Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1081 (2005) 31–35
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Enantioselective separation of rivastigmine by capillary electrophoresis with cyclodextrines

Silvia E. Lucangioli [a], Valeria Tripodi [a], Edmond Masrian [a], Silvia L. Scioscia [a], Clyde N. Carducci [b], Ernst Kenndler [c,*]

[a] *Department of Analytical and Physicochemistry, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentina*
[b] *National Academy of Pharmacy and Biochemistry, Buenos Aires, Argentina*
[c] *Institute for Analytical Chemistry, University of Vienna, Währingerstraße 38, A 1090 Vienna, Austria*

Available online 2 March 2005

Abstract

Different β-cyclodextrines (β-cyclodextrin, heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin, hydroxypropyl-β-cyclodextrin, and sulfated β-cyclodextrin) were investigated as additives for the enantioselective separation of the *R*-form from rivastigmine ((*S*)-*N*-ethyl-3-[(1-dimethyl-amino) ethyl]-*N*-methyl-phenyl carbamate), contained as impurity in this drug, which is used for the treatment of Alzheimer's disease. Electrophoresis was performed in an acidic background electrolyte (triethanolammonium phosphate, 75 mM, pH 2.5) with various concentrations of the additives. The electrophoretic mobilities measured are typical functions of the additive concentrations, with complex constants (obtained by fitting the appropriate binding curve on the data) ranging between about 180 and 770 M^{-1}. Best separation was obtained with 7.5 mM β-cyclodextrin, with the *R*-enantiomer as impurity migrating before the main *S*-compound. Intra- and interday reproducibility ($n = 6$ and 18, respectively) of migration time and peak area was in the low percentage range, linearity of the calibration line for the quantitation of the impurity in the range between 2.3 and 50 μg/ml, expressed by the linear correlation coefficient, was 0.9998. The limits of detection and quantitation, respectively, were 0.7 and 2.3 μg/ml, corresponding to 0.05 and 0.15%, m/m of the *R*- relative to the *S*-compound. Analysis can be carried out at 18 °C in less than 19 min.

Keywords: Rivastigmine; Chiral analysis; Electromigration; Electrokinetic chromatography; Capillary electrophoresis

1. Introduction

Separation, characterization and quantitation of enantiomers have become a relevant area not only in the synthesis process of pharmaceuticals, but also in quality control of bulk drug substances and final products. Due to the fact that a significant difference in the pharmacological activity and/or toxicological side effects of enantiomeric forms in racemic drugs the accomplishment of international requirements on chiral impurity profile is mandatory [1–8].

Development of methods for chiral analysis by capillary electromigration methods (CEM) has been of special interest in last decade. CEM in its different modes has turned out being a powerful tool for the separation of enantiomers because high efficiency and resolution in short time of analysis are its relevant features. Such methods with pseudostationary phases based on micelles and microemulsions or on soluble additives like cyclodextrins to resolve chiral compounds have been reported [2–15].

Cyclodextrins (CDs) are the most widely used chiral selectors in capillary electromigration methods, and native or derivatised CDs with different functional groups have been introduced with the purpose of improving the enantiomeric resolution of racemic compounds. In the present work neutral (β-cyclodextrin, CD; heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin, heptakis; hydroxypropyl-β-cyclodextrin, HP CD) and charged (sulfated β-cyclodextrin, sulfated CD) CDs in acidic buffer were applied in order to quantify the R-impurity of rivastigmine. Rivastigmine (*S*)-*N*-ethyl-3-[(1-

* Corresponding author. Tel.: +43 1 4277 523 05; fax: +43 1 4277 9523.
E-mail address: ernst.kenndler@univie.ac.at (E. Kenndler).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.02.038

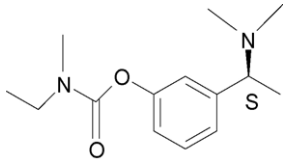

Fig. 1. Chemical structure of rivastigmine.

dimethyl-amino) ethyl]-*N*-methyl-phenyl carbamate (Fig. 1) is an acethylcholinesterase inhibitor of the carbamate type, approved for the treatment of Alzheimer's disease [16,17]. Very few papers dealt with the determination of rivastigmine and its metabolites by GC–MS and LC–MS in preclinical and clinical studies [18,19].

