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Chiral separations by non-aqueous capillary electrophoresis in DMSO-based background electrolytes

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ABSTRACT

Capillary electrophoresis (CE) is a powerful technique for enantioseparations due to its high separation efficiency, high versatility, speed of analysis and low consumption of samples and reagents. Non-aqueous capillary electrophoresis (NACE) appears as a promising technique to perform enantioseparations when the drugs, chiral selectors or samples are non-water soluble. Chiral separations have been performed by NACE mainly using alcoholic solvents as BGEs, with problems of current breakdowns and changes in the BGE composition, due to their high volatility.

In this work, the suitability of DMSO as BGE in NACE has been evaluated. Different experimental variables affecting the enantioresolution of three drugs have been evaluated, finally achieving complete enantioresolution of two drugs (verapamil, $Rs=1.5$ and pindolol, $Rs=2.0$) and partial resolution of the third one (fenfluramine, $Rs=1.2$). DMSO has been demonstrated to be a good alternative to methanolic BGEs in NACE.

1. Introduction

Up to 50% of the commercialized pharmacologically active compounds are chiral, and differences in the pharmacological activity and/or the pharmacokinetics of their enantiomers are possible, due to the interactions with biomacromolecules such as proteins, enzymes, receptors or carriers that are optically active [1]. Several drugs have been recently commercialized as pure enantiomers, after discovering that their pharmacological activity was, mainly or exclusively, due to one of the enantiomers [2–5]. Also, due to the potential differences between the activities of a pair of enantiomers, the regulatory authorities declare that enantioselective studies of all new drugs, including single enantiomers and racemates, are mandatory. For all these reasons, the development of methods for the chiral analysis of drugs is of growing interest for the pharmaceutical industry [6].

Capillary electrophoresis (CE) is a powerful technique for enantioseparations due to its intrinsic characteristics such as high separation efficiency, high versatility, speed of analysis, low consumption of samples and reagents and low environmental impact [7]. The most common modality of CE employed for enantioseparations is electrokinetic chromatography (EKC), in which a chiral selector is added to the background electrolyte (BGE) and acts as a pseudostationary phase. Usually, BGEs are aqueous and the chiral selectors employed are water soluble.

On the other hand, many candidate drugs are non-water soluble. This is actually one of the biggest problems for the pharmaceutical industry these days: the low solubility and dissolution rate of new pharmacologically active compounds. Alternatives to the classical EKC have been proposed in order to perform the chiral analysis of non-water soluble compounds: micellar electrokinetic chromatography (MEKC) and non-aqueous capillary electrophoresis (NACE) [8]. The use of non-aqueous solvents in CE (NACE) was introduced by Walbroehl and Jorgenson in 1984 [9] and the first chiral NACE application was presented in 1996 [10]. Chiral NACE can be considered as a complementary mode to aqueous separation since it facilitates the use of chiral selectors with a low solubility in water [11]. Another advantage is that the use of organic solvents enables poorly water soluble substances to be analyzed, and allows direct injection of organic extracts obtained after sample treatment [12]. The use of organic solvents in the background electrolyte (BGE) can enhance the resolution or the selectivity by tuning of the BGE composition, since different organic solvents have different acid–base behavior, which strongly affects the charge of the analytes, a key parameter for electrophoretic separations [8]. Also, the selector–selectand interactions are different depending on the solvent medium. Another advantage of NACE over aqueous CE is that low current values are obtained, so it is possible to apply high electric field strengths and high ionic strength buffers with no Joule heating, resulting in high separation efficiencies [8,12].

