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Research Article

Enantioselective separation of the novel antidepressant mirtazapine and its main metabolites by CEC

In this work, the simultaneous enantioseparation of the second-generation antidepressant drug mirtazapine and its main metabolites 8-hydroxymirtazapine and *N*-desmethylmirtazapine by chiral CEC is reported. The separation of all enantiomers under study was achieved employing a capillary column packed with a vancomycin-modified diol stationary phase. With the aim to optimize the separation of the three pairs of enantiomers in the same run, different experimental parameters were studied including the mobile phase composition (buffer concentration and pH, organic modifier type and ratio, and water content), stationary phase composition, and capillary temperature. A capillary column packed with vancomycin mixed with silica particles in the ratio (3:1) and a mobile phase composed of 100 mM ammonium acetate buffer (pH 6)/H_2O/MeOH/ACN (5:15:30:50, by vol.) allowed the complete enantioresolution of each pair of enantiomers but not the simultaneous separation of all the studied compounds. For this purpose, a packing bed composed of vancomycin-CSP only was tested and the baseline resolution of the three couples of enantiomers was achieved in a single run in less than 30 min, setting the applied voltage and temperature at 25 kV and 20°C, respectively. In order to show the potential applicability of the developed CEC method to biomedical analysis, a study concerning precision, sensitivity, and linearity was performed. The method was then applied to the separation of the enantiomers in a human urine sample spiked with the studied compounds after suitable SPE procedure with strong cation-exchange (SCX) cartridges.

Keywords:
CEC / Human urine / Metabolites / Mirtazapine / Solid-phase extraction
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1 Introduction

Over the last few decades, a great interest has arisen in the separation and quantitation of chiral drugs in pharmaceutical and biomedical fields. The stereochemistry of compounds with respect to their interaction with biological matrices is generally recognized in terms of enantiomers that differ in their pharmacodynamic and pharmacokinetic properties resulting in different pharmacological and toxicological activities [1, 2]. Despite this fact many drugs are nowadays marketed as racemic mixtures. For this reason the pharmaceutical regulatory authorities such as the US Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA) require the synthesis and use of drugs as single enantiomers [3] which should provide better therapeutic results and fewer toxic effects. Thus sensitive, efficient, accurate, and fast analytical methods are needed to determine chiral drugs in pharmaceutical preparations and biological matrices and to control the stereochemical purity of the compounds which is of critical importance in chiral drug synthesis and development. Moreover, it is of utmost importance in the determination and monitoring of the drug metabolites for therapy optimization.

Diverse separation techniques including HPLC and CE have been employed for the enantioselective determination of drugs and their metabolites. HPLC is currently the method mostly used for enantiomeric analysis by means of chiral stationary phases (CSPs) or chiral additives in the mobile

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Abbreviations: **CSP**, chiral stationary phase; **DMR**, *N*-desmethylmirtazapine; **8-OH-M**, 8-hydroxymirtazapine; **MRT**, mirtazapine

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phase [4]. Regardless of the robustness of the chromatographic system, different drawbacks concerning the high cost of the chiral columns and the use of large amounts of organic solvents are of certain importance. On the other hand, CE has become a powerful technique especially for the analytical enantioseparations. Its high separation power and flexibility obtained in short analysis time, as well as the use of very small amounts of samples, which is a fundamental need in the analysis of biological fluids [5–7], have been the main factors of this success.

Since Jorgenson and Lukacs [8] demonstrated the potential of CEC, this relatively new technique has become an attractive method combining the best properties of high speed and efficiency of CE with high selectivity and the increased sample loading of HPLC. The advantages arise from the electroosmotic transport of the mobile phase through the column which allows the reduction of plate heights due to the plug-like flow, the use of smaller particles, and longer columns. In accordance with the features mentioned above, CEC has received considerable attention in the separation of enantiomers, and has evolved as a powerful technique in the analysis of chiral and nonchiral pharmaceutical compounds [9–12].

Enantioseparations have been performed employing open-tubular [13], packed [14], or monolithic columns [15]. Most of the applications of CEC in chiral separations have been carried out with packed columns using CSPs including silica gel-modified with CD derivatives, Pirkle-type phases, ion exchangers, proteins, cellulose derivatives, and macrocyclic antibiotics [16–25]. Although it has been demonstrated that CEC can be successfully applied to the analysis of enantiomers, only a few applications are reported in the literature concerning the determination of chiral compounds in pharmaceutical preparations and biological fluids [26–28].