It is the goal of the present paper to evaluate the suitability of different CDs for chiral resolution of the enantiomers of rivastigmine, especially to quantify the *R*-form as impurity of the *S*-enantiomer. The evaluation is based on the mobility of the two enantiomers, and on the separation selectivity expressed by the ratio of the mobilities. Analysis of the impurity, which should allow its quantitation in pharmaceuticals at a 0.1% level, is characterised by the according figures of merit.

2. Experimental

2.1. Reagents

Rivastigmine hydrogentartrate (*S*)-*N*-ethyl-3-[(1-dimethyl-amino)ethyl]-*N*-methyl-phenylcarbamate hydrogentartrate) and its *R*-enantiomer was a gift of Raffo Laboratories (Argentina). β-cyclodextrin, heptakis (2,3,6-tri-*o*-methyl)-β-cyclodextrin and sulfated β-cyclodextrin were purchased from Sigma (St. Louis, MO, USA). Sulfated CD is not a single isomer, it has between 7 and 11 (average 9) sulfate groups per cyclodextrins molecule. Its average molecular mass is 1990. Hydroxypropyl-β-cyclodextrin was obtained from Beckman (Fullerton, CA, USA). Triethanolamine was purchased from Baker, Co. (Phillipsburg, NJ, USA). Eighty-five percent phosphoric acid and methanol (HPLC grade) were supplied by E. Merck (Darmstadt, Germany). Ultrapure water was obtained by an EASY pure^{TM} RF equipment (Barnstead, Dubuque, IA, USA). All solutions were filtered through a 0.45 μm nylon membrane (Micron Separations Inc., Westboro, MA, USA) and degassed before use.

2.2. Instrumentation

All CE separations were performed with a capillary ion analyzer (Waters Corp., Milford, MA, USA) and data were processed by an Empower Pro software (Waters). An uncoated fused-silica capillary of 60 cm length (53 cm to detector) and 50 μm i.d. (Waters) was employed.

2.3. Electrophoretic system

The separations were performed in 75 mM triethanolammonium phosphate buffer at pH 2.5 with varying concentrations of CD. The voltage applied was +25 kV, hydrostatic injection (10 cm height) was for 10s, resulting in an about 1.3 mm long injection zone with 2.6 nl volume. UV detection was set at 214 nm.

The capillary was rinsed at the beginning of each day with 0.1 M potassium hydroxide for 5 min, washed with water for 10 min and then with BGE for 20 min. Between runs, the capillary was conditioned with BGE during 3 min. At the end of the day, the capillary was flushed with 0.1 M potassium hydroxide for 5 min and finally with water for 10 min.

2.4. Stock and standard solutions

Stock solutions of rivastigmine hydrogentartrate and the *R*-impurity (containing 1.0 mg/ml each) were prepared in deionised water. Standard solutions of 30 μg/ml (75 μmol/l) of each compound were obtained by appropriate dilution with deionised water.

3. Results and discussion

3.1. Mobilities, selectivity, resolution

In order to achieve enantiomeric separation of rivastigmine and the *R*-impurity several analytical parameters can be modified. Different background electrolytes (BGEs) assayed showed triethanolammonium phosphate at pH 2.5 being the most favourable. In the examined concentration range between 25 and 100 mM best peak shapes were obtained at 75 mM. With this BGE different β-cyclodextrins were applied to achieve chiral resolution, uncharged ones such as native β-CD, heptakis-β-CD, HP-β-CD and the charged sulfated β-CD. With neutral or charged CDs the enantiomers exhibited different migration order. With the neutral additives the R-form had a higher mobility than the *S*-form of rivastigmine; this is in contrast to sulfated β-CD, which gave an inversion of the migration order of the enantiomers.