Several methods for the chiral separation by NACE have been published until now [11,13–19], mainly carried out in alcoholic and

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acetonitrile-based electrolytes, with the disadvantage of high volatility of the solvents which may affect the BGE composition during the run and cause current breakdowns. Also, methanol (MeOH) may be unable to assure the dissolution of poorly water-soluble and hydrophobic drugs. The use of dimethyl sulfoxide (DMSO) in NACE has been reported only a few times, mainly in chip-CE applications [20,21] and only in one case for a chiral separation [22], where few amino acids were partially or completely separated employing a cationic cyclodextrin. However, DMSO has good properties to be employed as a background electrolyte in NACE, since it has a high dielectric constant (ε=46.45), and it is a non-toxic, non-hazardous, non-volatile solvent [20]. Also, the stability of samples in DMSO is higher than in alcoholic solvents due to its aprotic character. On the other hand, there is an important drawback of DMSO for chiral NACE: selector–selectand interactions, which are mainly intermolecular forces, are very weak in this medium, so achieving the enantioseparation may be difficult and a fine adjustment of the conditions becomes necessary.

In this work, the suitability of DMSO as BGE for performing chiral separations in NACE is evaluated, as an alternative that can be employed when the use of classical methanolic BGEs is not possible. An anionic cyclodextrin, carboxymethyl-γ-cyclodextrin (CM-γ-CD) has been employed as chiral selector, and the enantioseparation of three racemic drugs with different molecular sizes and functional groups (verapamil, pindolol and fenfluramine) has been studied. The influence of some experimental variables on the enantioseparation of these three drugs has been evaluated.

2. Materials and methods

2.1. Instrumentation

A Beckman P/ACE MDQ Capillary Electrophoresis System equipped with a diode array detector (Beckman Coulter, Fullerton, CA, USA), and 32Karat software version 8.0 was used throughout. A 75 μm inner diameter (i.d.) fused-silica capillary with total and effective lengths of 60.2 and 50 cm, respectively, was employed (Beckman Coulter, Fullerton, CA, USA). Detection wavelength was fixed at 270 nm due to the absorbance of UV light by DMSO at lower wavelengths (the cut-off wavelength for DMSO has been fixed around 260 nm in the literature). Sample tray temperature in the CE system was set at 20 °C for all experiments.

2.2. Chemicals and reagents

All reagents were of analytical grade. DMSO, MeOH, ammonium acetate (NH_4Ac), acetic acid (HAc) and sodium hydroxide (NaOH) were from Scharlau (Barcelona, Spain). Carboxymethyl-γ-cyclodextrin (average degree of substitution ~3–5, acidic form) was from Cyclolab (Budapest, Hungary). Fenfluramine hydrochloride and pindolol were kindly provided by Prof. Y. Vander Heyden, Vrije Universiteit Brussel, and verapamil hydrochloride by Prof. J. Crommen, University of Liège.

Background electrolytes were prepared by weighing or measuring the adequate amount of all the additives and bringing to final volume with DMSO. Stock solutions (1 mg mL^{-1}) of the racemic drugs were prepared by weighing the adequate amount of the drugs and bringing to 10 mL with DMSO. Working solutions (0.2 mg mL^{-1}) were prepared by dilutions of the stock solutions in DMSO. BGEs and drug solutions were stable at room temperature for at least 2 weeks. They were filtered through 0.45 μm pore size nylon membranes (Micron Separations, Westborough, MA, USA) prior to their injection in the CE system.

2.3. Methodology

2.3.1. Capillary conditioning

New capillaries were conditioned for 15 min by rinsing with 1 M NaOH at 60 °C. Then, they were rinsed for 5 min with deionized water and 10 min with BGE at 25 °C. At the beginning and end of each day, the capillary was rinsed with 1 M NaOH for 5 min, water for 5 min, and methanol for 5 min. Between runs, the capillary was rinsed for 2 min with methanol and 2 min with BGE. All steps were carried out at 20 psi.

2.3.2. Procedure for the study of enantioseparations by NACE

The initial composition of the background electrolyte was as follows: DMSO–MeOH 80:20, with 50 mM NH_4Ac, 1 M HAc and 40 mM CM-γ-CD. Initial experiments were carried out at 25 °C, applying a voltage of 20 kV (normal polarity). Experimental conditions for the enantioseparations were varied one by one, keeping the others at fixed values. After filling the capillary with the BGE, drug samples were injected hydrodynamically by applying 0.5 psi for 5 s. Separations were performed with normal polarity in all cases.