Mirtazapine (1,2,3,4,10,14b-hexahydro-2-methylpyrazino[2,1-a]-pyrido[2,3-c][2-benzazepine], MRT) is a recent second-generation antidepressant drug belonging to the class of noradrenergic and specific serotonergic antidepressants (NaSSAs). Its mechanism of action involves the release of serotonin and norepinephrine due to the antagonism of autoreceptors and α_2 adrenergic heteroreceptors and due to the blockade of 5-HT_2 and 5-HT_3 receptors, allowing good efficacy in the treatment of patients who are nonresponder to other second-generation antidepressants [29]. MRT is employed against anxiety [30] and post-traumatic disorder [31]. Recently, one study has even suggested that MRT could be used in movement impairment, such as Parkinson's disease or acatisia caused by neuroleptic drugs [32]. MRT is biotransformed in the liver by action of Cytochrome CYP450 enzymes and the main metabolites formed are *N*-desmethylmirtazapine (DMR) and 8-hydroxymirtazapine (8-OH-M). DMR possesses pharmacological activity whereas the activity of 8-OH-M has not yet been elucidated.

MRT is a chiral drug administered as a racemic mixture even though the two enantiomers exhibit different pharmacological activities. The S(+) form is the more potent α-adrenoceptor antagonist of the two enantiomers [33]. Furthermore, the S(+) and R(−) forms tend to be metabolized by different CYP 450 isozymes. For this reason the chiral determination of MRT and its metabolites is important in pharmacokinetic and pharmacodynamic studies and to evaluate the clinical response of patients during therapeutic treatment.

Only a few HPLC methods have been reported in the literature concerning the enantiomeric separation of MRT as the parent drug [34, 35] or simultaneously with the metabolites [36]. A chiral CE method was optimized in order to determine MRT and DMR in human plasma [37]. Recently, a nano-LC method coupled with spectrophotometric and mass spectrometric detection has also been developed by our group for the chiral separation of MRT and its metabolites in a single run [38].

In the present paper, a chiral CEC method based on the use of the glycopeptide antibiotic vancomycin is proposed for the simultaneous separation of MRT and its main metabolites. To the best of our knowledge, no chiral separation of these analytes by CEC has been reported so far. Several experimental conditions were studied with the aim of optimizing a rapid and highly efficient CEC method of the selected compounds in biological samples demonstrating the real applicability of this powerful technique in the fields of pharmaceutical and biomedical analysis.

2 Materials and methods

2.1 Chemicals

Ammonia solution (30%) and acetic acid were purchased from Carlo Erba (Milan, Italy). HPLC-grade ACN, methanol, *n*-propanol, and 2-propanol were from Carlo Erba. Distilled water was deionized by using the Milli-Q system (Millipore, Bredford, MA, USA). MRT, DMR, and 8-OH-M pure compounds were kindly provided by Organon (Oss, The Netherlands). Lichrospher 100RP_{18} (d_p 5 μm) and Lichrospher diol silica (d_p 5 μm) were from Merck (Darmstadt, Germany).

Stock standard solutions were prepared by dissolving each drug at a concentration of 1 mg/mL in methanol and stored at −18°C. Further dilutions were daily prepared with mobile phase to obtain a final concentration of 10 μg/mL for MRT, 8-OH-M, and 20 μg/mL for DMR.

2.2 Apparatus

The CEC experiments were carried out on an Agilent Technologies Capillary Electrophoresis System (Agilent Technologies, Waldbronn, Germany) equipped with a diode array UV detector and an external nitrogen pump. The separations were performed on fused-silica capillaries, 75 μm id, 375 μm od (Composite Metal Services, Hallow, Worcester., UK) packed with vancomycin stationary phase. Both ends of the capillary were pressurized at 10 bar during runs and the air

thermostated at 20°C. The analysis was performed in positive polarity mode and the applied voltage was 25 kV. The analytes were detected at 200 nm. Samples were injected by pressure applying 10 bar × 0.5 min followed by a plug of mobile phase at 10 bar × 0.2 min.

2.3 Preparation of packed capillary column

CSP containing vancomycin as chiral selector was synthesized according to the method reported in ref. [23] by chemically bonding the antibiotic with silica diol particles. The capillary columns were packed in our laboratory following the procedure consisting of three steps used previously [39].

Briefly, one end of the capillary was connected to a mechanical temporary frit to retain the packing material and the other end to a stainless-steel HPLC precolumn which was used as reservoir for the slurry. The suspension was pumped into the capillary by an LC pump. The slurry was prepared by adding a few milligrams of stationary phase to 1 mL of acetone. Firstly the capillary was packed with Lichrospher 100 RP_{18} for about 10 cm. The column was flushed with distilled water for 30 min to remove the packing solvent from the column. The frit was prepared sintering the C_{18} particles by using a heating coil for 6 s at 1100°C. The capillary was cut close to the end of the frit, connected to the pump, and flushed with water in order to eliminate the excess of reversed stationary phase.

The capillary was connected to the precolumn from the empty side and filled with vancomycin-CSP/silica in the ratio 3:1 w/w or vancomycin-CSP only suspended in acetone for 23 cm. Finally, the capillary was packed again with C_{18} particles for several centimeters and flushed with water to prepare the end frit as previously described.

