ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 863–870

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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# Analysis and enantioresolution of donepezil by capillary electrophoresis

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Received 16 May 2000; received in revised form 25 October 2000; accepted 27 October 2000

**Abstract**

The analysis of donepezil, a centrally acting acetylcholine esterase inhibitor, is described by a CZE method suitable for applications in pharmaceutical field. A rapid migration of the analyte was obtained under acidic conditions (pH 3.0); with detection wavelength of 320 nm a LOD of $0.8 \times 10^{-3}$ mg/ml was provided. Applications on real sample (pharmaceuticals) were carried out using two different instruments with comparable results in terms of reproducibility and accuracy. The use of chiral selectors in the running buffer allowed the enantioseparation of donepezil; charged cyclodextrins (carboxymethyl-β-cyclodextrin and sulfated-β-cyclodextrin) were suitable for the chiral resolution of the analyte. Interesting results were also obtained using human serum albumin. The protein-based CE enantioseparation was carried out at pH 7.4 avoiding the partial filling technique due to the good absorptivity of donepezil at 320 nm. Interestingly, the use of bicine as BGE provided a significative improvement in the enantioresolution compared to that obtained by phosphate buffer. 

*Keywords:* Capillary electrophoresis; Donepezil; Enantioresolution; Charged cyclodextrins; Human serum albumin

## 1. Introduction

Donepezil, (*R*, *S*)-1-benzyl-4-[(5,6-dimethoxy-1-indanon)2-yl] methylpiperidine hydrochloride (E2020) (see Fig. 1) is the second drug approved by the U.S. FDA for the treatment of mild to moderate Alzheimer's disease. Structurally, it belongs to a new class of acetylcholinesterase inhibitors having an *N*-benzylpiperidine and an indanone moiety [1,2].

A structural feature of the compound is the presence of a chiral center adjacent to a carbonyl group which makes each enantiomer easily racemizable via keto-enol intermediate [3–5]. The reported analytical techniques to obtain the enantioresolution of donepezil were based on the use of liquid chromatography (HPLC) with ovomucoid-bonded column [4] and avidin conjugated column [5–7]. Currently, enantiomers of donepezil are determined to establish their pharmacokinetics profiles; detection obtained coupling the chiral liquid chromatography with mass spectrometry allowed each enantiomer to be determined at plasma level. From these studies, after

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0731-7085/01/$ - see front matter 
PII: S0731-7085(00)00554-9

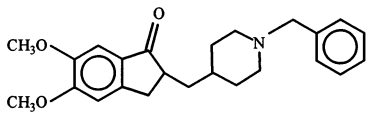

Fig. 1. Structure of Donepezil.

the drug administration, the mean human plasma levels of *S*-donepezil appear to be higher than those of *R*-donepezil [7].

Capillary electrophoresis (CE) offers the opportunity for useful alternative to HPLC separations, particularly regarding the analysis of compounds of pharmaceutical interest and a number of CE applications are nowadays available [8,9] including the enantioresolution of chiral drugs. The reported methods are based on the use of different types of chiral selectors, among which cyclodextrins are probably the most extensively used chiral additives in the background electrolyte [10]. Recently, however a number of papers have been focused on the use of proteins as chiral mobile phase modifiers. The several aspects of their use have been reviewed and successful results were obtained employing human serum albumin (HSA) [11–13] bovine serum albumin (BSA) [12–14] and $\alpha_1$-acid glycoprotein ($\alpha$-AGP) [12,13,15] in free solution or immobilized within the capillary.

Since CE methods for the determination of donepezil were not yet described, the present study was aimed to develop a rapid and reliable capillary electrophoretic system suitable for the analysis of donepezil hydrochloride in commercially available drugs. The enantioresolution of the drug was also considered using and comparing the performances of charged cyclodextrins and HSA as chiral selectors. The effects of chiral selectors concentration, the buffer pH and composition on the enantioresolution of donepezil were evaluated leading to a set of opportunity in the choice of the more suitable CE chiral system.