3. Results and discussion

The selection of initial conditions for the study (see Section 2.3.2.) was based on an overview of the literature about chiral NACE, in which buffering is done mainly using ammonium acetate/acetic acid systems. As we expected low current values and a possible lack of solubility of the buffer salts by using pure DMSO, a percentage of MeOH was added to the BGE. CM-γ-CD (acidic form) was selected as chiral selector due to its good solubility in the DMSO medium. It is important to use the acidic form of the CD because the sodium salt may be insoluble in non-aqueous solvents.

Using these initial conditions (BGE: DMSO–MeOH 80:20, with 50 mM NH_4Ac, 1 M HAc and 40 mM CM-γ-CD; capillary temperature, 25 °C and applied voltage, 20 kV), partial resolutions of 0.65 for verapamil (VER) and 0.69 for pindolol (PIN) were obtained. For fenfluramine (FEN), a little split of the peak was observed, but an *Rs* value could not be calculated. The calculation of *Rs* values was done using the following expression:

$$Rs = \frac{2(t_2 - t_1)}{W_2 + W_1} \quad (1)$$

where t_1 and t_2 are, respectively, the migration times of the first (E_1) and second (E_2) eluted enantiomers, and W_1 and W_2 are the peak widths at peak base.

An average current of ~12.3 μA was obtained with these conditions. The current was stable throughout the electrophoretic development.

A univariate optimization of the experimental conditions for the enantioseparation of three racemic drugs by NACE was carried out. This univariate procedure allows to see the effects of the different experimental variables one by one, and to better understand the mechanisms and effects responsible for the chiral separation. We consider that the study of the individual influences of variables in NACE is important, since there is still a lack of knowledge about the behavior of organic solvents and different buffer additives [12], and the way they can affect electrophoretic parameters such as peak efficiencies, selectivity or resolution. Also, as chiral NACE applications have been mainly studied with methanolic BGEs, the effects that could happen in a DMSO medium are still more unknown. So, taking as starting point the partial *Rs* obtained with the initial conditions, all experimental variables were adjusted in order to improve the *Rs* of enantiomers.

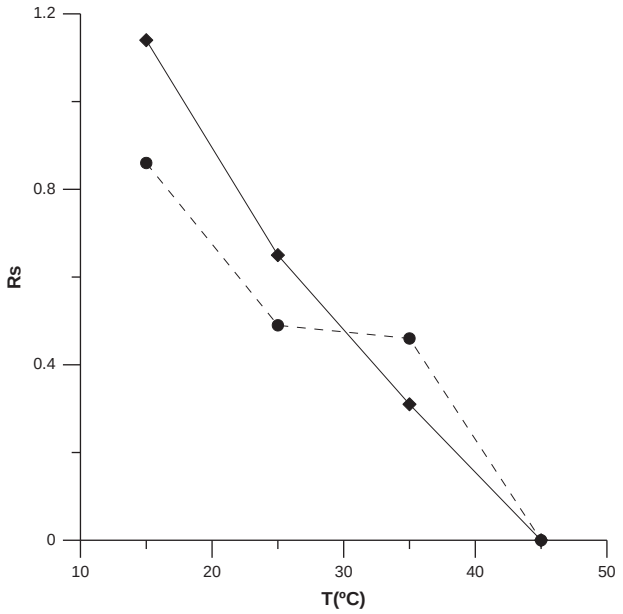

Fig. 1. Effect of the capillary temperature on the enantioresolution of VER (⬥, solid line) and PIN (●, dashed line). Other experimental conditions: BGE DMSO–MeOH 80:20, 50 mM NH_4Ac, 1 M HAc, 40 mM CM-γ-CD, applied voltage 25 kV.

