is a heterogeneous mixture of isoforms of different electrophoretic mobilities. A negative pressure equal to –50 mbar was sufficient to move the protein toward the anode when the selector concentration was 200 μM but at higher concentrations the viscosity of the RfBP increased, and in order to minimize this prerun time, a combination of negative pressure and positive voltage was optimized. The UV signal drop profiles obtained when using positive voltage (A) and negative pressure (B) during the plug building process are reported in Fig. 3. Tables 3a and b show that RDS% values of the "return velocity" are still high but this drawback would now be overcome by on-line monitoring. Once the plug has been built, the analyte is injected and positive voltage is applied. During the run the analyte experiences a nonhomogeneous system, that is, the protein separation zone followed by neat buffer, and furthermore the effective protein plug length (PL_{eff}) will be shorter than the applied one

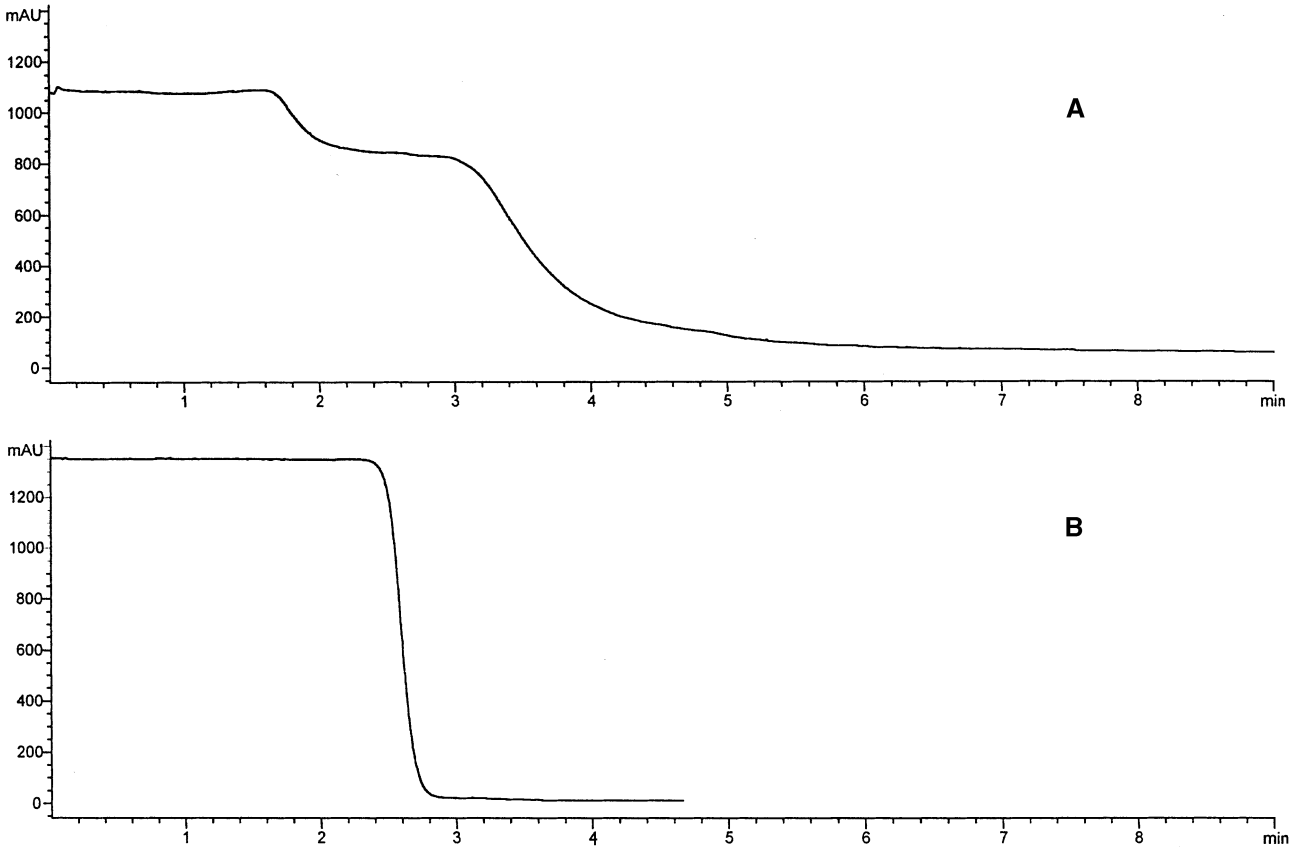


Figure 3. UV signal downslope profiles obtained when using (A) +20 kV and (B) –50 mbar during the plug building process. Protein concentration, 200 μM.