## 2. Experimental

### 2.1. Materials

Donepezil hydrochloride was a kind gift from Pfizer Italiana (Rome, Italy), promethazine hydrochloride used as internal standard was kindly supplied from Carlo Erba Reagenti (Milan, Italy). β-cyclodextrin, γ-cyclodextrin, hydroxypropyl-β-cyclodextrin (HPCD), Carboxymethyl-β-cyclodextrin (CMCD), citric acid and *N*,*N*-bis-(2-hydroxyethyl)glycine (bicine) were from Fluka (Buchs, Switzerland). Sulfated-β-cyclodextrin, dimethyl-β-cyclodextrin (DMCD) and human serum albumin (essentially fatty acid free) were from Sigma-Aldrich (Milan, Italy). Phosphoric acid, sodium hydroxide, *Tris*(hydroxymethyl)-aminomethane (*Tris* base), sodium chloride, sodium phosphate, dimethylsulfoxide (marker of the electroosmotic flow) and all the other chemicals were from Carlo Erba Reagenti (Milan, Italy). Purified water Milli-RX (Millipore, Milford, MA) was used to prepare buffers and standard solutions.

### 2.2. Apparatus

All separations were carried out using a $^{3D}$CE Capillary Electrophoresis system (Hewlett-Packard, Waldbronn, Germany), equipped with a diode array detector. The data were collected on a PC using the $^{3D}$CE-ChemStation software ver. A 06.

Fused-silica capillaries (Hewlett-Packard) 48.5 cm in length (40 cm effective length) × 50 μm I.D. were used. The applied voltage was held constant at different values ranging within 10–30 kV and the detection wavelength was adjusted to 320 nm. All the electrophoretic runs were carried out at a constant temperature (15–45°C); the samples were introduced hydrodynamically for 10 s (injection pressure 5 kPa). Before the injection into the CE system, each solution (running buffer and sample solutions) was subjected to filtration through on membrane 0.2 μm GyroDisc (Orange Scientific, Waterloo, Belgium) with the exception of HSA solutions.

A Biofocus 2000 system (BioRad, Hercules, CA) for capillary electrophoresis was also used for the analysis of donepezil in pharmaceutical samples. Untreated fused-silica capillary tube of 50 cm total length (effective length 43.5 cm) × 50 μm I.D. was employed. The electrophoretic runs were carried out at 40°C and applying 25 kV; the

injections were made hydrodynamically at 20 psi × s. The detection wavelength was 320 nm.

### 2.3. Solutions

Quantitation of donepezil in pharmaceutical formulations was performed in a running buffer (BGE) solution of phosphate 25 mM, pH 3.0. In the chiral separations different BGE were used. A 25 mM citric acid solution was adjusted to the desired pH values (pH 3.0) with *Tris* base and the appropriate amount of CMCD (1–5 mM) was then dissolved to obtain chiral running buffers.

Phosphate buffer 20 mM pH 7.0 was used to dissolve SβCD (0.4–4.5 mM); finally a bicine solution (100 mM) was prepared dissolving the appropriate amount of the solid compound in the presence of 50 mM sodium chloride and adjusting the pH value to 7.4 with sodium hydroxide. HSA was then dissolved in the range 15–45 μM.

The samples injected for the chiral analysis were aqueous solutions of donepezil hydrochloride 0.03 mg/ml.

### 2.4. Calibration graph

Donepezil hydrochloride aqueous solutions in the concentration range of 0.02–0.07 mg/ml were prepared for the evaluation of the response linearity. Each final solution contained 0.12 mg/ml of promethazine hydrochloride as internal standard. The corrected peak area (area/migration time) ratios ($Y$) of the analyte to the internal standard were plotted against the corresponding analyte concentration ($C$) to obtain the calibration graph.

### 2.5. Analysis of pharmaceutical formulation

A powdered sample of drug formulation (tablet) equivalent to about 1.7 mg of donepezil hydrochloride was dispersed in a 5 ml mixture of 25 mM phosphate buffer pH 3.0 — water 1:2 (v/v); after stirring (3 min), 1 ml aliquot of the filtered (0.2 μm membrane) solution was added to 1 ml of internal standard solution (1.2 mg/ml) and diluted to 10 ml with water. The samples obtained were directly injected into the CE system.