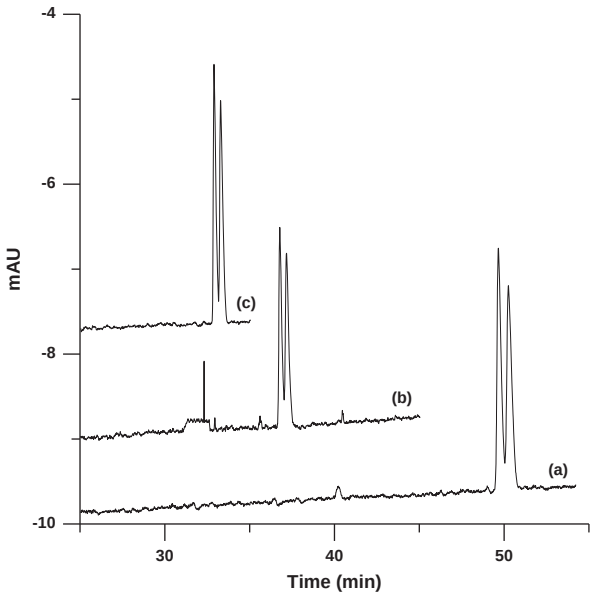

Fig. 2. Electropherograms corresponding to the enantioseparation of VER at three separation voltages: 20 kV (a), 25 kV (b) and 30 kV (c). Capillary temperature: 15 °C. Other experimental conditions as in Fig. 1.

3.1. Effect of the capillary temperature

Capillary temperature is a key parameter for enantioseparations since it affects the mobility of the enantiomers and also the thermodynamic complexation equilibrium between the enantiomers and the chiral selector. In this work, capillary temperature was varied between 15 and 45 °C. As shown in Fig. 1 for VER and PIN, the enantioresolution of both drugs increases when the temperature is decreased. A similar effect was observed for FEN, with an increase in the peak split, but *Rs* values could not be calculated. This effect can be explained because a decrease in capillary temperature will increase the viscosity of the BGE, thus decreasing the mobility of the enantiomers and the EOF, with the consequence of higher *Rs*. Longer migration times are obtained in all cases with low temperatures: the migration time of the second enantiomer increases from 20.5 min at 45 °C to 37.1 min at 15 °C in the case of VER, from 16.6 (45 °C) to 28.6 min (15 °C) for PIN, and from 15.5 (45 °C) to 27.1 (15 °C) for FEN. A separation temperature of 15 °C was selected in all cases.

3.2. Effect of the separation voltage

As a consequence of the low currents obtained in NACE, high voltages can be employed thus improving the migration time of the enantiomers and the peaks' efficiency. Here the voltage ranged between 20 and 30 kV.

The increase in separation voltage considerably reduced the migration times of all the drugs, changing from 35–50 min with 20 kV to 23–33 min with 30 kV. This is due to the considerable increase of the current (from 8.2 μA at 20 kV to 12.6 μA at 30 kV). Also, the enantioresolution was slightly improved when higher voltages were employed. Fig. 2 shows the electropherograms corresponding to the separation of verapamil enantiomers at the three applied voltages, where the effect of the voltage on the *Rs* and migration time can be seen.

3.3. Effect of the CM-γ-CD concentration

The concentration of chiral selector is usually the most influential variable on enantioseparations. When cyclodextrins are employed as chiral selectors, the separation mechanism is the formation of inclusion complexes of the enantiomers in the hydrophobic cavity of the cyclodextrin. The complexation equilibrium is affected by the concentrations of both the drug enantiomers and the cyclodextrins, so an increase in the CD concentration will favor the formation of the diastereomeric complexes responsible for the chiral separation. This is especially important in NACE because the interaction between the enantiomers and the CD is less favoured in organic media than in water.

In this work, the concentration of CD is changed between 10 and 70 mM for all drugs. Fig. 3 shows the enantioresolution values obtained for VER, PIN and FEN with all the CD concentrations. As shown in the figure, 10 mM CM-γ-CD was too low to provide any enantioresolution of drugs. With 20 mM, a partial *Rs* value is obtained for VER, but still no *Rs* was found for PIN and FEN. The maximum *Rs* value was obtained for PIN and FEN with 40 mM CM-γ-CD, decreasing with higher CD concentrations. For VER, the *Rs* was slightly higher with 50 mM CM-γ-CD, but 40 mM was enough to provide good enantioresolution, especially when combined with the best conditions selected in other variables. So, as the CD concentration is the most critical variable in terms of analysis cost, 40 mM was selected as the best concentration for the three drugs studied.