The detection window was prepared by removing the polyimide layer with a razor. The effective and total lengths of the capillary were 24.5 and 33 cm, respectively.

2.4 Sample pretreatment: SPE procedure

For the SPE procedure, two different cartridges namely Oasis HLB (hydrophilic–lipophilic balance) from Waters (Milford, MA, USA) and SCX Supelclean from Supelco (Bellefonte, PA, USA) were tested. The SCX Supelclean (500 mg, 3 mL) has been the most suitable SPE system for the extraction of MRT and its metabolites in human urine samples.

The analytes under study were extracted according to the method described in ref. [40] with minor modifications. Urine sample was spiked with a mixture of MRT and metabolites in order to obtain a final concentration of 10 μg/mL for MRT and 8-OH-M and 20 μg/mL for DMR. Spiked urine (1 mL) was added to 100 μL of 100 mM phosphoric acid. The cartridge was activated with 3 mL of methanol, conditioned with 3 mL of double distilled water and 3 mL of phosphoric acid (10 mM). The sample was loaded onto the cartridge at 1 mL/min and the cartridge was left to dry for 5 min. The cartridge was then rinsed with phosphoric acid (10 mM, 2 mL), acetic acid (100 mM, 1 mL), and methanol (2 mL).

The analytes were eluted with 4 mL of methanol containing 3% v/v of ammonium hydroxide (1 M). The eluate was evaporated to dryness under nitrogen and the residue was dissolved in 500 μL of methanol and stored at −18°C. During the experiments the urine sample was centrifuged and injected into the electrochromatographic system.

3 Results and discussion

3.1 Optimization of electrochromatographic conditions

The analytes under study possess very similar chemical structures (see Fig. 1) differing each other for an hydroxyl or methyl group. Therefore, the use of a chiral selector which exhibits a high enantiodiscrimination capability for structurally related compounds is needed.

Taking into account the great enantiomeric resolution properties of vancomycin-CSP in CEC towards different basic compounds demonstrated by Fanali and co-workers [23], we were encouraged to employ the macrocyclic antibiotic vancomycin as chiral selector for the enantiomeric resolution of MRT and its metabolites. Vancomycin is characterized by several asymmetric centers and functional groups responsible for stereoselective interactions based on electrostatic, dipole–dipole, π–π, inclusion complexation, hydrophobic interactions, and hydrogen bonds.

Preliminary electrochromatographic experiments were performed by using a capillary column packed for 23 cm with vancomycin-CSP mixed with silica particles in the ratio 3:1 w/w.

A mobile phase composed of 20% of aqueous ammonium acetate buffer pH 6 and 80% of organic modifier containing ACN and methanol was selected to carry out initial CEC runs.

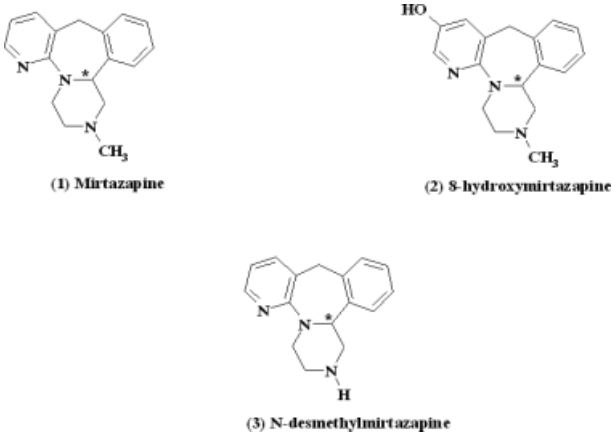

Figure 1. Chemical structures of the racemic analytes. (1) MRT, (2) 8-OH-M, and (3) DMR. (Asterisks mark the chiral center).

3.1.1 Influence of organic modifier concentration

Among the mobile phase parameters, the organic modifier content is an important factor because it affects the enantioresolution of the studied compounds. By maintaining a constant concentration of the ammonium acetate buffer at 5 mM, the MeOH/ACN ratio was modified increasing the content of methanol in the range of 20–50%. As expected, a modification of the ratio of the organic solvents caused alterations of the flow velocity due to the variation of the medium viscosity and the dielectric constant. Since the viscosity of methanol is higher than that of ACN, an increase in the content of methanol provided a decrease in the EOF and thus longer analysis times for all the studied compounds without considerably influencing the retention factor of the analytes (results not shown). The increase in methanol content in the mobile phase can influence the analyte interaction with the CSP *via* a competing or promoter effect. Figure 2 shows the effect of the increase in methanol concentration on the enantioresolution R_s of the studied compounds. As can be observed, a general enhancement of R_s factor for all the three racemic compounds was obtained. However, the increase in R_s was less relevant in the case of DMR, the most polar compound, which exhibits a higher affinity for methanol due to the presence of an NH– group on its chemical structure that may be responsible for hydrogen bonds and dipole–dipole interactions with the organic solvent. The highest values of R_s were observed with 50% MeOH, however with this eluent composition too long retention times were obtained. In order to improve peak efficiency and to speed the analyses, an MeOH/ACN mixture of 30:50 was selected for further experiments.