## 3. Results and discussion

### 3.1. Quantitation of donepezil

In preliminary studies, the stability of donepezil was investigated in aqueous acidic and basic medium under the exposure to UVA and UVB radiations from a solar simulator equipped with a 150 W xenon arc lamp. Moreover, stock solutions (2.5 mg/ml) of donepezil in water were warmed to 70°C for at least 30 min. No evidence of degradation products were observed injecting the irradiated or warmed solutions into the CE system also using detection wavelength of 200 nm with high absorbance and signal-to-noise values.

#### 3.1.1. CZE conditions

According to the p$K_a$ value of donepezil (around 9) [5], protonation of the piperidinic nitrogen is obtained at pH values below 8.0. The running buffer was chosen in order to have a rapid analyte migration and adequate electroosmotic flow stability [16]; thus buffer pH ranging within 4–8 were avoided and a 50 mM phosphate running buffer (pH 3.0) was used to obtain a more stable solute mobility. Under these experimental conditions ($T = 40$°C; 20 kV) the measured EOF using the neutral marker dimethylsulfoxide was $8 \times 10^{-5}$ cm$^2$ s$^{-1}$ V$^{-1}$ and the cathodic migration of the analyte was obtained in very short time ($\approx 3$ min). Donepezil showed a good detectability for the presence in its UV spectrum of two absorbance maxima at wavelengths 270 and 320 nm and the detection sensitivity achieved using both of these signals was sufficient for quantitation of the analytes in commercially available drugs (tablets).

#### 3.1.2. Precision of the instrument

The interday precision of the CE assay was evaluated by comparing the closeness of the agreement between a series of 10 ($n = 10$) measurements of the donepezil corrected peak area (area/migration time) from a same drug standard solution. The RSD% was found to be 1.6% at 0.035 mg/ml level and 2.3% at 0.02 mg/ml level, respectively.

Although the precision of the determination was high enough, the introduction of an internal standard was considered to provide a compensation of the errors from injection and dilution to volume of the real samples [16,17]. Promethazine was chosen as suitable internal standard because of its stability in aqueous solution and for its good absorptivity at 320 nm. In the presence of the internal standard the precision calculated as the peak correct area of the sample against the peak correct area of the internal standard ratio was 1.1 (RSD%) at 0.035 mg/ml level.

### 3.1.3. Linearity

Linearity of the response was evaluated in the concentration range of 0.02–0.07 mg/ml; the linear regression analysis obtained plotting the corrected peak area (area/migration time) ratios ($Y$) of the analyte to the internal standard versus the concentration ($C$) showed excellent correlation coefficient ($r = 0.9998$) and the equation was: $Y = (-0.00561 \pm 0.05014) + (0.03968 \pm 0.00104) \quad C$, $n = 5$

### 3.1.4. Analysis of donepezil tablets

A commercially available formulation (tablets) containing donepezil hydrochloride (Aricept®) was analyzed. In the tablets the active ingredient is associated with excipients as lactose, maize starch, cellulose, hydroxypropyl cellulose and magnesium stearate. Other ingredients are present in the external coating: polyethylene glycol, hypromellose and titanium oxide. A powdered aliquot of the tablets was simply extracted using diluted pH 3.0 phosphate buffer; after stirring, the sample was filtered and injected in triplicate into the CE system after the addition of the internal standard solution; under the separation conditions no interferences were observed (Fig. 2). The peak correct area ratios (analyte to internal standard) were measured and the donepezil hydrochloride content in each sample was calculated by comparison with a standard donepezil hydrochloride solution (0.035 mg/ml).

### 3.1.5. Accuracy and precision

The accuracy, regarded as the closeness of agreement between the value accepted as conventional true value (claimed content of the tablets) and the found value was 99.3% (RSD% = 1.3; $n = 7$).