3.4. Effect of the percentage of methanol

The physicochemical properties of DMSO and MeOH are quite different: MeOH has lower dielectric constant (ε), much lower viscosity (η), lower boiling point, and lower autoprotolysis constant (pK_{auto}), which implies that more ions in solution will be found in methanolic BGEs [8]. So, the percentage of MeOH will modify considerably the physicochemical properties of the BGE and will strongly affect the enantioseparation. In this work, since our main aim was to employ a DMSO-based BGE, the percentage of MeOH was varied from 0% to 30%, so the main component of the BGE was DMSO in all conditions.

A decrease in MeOH percentage increases the migration times of all compounds. In the case of VER, best *Rs* is obtained with 20% MeOH, so it is selected as optimum. For PIN, there is a clear decrease in *Rs* when the MeOH percentage is decreased (Fig. 4(1), so 30% is

selected as best option since it combines better *Rs* with shorter migration time. The case of FEN (Fig. 4(2) is the opposite; the split of peaks is larger without MeOH so a pure DMSO BGE is selected as best option.

The differences between the three compounds in the optimum MeOH percentage should be due to a dual mechanism that affects the enantioresolution: on the one hand, the increase in MeOH increases the ε/η ratio of the BGE, which is a measure of the speed of the analysis, and decreases in consequence the migration times, so, if peaks migrate faster, less *Rs* can be expected *a priori* (this is the case of FEN). On the other hand, as DMSO is an aprotic solvent, the increase of MeOH can increase the ionization degree of the drugs, enhancing the interaction with the anionic CD (the case of PIN). For VER, both opposite effects lead to an intermediate BGE composition (DMSO–MeOH 80:20) as the best option.

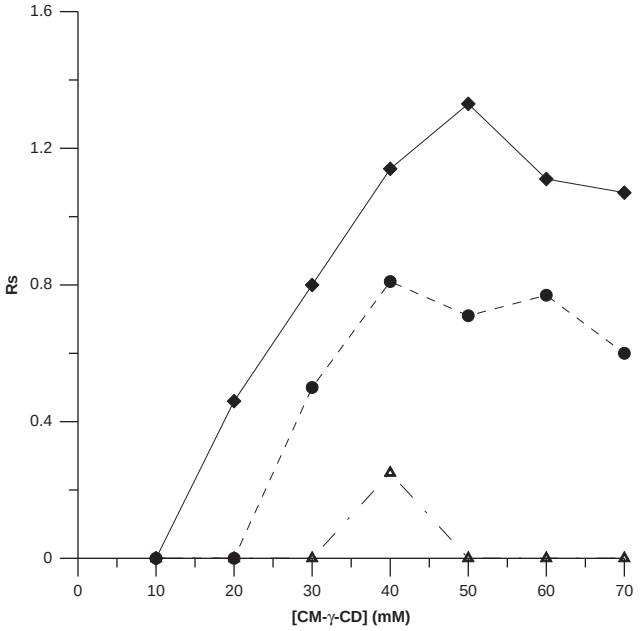

Fig. 3. Effect of CD concentration on the enantioresolution of VER (◆, solid line), PIN (●, dashed line) and FEN (▲, semi-dashed line). Capillary temperature 15 °C, applied voltage 30 kV. Other experimental conditions as in Figs. 1 and 2.