(PL_{app}), taking into account the plug migration while applying voltage. According to the equation described by Amini *et al.* [27]:

$$PL_{eff} = [v_s/(v_s - vPL_{RfBP})] \\ [PL_{app} + t_{mig}\, vPL_{RfBP} - (L_d\, vPL_{RfBP})/v_s)] \quad (2)$$

where v_s is the migration velocity of the solute in the absence of the separation zone , t_{mig} the migration time of the solute during the analysis, and L_d is the effective length of the capillary.

This is nevertheless a modification of the previously described equation, in that the vPL_{RfBP} is the protein plug electrokinetic migration velocity instead of the electrokinetic migration velocity of the protein, and PL_{app} is redefined according to Eq. (1). In order to calculate vPL_{RfBP}, the capillary was totally filled with the protein solution, the capillary ends were dipped in the running buffer, and a positive voltage (+20 kV) was applied. Table 4 reports the influence of RfBP plug concentrations on plug mobility. The lesser extent of shortening for a plug of higher concentration is evident. The selector consumption per run is 1.2 μL.

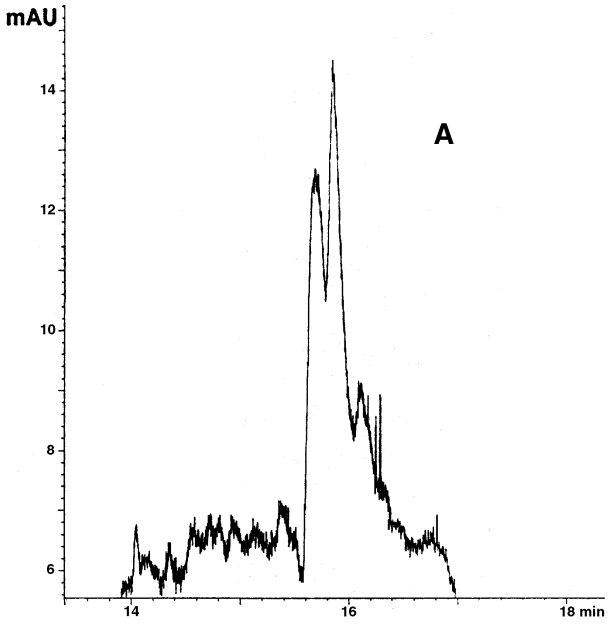
Table 3. (a) Precision of return velocity

qRfBP concentration (μM)	Return mode	v_r (cm/min)	RSD% $n = 10$
200	–50 mbar	3.199	2.52
300	–50 mbar +20 kV	5.538	2.46
400	–50 mbar +20 kV	4.040	1.35
700	–50 mbar +30 kV	6.430	2.38
900	–50 mbar +30 kV	6.080	1.23

(b) Interday and intraday precision of return velocity

	v_r (cm/min)	RSD% $n = 10$
Interday $n = 10$[a)]	5.538	2.46
Intraday $n = 10$	5.333	1.7

a) Two replicates on five consecutive days



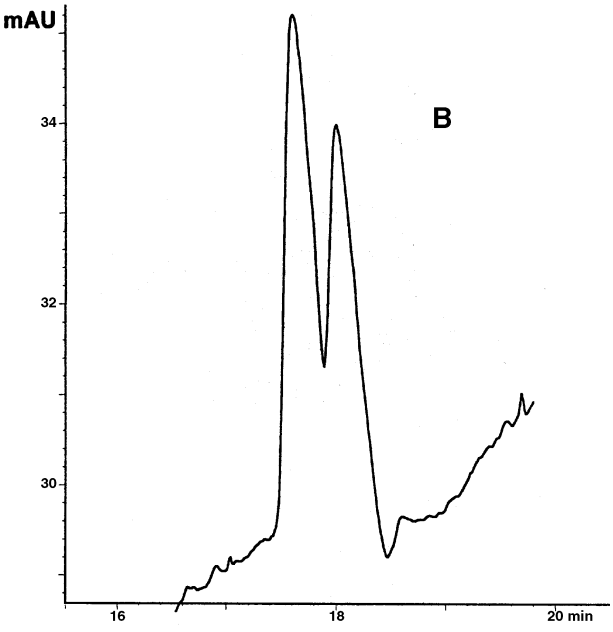


Table 4. Influence of plug concentrations on plug electrophoretic mobility

qRfBP concentration (μM)	vPL_{RfBP} (cm/min)
200	2.23
300	1.73
400	1.09
700	0.52
900	0.51