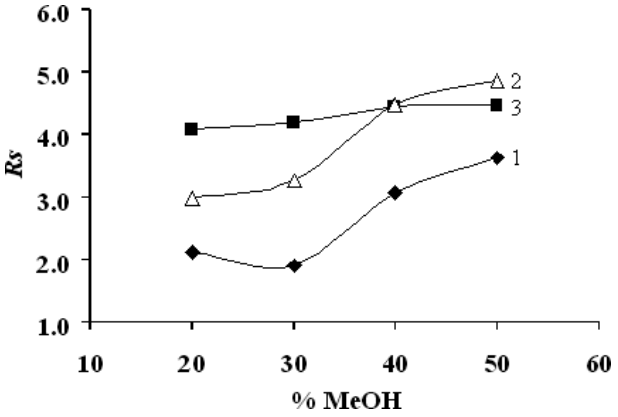

Figure 2. Dependence of the enantioresolution factor R_s of the studied analytes on the methanol content in the mobile phase. Capillary column, 75 μm id, 33 cm total length, 23 cm packed length, 24.5 cm effective length; stationary phase, vancomycin-CSP-silica (3:1 w/w); mobile phase, 20% aqueous ammonium acetate buffer (pH 6.0) and 80% of organic modifier containing MeOH/ACN v/v at different ratios; applied voltage, 25 kV; capillary temperature, 20°C; pressurized column at both ends with 10 bar; injection by pressure at 10 bar, 0.5 min, followed by a plug of mobile phase at 10 bar, 0.2 min. (1) MRT, (2) 8-OH-M, and (3) DMR.

3.1.2 Influence of the pH and ionic strength of the buffer in the mobile phase

In order to optimize the enantiomeric separation of the analytes under study, the effect of buffer pH on resolution and retention factors was studied in the range between 4 and 6. Due to the zwitterionic nature of vancomycin, a change in the pH buffer can modify its charge affecting the enantioresolution and selectivity of the chiral selector. Besides, a change in the pH can also modify the charge of the analytes, as well as their electrophoretic mobilities. Therefore, the buffer pH plays a key role in the electrostatic interactions involved in the resolution mechanism.

Variation of R_s in function of buffer pH appears to depend on the protonation of the analytes which are positively charged (the pK_a values were 7.40, 7.41, and 8.26 for MRT, 8-OH-M, and DMR, respectively) in the pH range studied and on the dissociation of the carboxylic group present in the chemical structure of the vancomycin. By increasing the pH a general increase in the retention factors k and R_s was observed for all the analyzed compounds reaching a maximum value at pH 6 due to the highest electrostatic interaction analyte–chiral selector obtained (data not shown). At lower pH values only a partial enantioresolution of the analytes was achieved, except for DMR which is more positively charged (absence of the methyl group) than the other analytes.

The effect related to variations of the ionic strength of the mobile phase was also considered. Retention factors of MRT and its metabolites were determined at different ionic strengths of the ammonium acetate buffer (pH 6), maintaining constant the percentage of the organic modifier (80%). EOF, which has an inverse relationship to the square root of the buffer concentration, decreased as the ionic strength increased with a reduction in the retention times and retention factors of analytes (results shown in Fig. 3a). The dependence of the resolution R_s for the chiral drugs in function of the ionic strength was also investigated. As can be observed in Fig. 3b, R_s factor decreased by raising the buffer ionic strength because of the effect of the cation which competes with the analytes for the chargeable sites of the vancomycin. Among the buffer concentration studied, a mobile phase with an ionic strength of 5 mM provided the best results in terms of analyte enantioseparation.

3.1.3 Effect of variation of water content

The study was performed by using a mobile phase composed of a constant concentration of methanol (30%) and modifying the ratio ACN/H_2O. Figure 4 shows the influence of the variation of the water content on the R_s factor of the racemic compounds. MRT and DMR were best resolved with 15% of water whereas a less value of R_s was achieved with 8-OH-M because of the presence of the OH-group on its chemical structure which exhibited a higher affinity for the mobile phase. Higher amounts of water (20%) in the mobile phase