3.5. Effect of the NH_4Ac and HAc concentration

The electrophoretic and electroosmotic mobilities are inversely proportional to the concentration of salts in the BGE [23], so longer

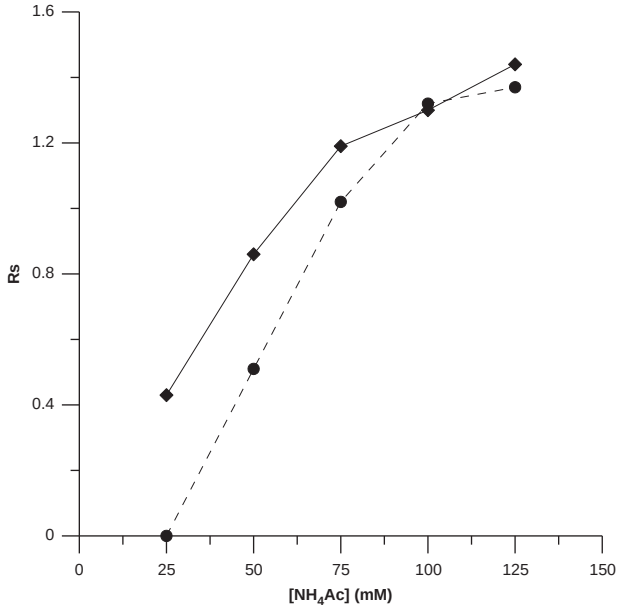

Fig. 5. Effect of [NH_4Ac] on the enantioresolution of VER (◆, solid line) and PIN (●, dashed line). MeOH percentage: 20%. Other experimental conditions as in Fig. 4.

Table 1
Experimental conditions selected for the enantioseparation of each drug by NACE, and the corresponding electrophoretic parameters obtained in each case.

	VER	PIN	FEN
Temperature (°C)	15	15	15
Voltage (kV)	30	30	30
[CM-γ-CD] (mM)	40	40	40
% MeOH	20	30	0
[NH_4Ac] (mM)	125	125	125
[HAc] (M)	1	0.5	0.5
Rs	**1.5**	**2.0**	**1.2**
Analysis time (min)	**43**	**25**	**42**
Current (μA)	**25**	**29**	**17**

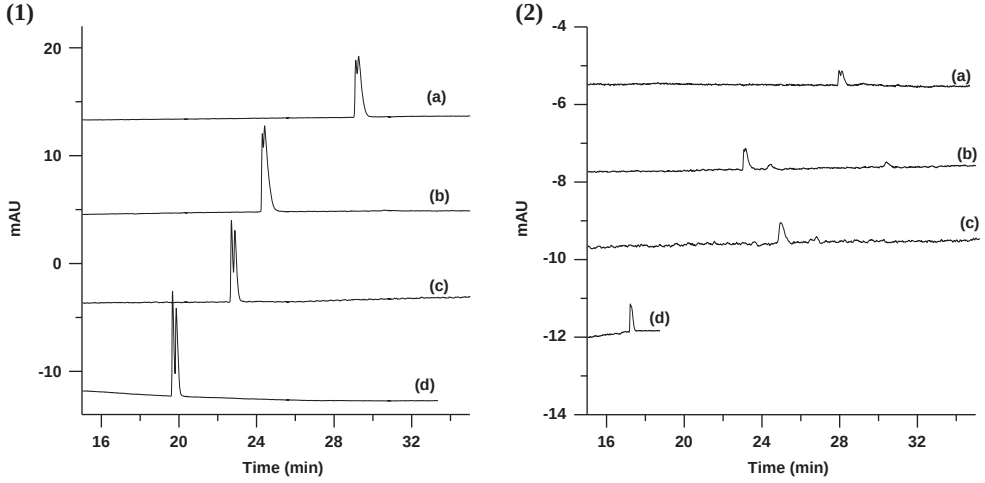

Fig. 4. Electropherograms corresponding to the enantioseparation of PIN (1) and FEN (2), with different MeOH percentages in the BGE: 0% (a), 10% (b), 20% (c) and 30% (d). [CM-γ-CD], 40 mM. Other experimental conditions as in Fig. 3.