The according effective mobilities of the analytes (the total, measured mobilities corrected by that of the electroosmotic flow) are shown in Fig. 2 in dependence on the CD concentration. Typical binding curves are obtained, with effective mobilities decreasing from a value of 22.54×10^{-9} m^2 V^{-1} s^{-1} of both enantiomers in the CD-free buffer. In this acidic BGE the analytes carry a positive charge due to the protonation of the tertiary amino group (see Fig. 1); note that the nitrogen in the amido group is not sufficiently basic for protonation. The effect of the complexing agent on the mobility follows the sequence HP-, heptakis-, native, sulfated CD. The most pronounced influence is found for the sulfated CD, which can be related to the strong ion-ion interaction between the cationic analyte and the anionic com-

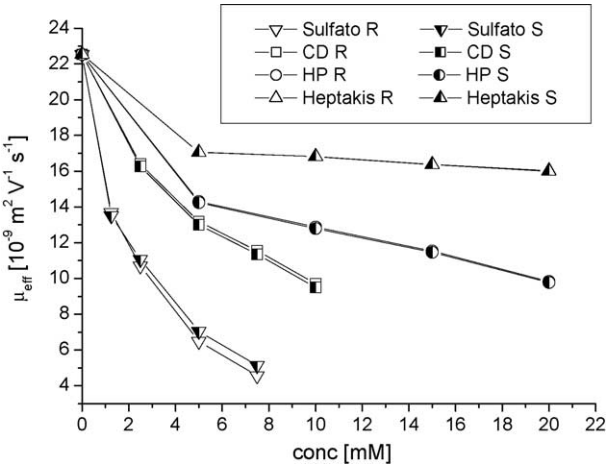

Fig. 2. Effective mobilities of the *R*- and the *S*-enantiomer of rivastigmine in dependence on the concentration of the different cyclodextrins. Temperature 25 °C. Abbreviations: CD, unmodified β-cyclodextrin; heptakis, heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin; HP, hydroxypropyl-β-cyclodextrin; sulfato, sulfated β-cyclodextrin.

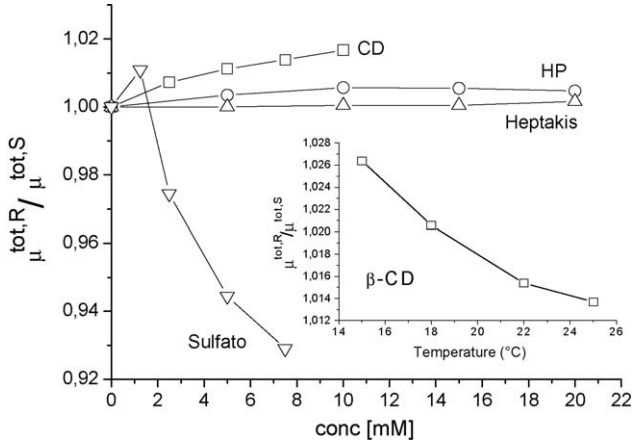

Fig. 3. Ratio of the total mobility of the *R*- to the *S*-enantiomer of rivastigmine in dependence on the concentration of the different cyclodextrins. Insert: temperature dependence of the ratio for unmodified CD. Abbreviations as in Fig. 2.

plexing agent, and to the fact that the charge of the complex is inversed to negative (the complexes with the neutral CDs are still cationic).

A rough derivation of the complex constants, K_C, by fitting the mobility data to the well-known function $\mu_A^{eff} = (\mu_{A+} + \mu_{AC+}K_C[C])/(1 + K_C[C])$ (for 1:1 complex formation) gives values between 180 and 770 M^{-1}. In this equation μ_A^{eff}, $\mu_{A+}$and $\mu_{AC+}$are the (EOF-corrected) effective mobility of the analyte, A, of the free analyte cation, A^+, and of the complex, AC^+. [C] is the equilibrium concentration of the complexing agent. We give the range of the K_C values rather than the more detailed data, because we do not overestimate the quality of these complex constants. They are derived with a number of simplifications: (i) we have not considered the change of the viscosity of the BGE, and its influence on the analyte mobility (albeit the increase in viscosity is only few percent). (ii) We have also not taken the equilibrium concentrations of CD, but the initial concentration (which is acceptable because the additive is present in a large excess compared to the analyte). (iii) The number of data points is rather small. For the sulfated CD no K_C was derived because it is a mixture of different isomers, and not a single one.