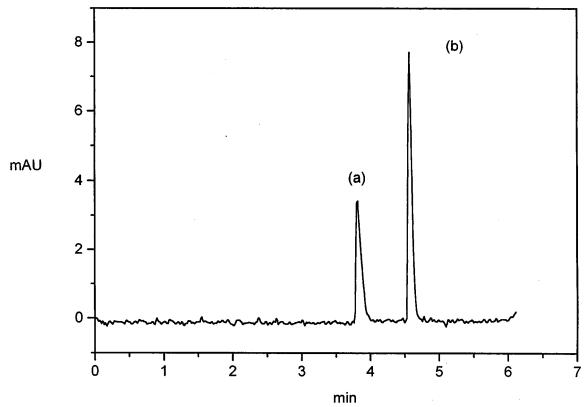


Fig. 2. Electropherogram of a donepezil commercial sample analysed under the following conditions: fused-silica capillary (48.5 cm total length); 25 mM phosphate buffer (pH 3.0); separation voltage 20 kV; temperature 40°C; hydrodynamic injection (50 mbar) for 10 s; detection at 320 nm. (a) promethazine (internal standard); (b) donepezil.

Applying the same procedure, the samples were subjected to analysis by a different operator and using a different apparatus (Bio Rad CE system). Under the chosen experimental conditions (described in the Experimental section), the accuracy resulted to be 100.8% with a RSD% 2.0 ($n = 5$). The results obtained using the two different instrumentations were compared by applying the $F$-test and $t$-test at the 95% confidence level; no significant differences were found.

The accuracy of the method was also verified by recovery studies analysing samples fortified by known quantities of the drug (20%) [18]; essentially quantitative recoveries were found (100.3%, RSD% = 0.8%; $n = 7$). In all these analyses, the precision of the whole method (extraction and CE assay), as expressed by the reported RSD% values, was found to be quite satisfactory.

### 3.1.6. Sensitivity

The limit of detections (LOD) of donepezil using both the instruments were evaluated by progressive dilution of pharmaceutical samples until $S/N = 3$ were reached. LOD measured at a wavelength of 320 nm using the instrument

equipped with a diode-array detector (Hewlett-Packard) with new deuterium lamp was found to be comparable to that achieved using a very long time operating lamp ($6 \times 10^{-3}$ mg/ml). The Bio Rad instrument equipped with a variable wavelength UV detector (with a brand new deuterium lamp) provided a LOD of $0.8 \times 10^{-3}$ mg/ml.

### 3.2. Chiral separation

Donepezil easily racemizes via keto-enol intermediate owing to the chiral center adjacent to a carbonyl group [3–7] and the commercially available dosage forms are containing the racemic drug. Nevertheless, a number of chiral liquid chromatographic systems have been described [4–7] for the importance to have stereoselective analytical methods able to provide the single enantiomer pharmacokinetic profile.

In the present work the attention was focused on the opportunity to obtain chiral resolution of donepezil using capillary electrophoretic approaches. First, as the LOD value of the proposed CE method can be considered quite good especially using UV — 210 nm detection ($0.2 \times 10^{-3}$ mg/ml), the method could be susceptible of applications to biological samples (plasma) after appropriate sample preparation involving a concentration step. Moreover fast and simple enantioresolution methods are requested in view of further investigation on enantioselective human serum albumin (HSA) binding of donepezil by frontal analysis (FA) studies [19–21]. For this purpose, enantioselectivity of cyclodextrins towards donepezil was evaluated in order to obtain chiral capillary electrophoretic conditions suitable to determine the single enantiomer concentration.

#### 3.2.1. Cyclodextrins as chiral selectors

With the aim to develop a fast electrophoretic chiral system, both neutral (β-cyclodextrin, dimethyl-β-cyclodextrin (DMCD), hydroxypropyl-β-cyclodextrin (HPCD) and γ-cyclodextrin) and charged (carboxymethyl-β-cyclodextrin CMCD and sulfated-β-cyclodextrin SβCD) cyclodextrins were used as chiral BGE additives. Among the neutral cyclodextrins only DMCD provided a partial chiral separation whereas both the charged cyclodextrins available, under acidic conditions offered opportunity for complete enantioresolution. Actually, negatively charged cyclodextrins are expected to give the best resolving power when the analytes are positively charged, due to the opposite electrophoretic mobility of the chiral additive respect to the analyte and to strong electrostatic forces which can be involved between the chiral selector and the selectand [22–24].