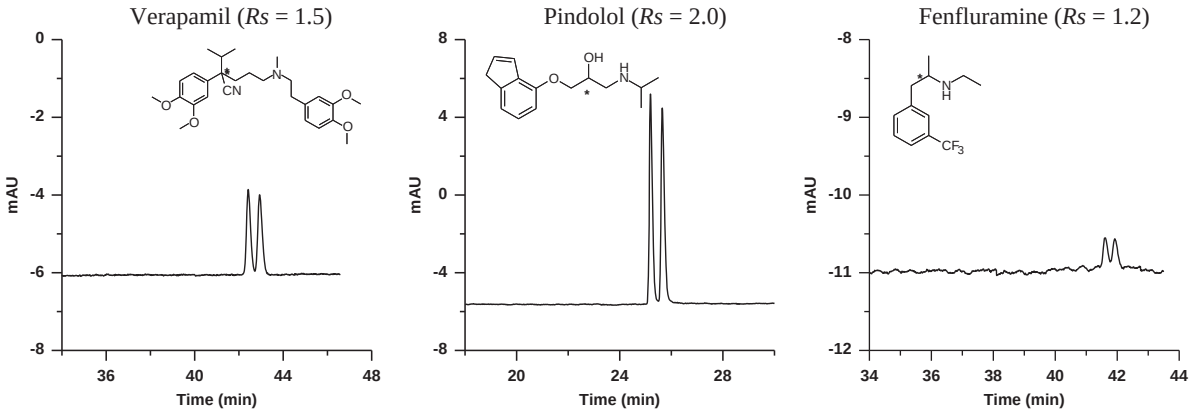

Fig. 6. Electropherograms corresponding to the selected enantioresolution conditions for VER, PIN and FEN. Experimental conditions detailed in Table 1.

migration times and better selectivities are expected when the buffer concentration is increased. The NH_4Ac concentration ranged in this work from 25 to 125 mM (a BGE without NH_4Ac was also tested, but no stable current was obtained). Better *Rs* values but longer migration times were obtained with higher NH_4Ac concentrations, as expected. Due to the notable improvement on the *Rs* of enantiomers (Fig. 5), 125 mM was selected as the best NH_4Ac concentration for all tested compounds.

The HAc concentration was varied between 0.5 and 1.5 M. The differences in HAc concentration did not affect so much the migration time and the resolution of enantiomers. Slightly better *Rs* values (or peak split, in the case of FEN) were found using 1 M HAc in the case of VER and 0.5 M for PIN and FEN, so these conditions were selected.

3.6. Selected conditions

Table 1 summarizes the experimental variables studied and the best conditions selected for the enantioseparation of VER, PIN and FEN in this DMSO-based NACE. It also shows the *Rs* values obtained for the three drugs, the analysis times of the three enantioseparations (migration times of the second eluted enantiomers), and the current values obtained. As can be seen in Table 1, complete enantioresolution of VER and PIN and partial *Rs* of FEN were obtained. The electropherograms corresponding to these best experimental conditions are shown in Fig. 6, together with the chemical structures of the three drugs.

4. Conclusions

In this work, the suitability of DMSO to be employed as BGE in NACE has been evaluated. Different experimental conditions were tested in order to achieve the best enantioseparation conditions for three racemic drugs: verapamil, pindolol and fenfluramine. Complete enantioresolution was obtained for VER and PIN while a partial *Rs* value was obtained for FEN. Best enantioresolutions were obtained with low capillary temperatures, high separation voltages and high concentration of salts in the BGE, in general terms. For the other variables, individual effects may be considered. The analysis times were lower than 45 min in all cases (43 min for VER, 25 for PIN and 42 for FEN). These analysis times are *a priori* long for an electrophoretic separation, due to the high viscosity and relatively low ε/η ratio of DMSO, but are comparable with chromatographic enantioseparations performed with chiral stationary phases.

It is also remarkable that in all the tested experimental conditions current values were stable and there were no current breakdowns, so DMSO has proved to be a good solvent to be employed in NACE, combined (or not) with a small percentage of MeOH in order to modulate the physicochemical properties of the BGE. DMSO appears to be a good alternative for NACE when, due to solubility, stability or volatility problems, the common methanolic BGEs cannot be employed.

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