Chiral separation selectivity will be expressed here as the ratio of the total mobilities of the two enantiomeric forms. We do not use the ratio of the EOF-corrected effective mobilities, because it is in fact the relative difference of the total mobilities which decides whether a pair of analytes can be separated or not under given conditions. These conditions determine a certain separation efficiency (plate height), and the EOF (which can play an important role for separation), having then a certain magnitude and direction.

The ratios of the total mobilities of the two enantiomers are depicted in Fig. 3. The largest influence on selectivity is observed for the charged CD. This means that the interactions are not only strong due to ionic forces, they are also pronouncedly stereoselective. However, in the higher concentration range of this additive the *S*-form migrates before the *R*-form, which is unfavourable for quantitation when the latter is present in the sample in minor quantities only. In the lower concentration range of this additive, where *R* migrates before *S* (see also ref. [20]), the resolution was not sufficient to separate the former enantiomers from the latter when present in minor concentration. For these reasons this additive, although being the most selective, was not used for further analysis.

From the uncharged CDs—with all of them the *R*-form migrates in front of the *S*-form—native CD implements the largest selectivity. The mobility ratio exceeds a value of 1.01 at even less than 7 mM additive concentration. We have therefore used further native CD at a concentration of 7.5 mM. It can be calculated that a plate number of about 22,000 is needed to resolve two equally sized peaks at baseline, a number which should easily be achievable. As in the present case a small peak (from the impurity) has to be separated from a large peak, larger plate numbers are needed. Indeed 270,000 plates are obtained under this condition at low concentrations (see below).

As mobility and complex constant are functions of the temperature, the temperature dependence of the selectivity was determined with the selected BGE in the range between 15 and 25 °C. The result is given as insert in Fig. 3. It can be seen that a decrease of the temperature led to an increase of the selectivity. However, as lowering the temperature results in prolonged analysis times, we have selected the temperature of 18 °C as a compromise for further analysis. The resulting electropherogram is given in Fig. 4, showing that under these conditions the enantiomers can be resolved better than with baseline separation in less than 20 min. This electropherogram was recorded after hydrostatic injection for 10 s from an aqueous solution, which turned out to be most favourable.

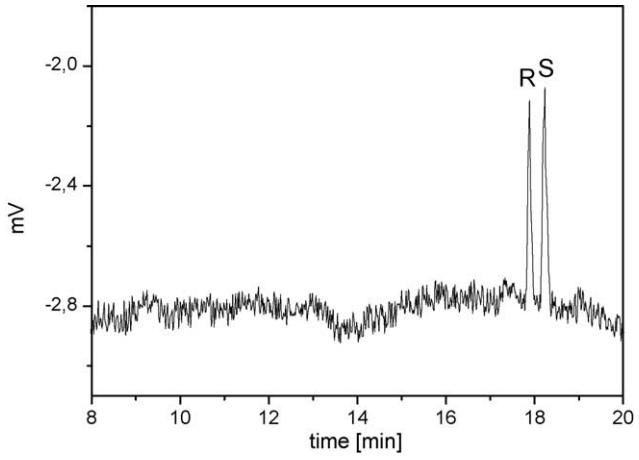

Fig. 4. Electropherogram of *S*- and *R*-enantiomers of rivastigmine. Experimental conditions: BGE, triethanolammonium phosphate, pH 2.5, 75 mM; additive unmodified β-CD, 7.5 mM. Uncoated fused-silica capillary, 60 cm length (53 cm to detector), 50 μm i.d. Voltage +25 kV. UV detection at 214 nm. Hydrostatic injection (10 cm height) for 10 s (2.6 nl). Temperature 18 °C. Analyte concentrations 30.0 μg/ml each.