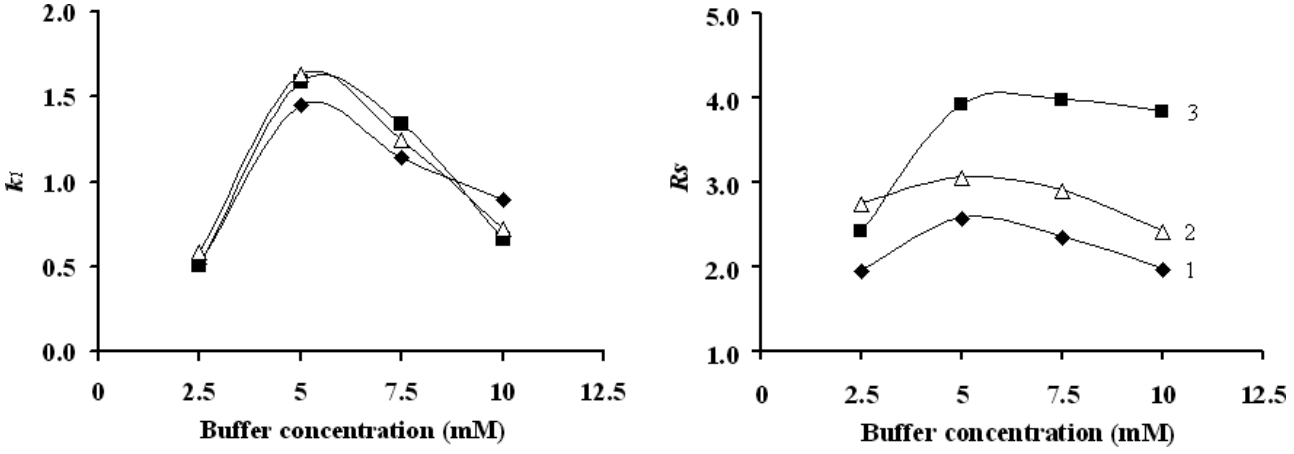

Figure 3. Influence of the ionic strength of the buffer on (a) retention factor k_1 (first-eluting enantiomer) and (b) enantioresolution factor R_s of the studied compounds. Mobile phase, 100 mM ammonium acetate (pH 6)/H_2O/MeOH/ACN (var.:var.:30:50, by vol.). Other experimental conditions as in Fig. 2. (1) MRT, (2) 8-OH-M, and (3) DMR.

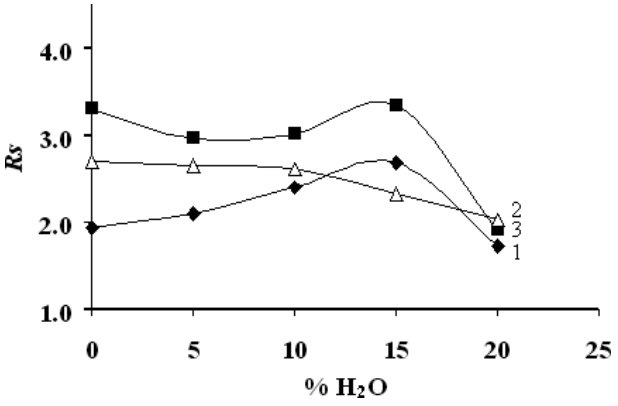

Figure 4. Effect of the water content in the mobile phase on the R_s factor of the analytes under study. Mobile phase, 100 mM ammonium acetate buffer (pH 6)/H_2O/MeOH/ACN (5:var.:30:var., by vol.). Other experimental conditions as in Fig. 2. (1) MRT, (2) 8-OH-M, and (3) DMR.

induced a general decrease in R_s for all analytes owing to the high polarity of water which has a competitor effect with the analytes for the vancomycin chiral sites. A water content of 15% was selected because it provided the highest enatioresolution of the analytes.

3.1.4 Effect of organic solvent type

Taking into account that the organic modifier in the mobile phase strongly affects the enantiocapability of the vancomycin and the viscosity of the mobile phase, the influence of different types of organic modifiers on the enantioresolution and on the retention factors k of the analytes was also investigated. By keeping the buffer, ACN, and water content constant (5:15:50, by vol.), different organic solvents as ethanol, *n*-propanol, and isopropanol were added (30%) to the mobile phase. It was observed that the retention times and the retention factors k increased with the less polar solvents. The highest values of R_s were achieved by using *n*-propanol with longer analysis time (Fig. 5). Methanol was then selected as the best organic alcohol which enabled the best enantioseparation of the analytes in shorter analysis time with a high separation efficiency.