*3.2.1.1. Carboxymethyl-β-cyclodextrin.* Using chargeable cyclodextrins as chiral mobile phase additives, particular importance should be given to the BGE pH values. Under acidic conditions (pH 3.0, *Tris* base–citric buffer), the analyte is completely protonated at the piperidinic nitrogen and its rapid cathodic migration is observed. Increasing the CMCD concentration over the 1–5 mM range, a progressive resolution increase was obtained (Fig. 3). When the CMCD was used at a concentration of 5 mM, the enantioresolution value was Rs = 2.7 (RSD 2.8%, $n = 5$). As shown, small concentration of the chiral selector allowed relatively high resolution values to be achieved. At the same time the high affinity of CMCD towards donepezil, led to obtain a poorely robust

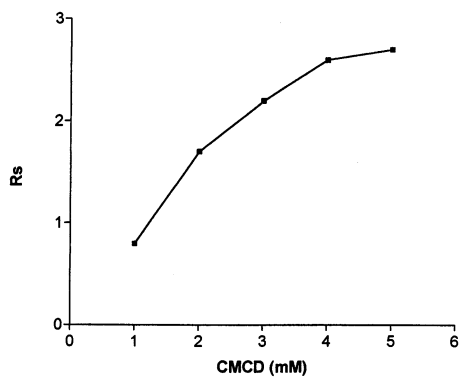


Fig. 3. Effect of carboxymethyl-β-cyclodextrin (CMCD) concentration on the enantioresolution of donepezil. Electrophoretic conditions: *Tris* base–citric acid (25 mM) running buffer (pH 3.0); temperature 15°C; voltage 30 kV; other conditions as in Fig. 2. The enantioresolution (Rs) values were the mean value of three replicate injections.

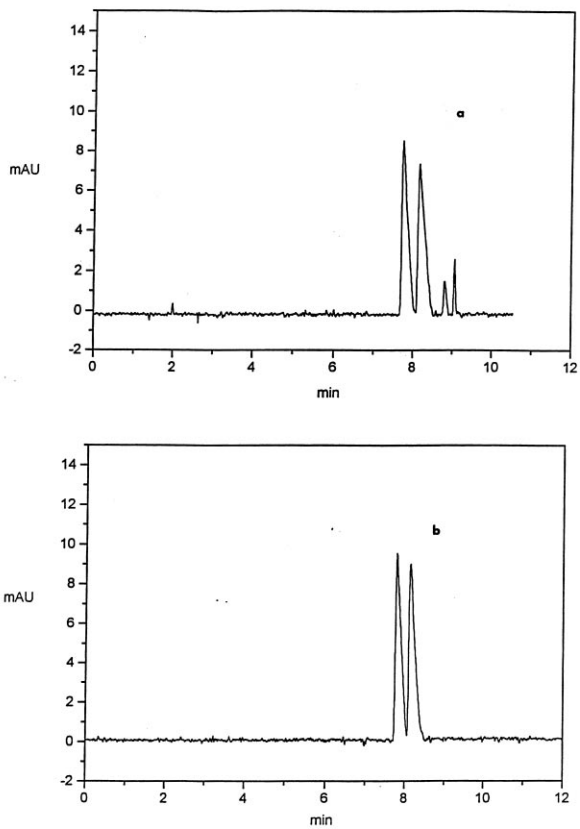


Fig. 4. Representative electropherogram of enantioresolution of donepezil using CMCD as chiral selector (3 mM). (a) hydrodynamic injection of sample: 10 s; (b) hydrodynamic injection of: methanol (5 s), sample (10 s) and methanol (5 s). Other conditions as in Fig. 3.

chiral method. Moreover starting from a concentration of 2 mM CMCD a couple of small peaks were observed immediately after the enantiomers peaks of the analyte. The recorded on-line spectra of these additional peaks were found to perfectly fit with the analyte peak spectrum. Since preliminarly the donepezil purity and stability were evaluated, these additional peaks were considered as a result of anomalous sampling as previously reported and referred as 'emersion peaks' [25]. In fact, using a sample zone comprised between two small plugs of an organic solvent (methanol) resulted in the removal of the emersion peaks phenomena (Fig. 4).