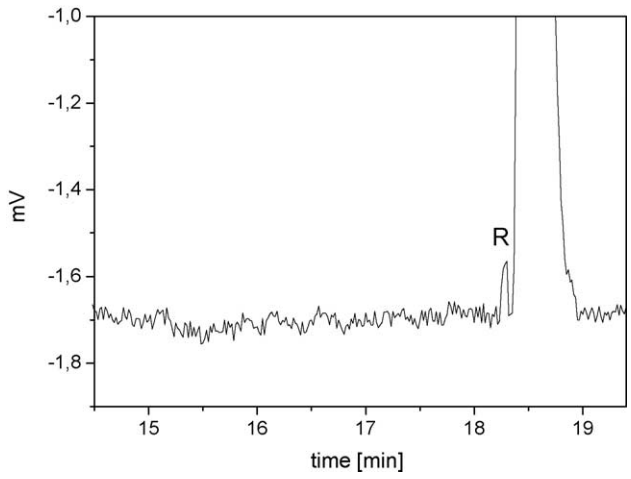

Fig. 5. Electropherogram of rivastigmine with 0.15% (m/m) *R*-impurity. Experimental condition as in Fig. 4.

3.2. Validation

For the validation of the method parameters such as precision, linearity, limits of detection (LOD) and quantification (LOQ) had to be determined. These parameters were determined according to ICH guidelines [1]. Precision evaluated for intra-day ($n = 6$) and inter-day assays ($n = 18$) was expressed as %RSD for migration times and normalized areas (Table 1). It can be seen that for the *R*-impurity (at the 0.5%, m/m level) they are 1.3% and 1.8% for the time, and 2.0 and 2.5% for the peak areas.

LOD and LOQ were determined as 3 and 10 manifolds of the standard deviation of the blank signal. The standard deviation of the noise was determined from six measurement of the heights of a peak from a 25 μg/ml rivastigmine sample. Three and ten times the standard deviation related to the sensitivity (expressed by peak height to concentration) gives the LOD (0.7 μg/ml) and the LOQ (2.3 μg/ml). In the enantiomer mixture the LOD and the LOQ were determined from a pure *S*-rivastigmine sample of 1.5 mg/ml concentration. The standard deviation of the noise at the migration time of the *R*-form from six replicate measurements was then related to the peak of the *R*-enantiomer in a mixture of 0.5% *R*- in 1.5 μg/ml solution of *S*-rivastigmine. The resulting LOD and LOQ values of the *R*-form was 0.05 and 0.15% (m/m), respectively, which means that the method is well suited for its quantitation in the purity range of pharmacological relevance.

Table 1
Figures of merit for the quantitation of rivastigmine and its *R*-impurity

Parameter	*R*-enantiomer	*S*-enantiomer
Precision %RDS[a]		
Intra-day ($n = 6$)		
Migration time	1.3	0.4
Peak area	2.0	1.2
Inter-day ($n = 18$)[b]		
Migration time	1.8	1.1
Peak area	2.5	1.6
Linear range (μg/ml)[c]	2.3–50	2.2–500
r	0.9998	0.9999
LOD (μg/ml)[c]	0.7 (0.05%)	0.7
LOQ (μg/ml)[c]	2.3 (0.15%)	2.2

[a] At the 0.5%, m/m level of the *R*-form.
[b] Three different days.
[c] As rivastigmine hydrogentartrate.

The linearity performed at five different concentration levels of rivastigmine over the mentioned range between 2.3 and 50 μg/ml for the impurity. The linear correlation coefficient is 0.9998. The peak areas of the R-impurity as the minor or trace compound was determined in the range between the LOQ (2.3 μg/ml) and 50 μg/ml in mixtures with the *S*-component (at a total of 1.5 mg/ml of rivastigmine). This range resulted in 0.15–5%, m/m of the *R*-form relative to the main component. The electropherogram for the sample containing 0.15% *R*-compound is shown in Fig. 5. It is clearly separated from the bulk drug, and can directly be quantified.