3.1.5 Effect of applied voltage and capillary temperature

Having concluded that the mobile phase composed of 100 mM ammonium acetate (pH 6)/H_2O/MeOH/ACN (5:15:30:50, by vol.) was the most suitable combination for

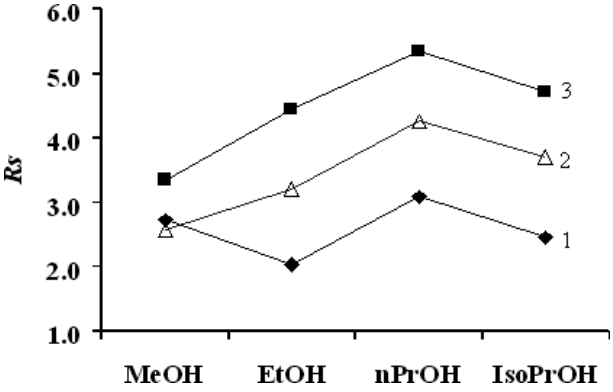

Figure 5. Effect of organic modifier type on the enantioresolution factor R_s of the studied analytes. Mobile phase, 100 mM ammonium acetate buffer (pH 6)/H_2O/ACN (5:15:50, by vol.) and 30% of different organic solvents. All other conditions were described in Fig. 2. (1) MRT, (2) 8-OH-M, and (3) DMR.

the complete resolution of the three couples of enantiomers, further experiments were performed to examine the effect of the applied voltage with the aim to minimize the separation time. When the applied voltage was varied in the range of 15–30 kV, a slight improvement of the peak efficiency and a decrease in the retention times were observed as the potential increased (results not shown). A voltage of 25 kV was then selected in order to reduce the analysis time without compromising the resolution.

Temperature was the final parameter evaluated in the optimization of this CEC method. Its effect on the enantioseparation of the analytes was studied over the range of 15–30°C. An increase in R_s factor was observed by increasing the temperature from 15 to 20°C. At higher temperatures, as expected, a general decrease in the retention times of the analytes was noticed with a simultaneous reduction in R_s factor. Based on these experiments, a run temperature of 20°C was chosen for the CEC developed method.

3.1.6 Effect of stationary phase

The optimization of all these parameters allowed the complete resolution of the chiral compounds, but not the simultaneous separation of all analytes (electrochromatogram shown in Fig. 6a). Thus in order to separate the six enantiomers completely, the effect of a different composition of the stationary phase was tested. A capillary column packed with a stationary phase containing vancomycin-CSP only for 23 cm was used to analyze the mixture of the three couples of enantiomers. Changing the composition of the packed bed an improvement of the chiral resolution was recorded obtaining the simultaneous separation of all the enantiomers that is reported in Fig. 6b. Satisfactory results were achieved, however a longer analysis time was observed due both to the higher amount of vancomycin and to the slight decrease in the EOF.

3.2 Method validation

Quantitative analysis is an important part of pharmaceutical and biological applications. Regarding this aspect the CEC optimized method was tested in terms of repeatability and reproducibility of the column. In Table 1, the intra- and interday repeatability data and the reproducibility of the column in terms of retention times and peak areas (RSD%) are reported. Intraday repeatability was determined for each enantiomer by assaying a standard mixture of MRT and its metabolites (the analytes concentrations are reported in Section 2.1) for six consecutive runs. Interday repeatability was assessed by analyzing the same mixture over five days.