Voltage, +20 kV

a) Protein plug electrokinetic velocity

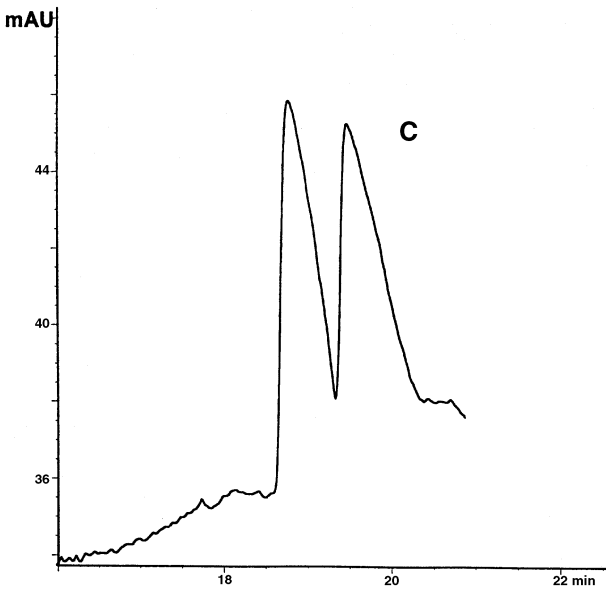


Figure 4. Influence of protein concentration. Analyte, 200 μM oxprenolol; plug length 10 cm; qRfBP concentrations, (A) 200 μM, (B) 300 μM, (C) 400 μM

3.4 Optimization of separation conditions

With the aim of improving the enantioseparations obtained with the CFT, we began considering different protein plug concentrations and different plug lengths, keeping constant the pH value (6.0), the voltage (+20 kV), and the analyte concentration (200 μM). When the enantioseparation of a racemate was optimized, the analyte concentration was further reduced. Protein concentration was varied in the range between 200–900 μM; no aggregation phenomena were observed at the highest concentration level, and it is possible to operate safely at concen-

Table 5. Comparison between efficiency in the partial filling technique and in the complete filling technique

	Partial filling technique		Complete filling technique	
Analyte	$N_1/m^{a)}$ PL_{app}	$N_2/m^{b)}$ PL_{app}	$N_1/m^{a)}$	$N_2/m^{b)}$
Oxprenolol	124 520 10 cm	114 200 10 cm	9511.8	5337.2
Prilocaine	113 671 20 cm	44 407 20 cm	5904.8	2705.1

Conditions: BGE, 100 mM phosphate buffer, pH 6.0; protein concentration, 300 μM (partial filling technique); BGE, 30 μM qRfBP in 50 mM phosphate buffer, pH 6.0; analyte concentration, 200 μM (complete filling technique)