*3.2.1.2. Sulfated-β-cyclodextrin (SβCD).* The employed cyclodextrin is described by the manufacturer as a mixture corresponding to an average substitution degree of 2/3 of the available hydroxy sites (substitution degree of 0.67). The anionic character allowed to work at neutral or weakly acidic pH values. Using a phosphate buffer (pH 7.0), the effect of the SβCD concentration on the enantioresolution of donepezil was investigated within the 0.4–4.5 mM range of selector. Moreover, the effect of the running buffer pH value on the enantioresolution was performed in the pH range of 4.5–7.5 and the best results were obtained at pH 7.0.

In order to obtain good migration time reproducibility (RSD 1.2%, $n = 5$), the capillary was rinsed with the running buffer (8 min) between the electrophoretic runs. As is shown in Fig. 5, a good enantioresolution value was obtained already at 1.7 mM concentration of SβCD; further, small differences in the resolution were observed in the range 0.2–4.5 mM of chiral selector. Regarding the concentration effect of the cyclodextrins, SβCD provided a more robust system than CMCD. In Fig. 6 a representative electropherogram of the chiral separation of donepezil using SβCD (4.5 mM, pH 7.0) is shown; the obtained

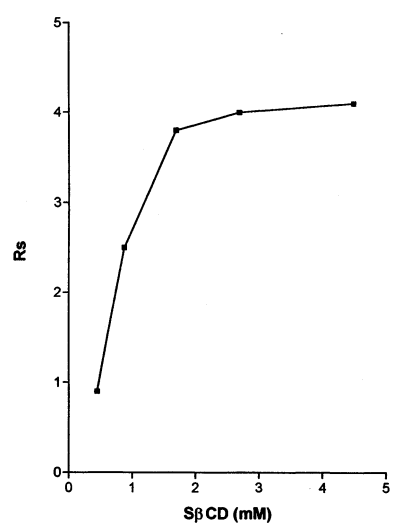


Fig. 5. Effect of sulfated-β-cyclodextrin (SβCD) concentration on the enantioresolution of donepezil. Electrophoretic conditions: 20 mM phosphate running buffer (pH 7.0); other conditions as in Fig. 3.

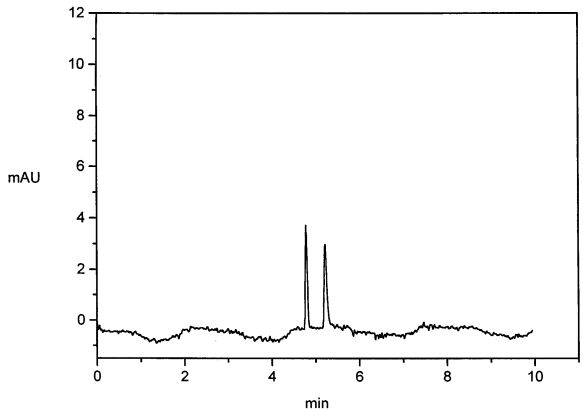


Fig. 6. Representative electropherogram of enantioresolution of donepezil using SβCD as chiral selector (4.5 mM). Other conditions as in Fig. 5.

enantioresolution value was Rs = 4.1 (RSD 2.2%, $n = 5$) and as it can be seen, the 'emersion peaks' phenomenon was not observed.

### 3.2.2. Human serum albumin (HSA) as chiral selector

The evaluation of the enantioselectivity of HSA towards donepezil was performed with the aim to offer further chiral CE methods of analysis for the studied drug. Moreover, the discovery of enantioselective interactions between donepezil and HSA makes interesting to carry out quantitative investigations of such interactions by affinity capillary electrophoresis (ACE) [19,26,27].