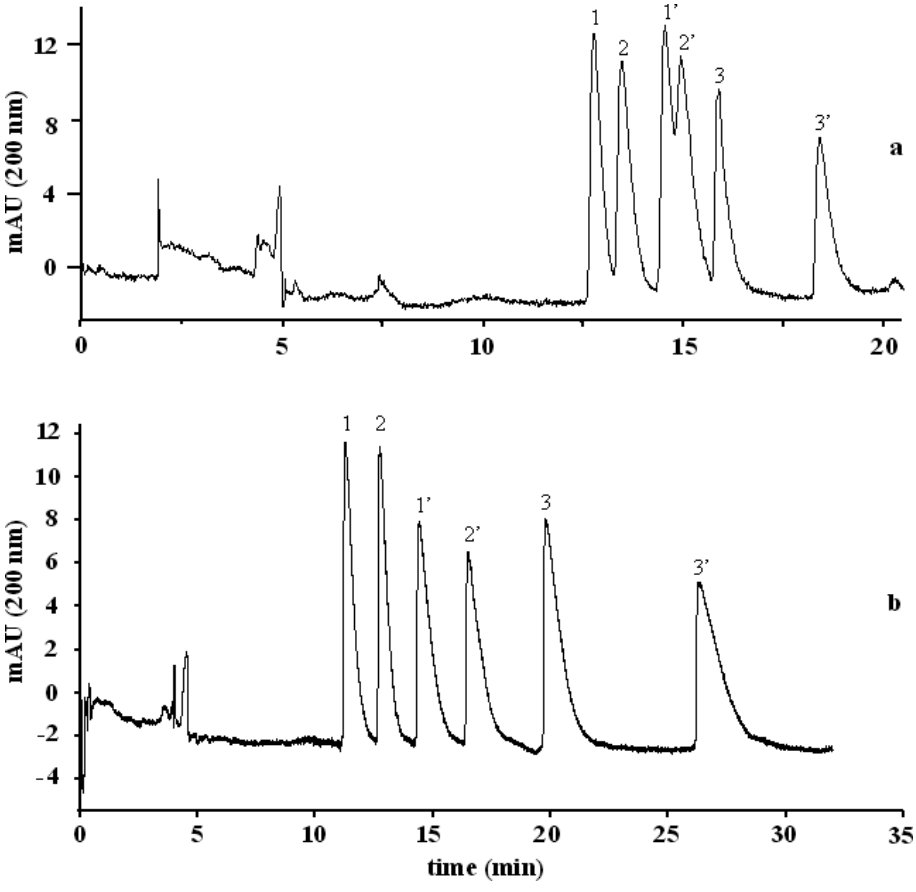

Figure 6. Comparison of the chiral CEC separations of MRT and its metabolites obtained by using different stationary phase compositions: (a) vancomycin-CSP silica (3:1 w/w) and (b) vancomycin-CSP. Compounds: (1) MRT_1, (1′) MRT_2; (2) 8-OH-M_1, (2′) 8-OH-M_2,; (3) DMR_1, (3′) DMR_2. Mobile phase, 100 mM ammonium acetate buffer (pH 6)/H_2O/MeOH/ACN (5:15:30:50, by vol.). For other CEC conditions, see Fig. 2.

Table 1. Precision data for retention times (t_R) and peak areas of the studied analytes

Analytes	Intraday repeatability (n = 6) RSD%		Interday repeatability (5 days) RSD%		Column-to-column reproducibility (n = 3) RSD%	
	t_R	Peak area	t_R	Peak area	t_R	Peak area
MRT_1	1.07	2.47	2.69	4.88	3.54	6.80
MRT_2	1.20	2.92	2.31	4.07	3.35	7.05
8-OH-M_1	1.26	2.41	2.61	3.88	3.80	6.78
8-OH-M_2	1.45	2.45	2.60	3.97	4.10	6.57
DMR_1	1.92	3.55	2.40	3.33	3.95	7.30
DMR_2	1.98	4.20	2.75	3.60	4.20	7.45
EOF	0.57	–	1.75	–	2.50	–

Electrochromatographic conditions: mobile phase, 100 mM ammonium acetate buffer (pH 6)/H_2O/MeOH/ACN (5:15:30:50, by vol.); stationary phase, vancomycin-CSP. Other conditions as in Fig. 2.

Besides in order to test column-to-column reproducibility, three chiral electrochromatographic columns were packed with the same procedure and tested with the mixture. The high RSD peak area values obtained were probably due to the laboratory-made packing procedure of the columns.

The LOD, S/N = 3 and LOQ, S/N = 10) were determined in order to investigate the sensitivity of the CEC method. The values of LOD and LOQ were 0.25 and 1 μg/mL, respectively for MRT and 8-OH-M; 0.5 and 2 μg/mL for DMR.

The method linearity was estimated plotting peak areas as a function of analyte concentration in μg/mL in the range of concentration between 0.5 and 10 μg/mL for MRT and 8-OH-M and in the range between 1 and 20 μg/mL for DMR. Standard solutions containing MRT, 8-OH-M, and DMR were prepared at six different concentration levels and were analyzed in duplicate. The regression equations as well as the relative squared correlation coefficients (r^2) for each enantiomer are reported in Table 2. As can be observed, good linearity was achieved over the studied concentration ranges. To the best of our knowledge, only two papers were found concerning the determination of MRT in urine samples in which the mean values were 0.62 and 1.31 μg/mL, respectively [41, 42]. These data were in accordance with the linearity range studied.

3.3 Analysis of biological samples

The application of the analytical method to a highly complex biological matrix such as human urine requires a reliable sample pretreatment to eliminate the interfering compounds.

In this work, the sample pretreatment was performed by using an SPE procedure which provides reproducible results with a good purification of the biological samples. Different kinds of sorbents were tested for the SPE procedure.

Oasis HLB (hydrophilic–lipophilic balance) cartridges from Waters were used according to the method developed from Mandrioli *et al.* [37] for the extraction of MRT and its metabolites in human plasma samples. When urine samples were analyzed, mean recovery values lower than 60% were achieved and thus a different sorbent (*i.e.*, SCX Supelclean) was used.

This cartridge was chosen as the most suitable SPE procedure in order to obtain better results. Figures 7a and b show the electrochromatograms of a blank urine sample without analyte spiking and a urine sample spiked with10 μg/mL of MRT and 8-OH-M, 20 μg/mL of DMR. As can be observed the enantiomers separation is comparable to that obtained with the standard mixture and the urine matrix does not interfere with the analyte peaks.

The developed procedure was tested for the recovery value at three different spiking levels (1–5–10 μg/mL for MRT and 8-OH-M; 2–10–20 μg/mL for DMR) in duplicate and measuring the average values for three repeated runs. Recoveries data were in the range of 85–88% for both MRT and 8-OH-M enantiomers and higher than 78% for DMR enantiomers.