a) First enantiomer

b) Second enantiomer

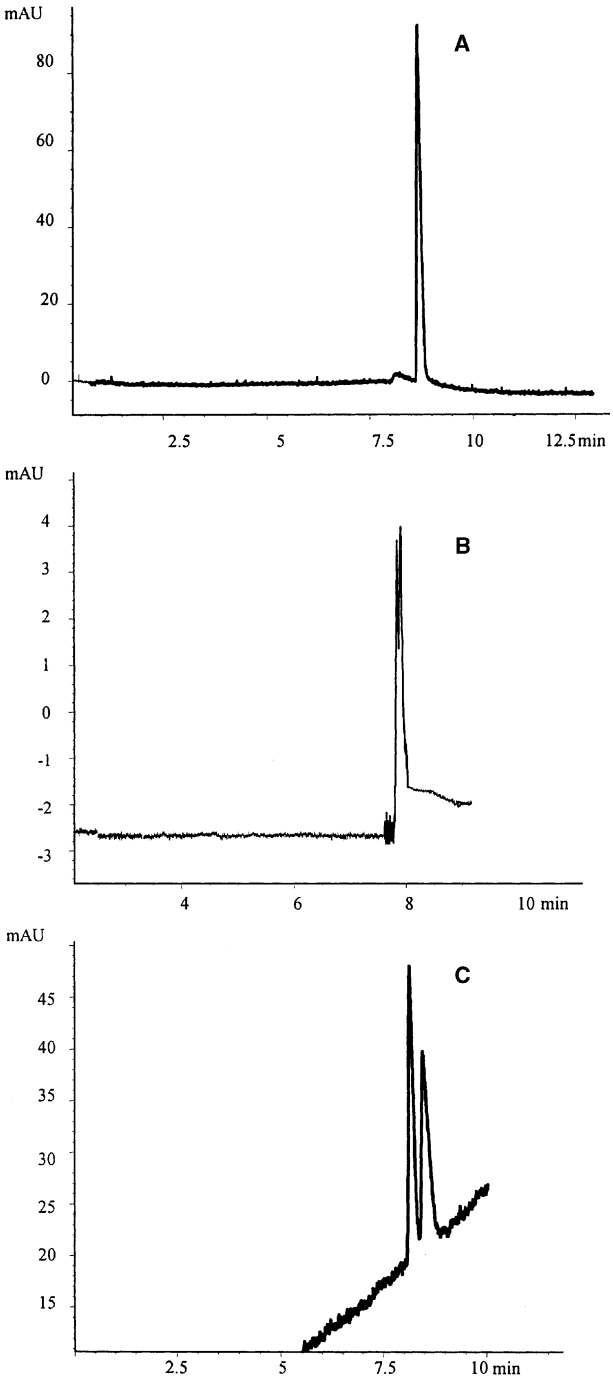


Figure 5. Influence of protein plug length. Analyte, 200 μM prilocaine; qRfBP concentration, 300 μM; plug lengths, (A) 5 cm, (B) 10 cm, (C) 20 cm; capillary length, 49.5 cm (41 cm effective length).

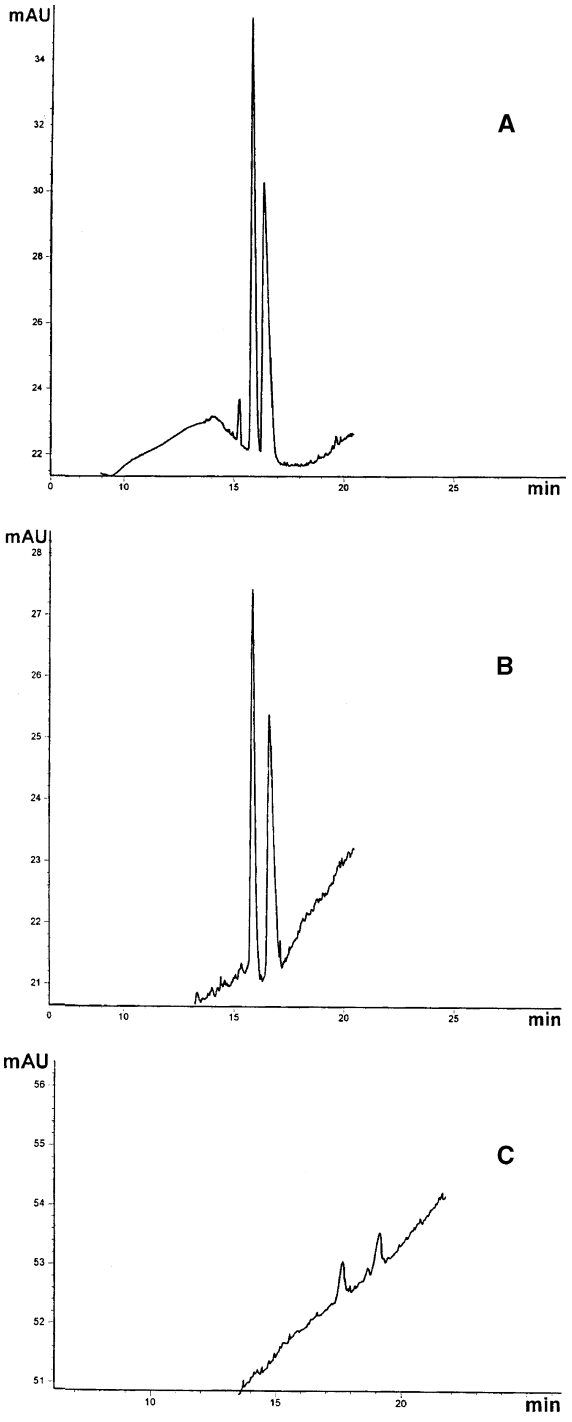


Figure 6. Influence of analyte concentration. qRfBP concentration, 300 μM. Plug length, 20 cm; analyte, (A) 200 μM, (B) 100 μM, (C) 5 μM prilocaine.

trations up to 1.2 mM. As shown in the example (Fig. 4), by increasing the protein concentration, resolution for oxprenolol increases although efficiency deteriorates. Selector plug length was investigated at 5, 10, 20, and 30 cm, and it was found that this parameter affected both enantioresolution and efficiency. This is clearly shown in Fig. 5, where prilocaine was best separated with a protein plug length of 20 cm.