For these purposes, the potentiality of HSA as chiral selector in the enantioresolution of donepezil was tried dissolving the protein in the running buffer (affinity CE mode) [12,13]. Some favourable conditions simplified the use of HSA as chiral additive: first, the relatively high molar absorptivity of the analyte at 320 nm, where the absorption of HSA is negligible, allowed to avoid the 'partial filling' technique [12,13,28]; moreover the p$K_a$ value of donepezil (about nine) allows to work under condition of pH even higher than seven. Relatively to the pH conditions, in neutral or near-neutral running buffer, HSA is strongly negative (p$I \approx 4.7$) [12] establishing the best environment for the separation of cationic racemates.

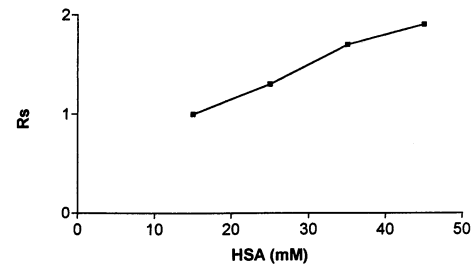


Fig. 7. Effect of human serum albumin (HSA) concentration on the enantioresolution of donepezil. Electrophoretic conditions: bicine (100 mM) with sodium chloride (50 mM) running buffer (pH 7.4); voltage 15 kV; temperature 35°C; other conditions as in Fig. 3.

Working at pH value of 7.4, a 50 mM phosphate running buffer was used to dissolve HSA at 50 μM concentration; under these conditions only a partial enantioresolution was obtained. Interestingly, a significant improvement of the enantioresolution was obtained employing *N*,*N*-bis(2-hydroxethyl)glycine (bicine) 100 mM as a BGE; in particular, at a pH value 7.4 with 50 mM sodium chloride, the chiral resolution of donepezil was obtained using HSA for concentration higher than 10 μM. The effect of the HSA concentration on the enantioresolution was investigated in the range 15–45 μM (Fig. 7); the baseline resolution of the two enantiomers was provided at 35 μM concentration (Fig. 8). Under the described conditions, good repeatability of the

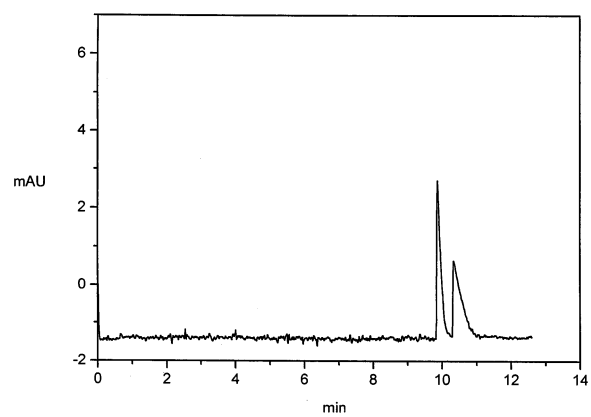


Fig. 8. Representative electropherogram of enantioresolution of donepezil using HSA as chiral selector (35 μM); other conditions as in Fig. 7.

migration time (RSD 0.85%, $n = 5$) and resolution (RSD% 2.5, $n = 5$) were obtained applying a rinsing cycle of the fused-silica capillary: sodium hydroxyde 0.1 M (2 min), water (3 min) and finally a brief conditioning with the running buffer (2 min).

## 4. Conclusion

Capillary electrophoresis has been proposed as a good alternative technique for the analysis of donepezil and a simply CZE method has been developed for the application to the quality control of a pharmaceutical formulation.

As the enantioseparation of the analyte has been previously considered a very important feature of pharmacokinetics methods, in the present paper different CE approaches to the chiral separation of donepezil were investigated. Using both negatively charged cyclodextrins and human serum albumin, successful results were reached. The proposed methods could find applications in the analysis of biological matrix after an appropriate sample preparation and concentration. Moreover, in view of the interesting behaviour of HSA towards donepezil, further studies will be addressed to the evaluation of useful displacers for a deepened knowledge of the enantioselective HSA-donepezil binding and interaction.

## Acknowledgements

Thanks are due to Barbara Mattei for her valuable technical assistance. This work was supported by a grant from MURST (Cofin. '98), Rome (Italy).

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