4. Conclusions

A method was developed to separate and quantify the *R*-impurity in *S*-rivastigmine, a drug applicable for the treatment of Alzheimer's disease. It was based on the electrophoretic separation of the analytes under acidic conditions, where they are present as cations. Enantioselective separation was implemented by the addition of cyclodextrins. From the four different β-cyclodextrins investigated (three neutral and one negatively charged), unmodified β-cyclodextrin turned out to be the most favourable in terms of resolution and analysis time, under the premise that the minor *R*-compound migrates in front of the major *S*-form. The constants for the complex

formation between the analytes and the neutral cyclodextrins ranges in the several hundred M^{-1} range. The analytical parameters (precision, linear range, LOD, LOQ) make the method well-suited for the quantitation of the enantioforms.

Note added in the proof

We would like to point out that after submission of the present work a paper with a similar topic has been published by A. Kavalirova et al., Anal. Chim. Acta 525 (2004) 43.

Acknowledgement

The authors wish to thank Raffo Laboratorios Argentina for providing the rivastigmine samples.

References

[1] http://www.fda.gov/cder/guidance/stereo.htm.May 1.
[2] S. Fanali, Z. Aturki, J. Chromatogr. A (1995) 297.
[3] N. Agyei, K. Gahm, A. Stalcup, Anal. Chem. Acta 307 (1995) 185.
[4] A. Stalcup, K. Gahm, Anal. Chem. 68 (1996) 1360.
[5] G. Gübitz, M. Schmid, J. Chromatogr. A 792 (1997) 179.
[6] K. Assi, A. Abushoffa, K. Altria, B. Clark, J. Chromatogr. A 817 (1998) 83.
[7] S.E. Lucangioli, L.G. Hermida, V.P. Tripodi, V.P. Rodriguez, E.E. Lopez, P.D. Rouge, C.N. Carducci, J. Chromatogr. A 871 (2000) 207.
[8] R. Mandrioli, S. Fanali, V. Pucci, M.A. Raggi, Electrophoresis 24 (2003) 2608.
[9] A. Enz, A. Chappuis, A. Dattler, Biomed. Chromatogr. 18 (2004) 160.
[10] F. Lelievre, P. Gareil, Anal. Chem. 69 (1997) 385.
[11] L. Labat, M. Deveaux, P. Dallet, J.P. Dubost, J. Chromatogr. B 773 (2002) 17.
[12] A.M. Abushoffa, M. Fillet, P. Hubert, J. Crommen, J. Chromatogr. A 948 (2002) 321.
[13] R. Pascoe, J.P. Foley, Analyst 127 (2002) 710.
[14] S. Rudaz, E. Calleri, L. Geiser, S. Cherkaoui, J. Prat, J.L. Veuthey, Electrophoresis 24 (2003) 2633.
[15] B. Chankvetadze, K. Lomsadze, N. Burjanadze, J. Breitkreutz, G. Pintore, M. Chessa, K. Bergander, G. Blaschke, Electrophoresis 24 (2003) 1083.
[16] R.J. Polinsky, Clin. Ther. 20 (1998) 634.
[17] W. Lojkowska, D. Ryglewicz, T. Jedrzejczak, S. Minc, T. Jakubowska, H. Jarosz, A. Bochynska, J. Neurol. Sci. 18 (1998) 785.
[18] F. Pommier, R. Frigola, J. Chromatogr. B 784 (2003) 301.
[19] Y. Sha, C. Deng, Z. Liu, T. Huang, B. Yang, G. Duan, J. Chromatogr. B. 806 (2004) 271.
[20] C.E. Lin, W.S. Liao, K.H. Chen, Electrophoresis 24 (2003) 3139.