Table 6. Electrophoretic parameters for the best enantioresolutions

Analytes (μM)	Plug length (cm)	Protein concentration (μM)	N_1/m[a)]	N_2/m[b)]	Resolution factor (R_s)	Selectivity factor (α)
Oxprenolol (100)	10	300	219 512	216 320	1.10	1.02
Prilocaine (200)	20	300	113 671	44 407	1.27	1.04
Bupivacaine (50)	30	900	223 094	142 148	1.10	1.02

a) First enantiomer
b) Second enantiomer

Table 7. Repeatability of migration times and peak areas for the separations of racemic prilocaine ($n = 8$)

	Migration time average (min)	RSD %	Peak area average	RSD %
First enantiomer	15.75	0.81	160.9	1.8
Second enantiomer	16.24	0.82	162.4	1.9

Analyte concentration, 200 μM; plug length, 20 cm; protein concentration, 300 μM

As expected, the efficiency obtained with the partial filling technique was much improved when compared with the results obtained with the CFT, as shown in Table 5. Oxprenolol, prilocaine and bupivacaine were baseline separated and the electrophoretic parameters, namely selectivity factors (α), efficiency (N) and resolution factors (R_s), are reported in Table 6. Figure 6 shows that by reducing the analyte concentration, the resolution is much improved. The partial filling technique allowed LOQ and LOD values for prilocaine of 160 ng/mL and 80 ng/mL, respectively (signal-to-noise ratio, 3:1). The repeatabilities of migration times and peak areas were measured for the separation of racemic prilocaine (Table 7). The relative standard deviations are below 1% and 2% for the retention times and peak areas, respectively. These good results are believed to be due to the overall good performance of the modified partial filling technique here reported. Amlodipine did not show enantioseparation in any of the conditions tested. When building a plug length equal to or longer than 10 cm, a strong retention was observed, at any protein concentration. In order to reduce this strong interaction, different percentages of methanol (1%, 5%, 10%) were added to the running buffer, thus obtaining shorter migration times but no enantioresolution. Bepridil, which already gave high retention with the CFT by using the partial filling technique, was practically not detected, even when adding 15% methanol to the running buffer. This compound could not be resolved in any of the conditions tested.

3.5 Performance of capillaries

In our experience, the performance, the lifetime, the between-batch and the batch-to-batch reproducibility of coated capillaries is still an issue that has not been thoroughly investigated so far. This is in fact one of the reasons why we employed run-by-run monitoring of the actual plug velocity, in order to have an "on-line" situation of the coated capillary wall conditions. As previously described, coated capillaries have a short lifetime. Amini *et al.* [27] reported a reproducible performance of a polyacrylamide-coated capillary for no more than 8–12 runs; Tanaka *et al.* [22] also confirmed that EOF reappeared during extended use; typically, the capillary had to be replaced after 100–150 runs. In this work, at the initial capillary lifetime, the EOF was less than 10^{-5} cm^2 V^{-1} s^{-1}, but it became necessary to change the capillary after 150 runs, that is, when EOF was larger than 10^{-5} cm^2 V^{-1} s^{-1}. As previously described by Tanaka *et al.* [22], the reappearance of EOF did not affect the enantioresolution. When quantitative measurement of peak area and retention times started to become irreproducible (*i.e.*, a peak height lowering was observed) we restored the capillary initial performance by cutting off one millimeter at both capillary ends.

4 Concluding remarks

We have presented a modified partial filling technique that allows a reliable and reproducible building of the applied selector plug length. The on-line monitoring of the actual plug length ultimately permits very good RSD% values of both migration times and peak areas. Detection limits, resolution and efficiency were much improved by the use of the partial filling technique when compared with the CFT data. Riboflavin binding protein extracted from quail egg white proved to be a satisfactory chiral selector for the basic racemates tested and further investigation on a wider number of analytes will be envisaged.

Ms. Elena Tabolotti is gratefully acknowledged for supervising the protein purification protocol.

Received Feruary 16, 1999

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