4 Concluding remarks

In this paper, the enantiodiscrimination of MRT and its metabolites by chiral CEC using vancomycin as CSP was demonstrated. Optimization of the enantioseparation requires a careful investigation of different physicochemical parameters of the mobile phase, such as organic modifier concentration, buffer type and pH, and the composition of the stationary phase. These parameters influence the EOF driving force, as well as the analyte retention factors and the enantiorecognition mechanism. The optimized method provided the complete and simultaneous enantioseparation of the racemic analytes in an acceptable analysis time (less than 30 min). The method was then validated achieving good results in terms of repeatability, linearity, and recovery values.

Table 2. Method linearity data

Analytes	Concentration range (six calibration levels) (μg/mL)	Regression equation	SD for the slope Sa	SD for the intercept Sb	Correlation coefficient, r^2
MRT_1	0.5–10	$y = 1.8138x - 0.0644$	0.036	2.069	0.9983
MRT_2	0.5–10	$y = 1.7895x - 0.2562$	0.034	1.931	0.9985
8-OH-M_1	0.5–10	$y = 1.9029x - 0.3776$	0.042	2.360	0.9980
8-OH-M_2	0.5–10	$y = 1.8002x - 0.3925$	0.035	1.980	0.9985
DMR_1	1–20	$y = 1.5306x - 2.6054$	0.038	4.298	0.9975
DMR_2	1–20	$y = 1.5111x - 4.7431$	0.035	3.961	0.9978

Regression equation, $y = ax + b$.
Experimental conditions as in Table 1.

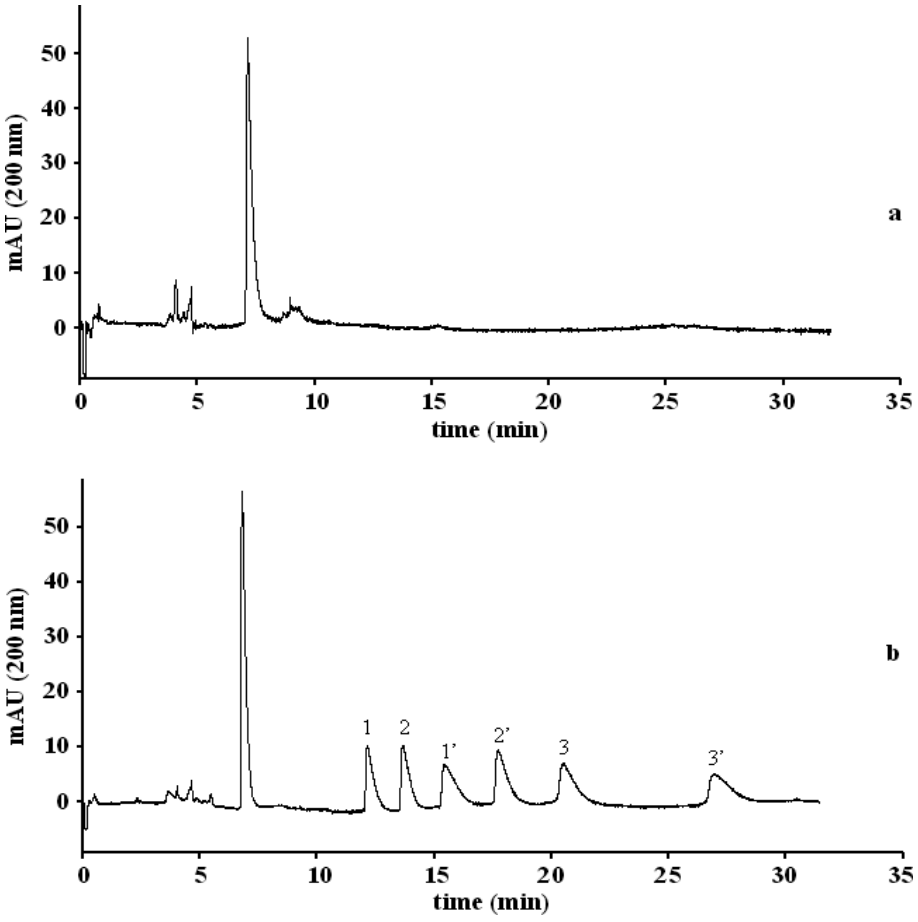

Figure 7. Electrochromatograms of (a) blank urine sample and (b) blank urine sample spiked with 10 μg/mL of racemic MRT and 8-OH-M and 20 μg/mL of racemic DMR. Both samples were subjected to the SPE procedure. Electrochromatographic conditions as in Fig. 6b.

The potential of the CEC method was shown performing the quantitative estimation of the antidepressant MRT and its metabolites in human urine samples spiked with the three racemic analytes, using a suitable SPE procedure for biological sample pretreatment. The satisfactory results obtained demonstrate the powerful applicability of this technique in the field of biological fluids analysis.

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