ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
23 (2000) 117–125

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Chiral resolution of the enantiomers of 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide using high-performance liquid chromatography on cellulose-based chiral stationary phases

Giuseppe Cannazza [a,*], Daniela Braghiroli [b], Mario Baraldi [a], Carlo Parenti [a]

[a] *Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia, Via Campi 183, 41100 Modena, Italy*

[b] *Dipartimento di Scienze del Farmaco, Università degli Studi 'G. D'Annunzio' Via Dei Vestini, 66013 Chieti, Italy*

Received 30 June 1999; received in revised form 22 October 1999; accepted 8 November 1999

Abstract

Analytical high-performance liquid chromatography (HPLC) methods using derivatized cellulose chiral stationary phases (CSPs) were developed for the separation of the enantiomers of 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±) IDRA21). In previous studies, (±) IDRA21 has been found to have an interesting inhibitory effect on the desensitization of α-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA) receptor and improve cognition in animals. This compound posses one chiral carbon atom, but very little information has been reported on the stereoselectivity of his activity. Therefore resolution of the enantiomers of this compound and subsequent identification of stereospecifity in his pharmacological actions are clearly matters of interest. The resolution were made under normal- and reversed-phase conditions using a mobile phase consisting of *n*-hexane:2-propanol (70/30, v/v) and water:acetonitrile (60/40, v/v) respectively, and a CSP of silica-based cellulose tris-3,5-dimethyl-phenylcarbammate (Chiralcel OD and Chiracel OD-R). The enantiomeric nature of eluates was confirmed by circular dichroism (CD) spectra. A baseline separation ($R_s > 1.5$) was obtained in both cases. Furthemore the isolation of optical isomers of (±) IDRA21 was perfomed using a semipreparative column packed with the same cellulose OD CSP.

Keywords: Benzothiadiazine; Enantioseparation; Cellulose-based chiral stationary phases; Enantiomerization; Semipreparative enantioseparation

1. Introduction

Recent studies on memory and cognition-enhancing compounds (nootropic drugs) implicate α-amino-2,3-dihydro-5-methyl-3-oxo-4-isoxazolepropanoic acid (AMPA) receptors as their target

* Corresponding author. Tel.: + 39-059-378562; fax: + 39-059-378560.
E-mail address: pippi@unimo.it (G. Cannazza)

0731-7085/00/$ - see front matter
PII: S0731-7085(00)00281-8

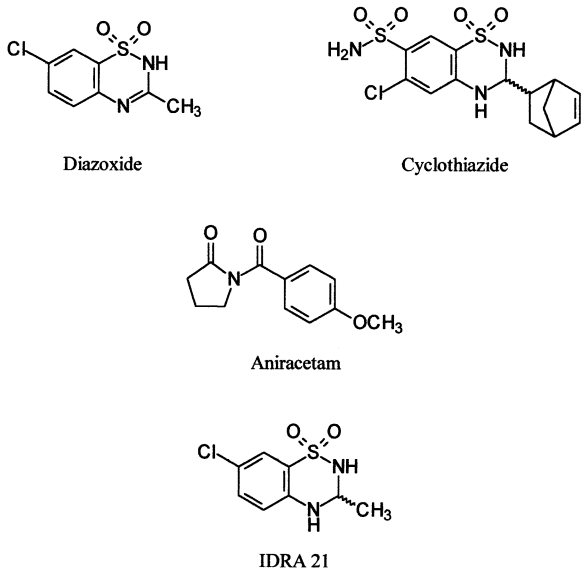

Fig. 1. Structures of some modulators of AMPA receptors.

of action. These compounds (i.e. diazoxide, cyclothiadiazide, aniracetam) (Fig. 1) are thought to work by potentiating glutamate synaptic currents through a removal of the receptor desentization. Cyclothiadiazide has been demonstrated to be the most potent compound in vitro able to remove desensitization from AMPA receptors [1,2] enhancing in this way synaptic transmission [3].

Since cyclothiadiazide does not cross the blood-brain barrier, the development of analogues of this compound that manage to reach the central nervous system and do not have peripheral effects gains importance.

Among the compounds related to cyclothiadiazide derivatives recently synthetized, (±) 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadia zine 1,1-dioxide ((±) IDRA21) (Fig. 1) shows physicochemical characteristics that allow it to cross the blood-brain barrier more efficiently than cyclothiadiazide, and behavioral studies evidentiate also its efficacy as a cognition-enhancer in animals [4–6]. The observed cognition-enhancing

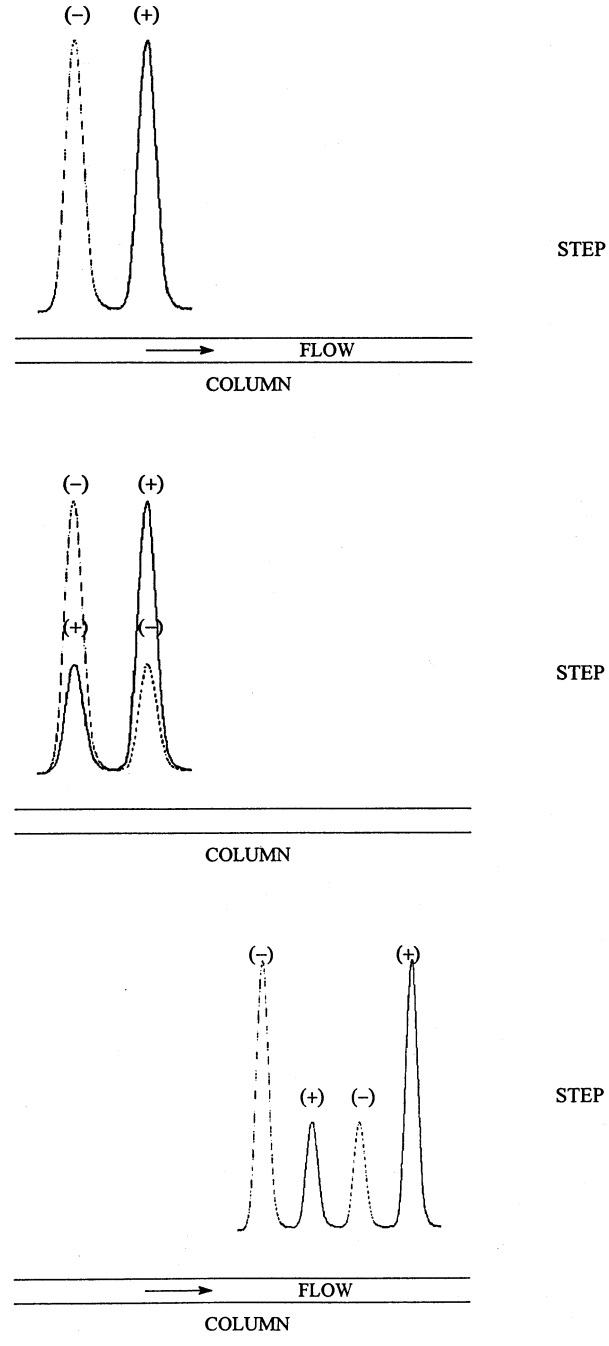

Fig. 2. On-column enantiomerization by the stopped-flow technique during chromatographic enantioseparation of 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±) IDRA21). Step 1. (±) IDRA21 was injected and a partial elution of enantiomers occured. Step 2. The mobile phase flow was stopped and the column was kept for a set time at a set temperature. Each enantiomer was interconverted in the other one in a time and temperature dependent manner. Step 3. The column was cooled back to the previous temperature and the original flow rate was reinforced leading to enantiomers separation.

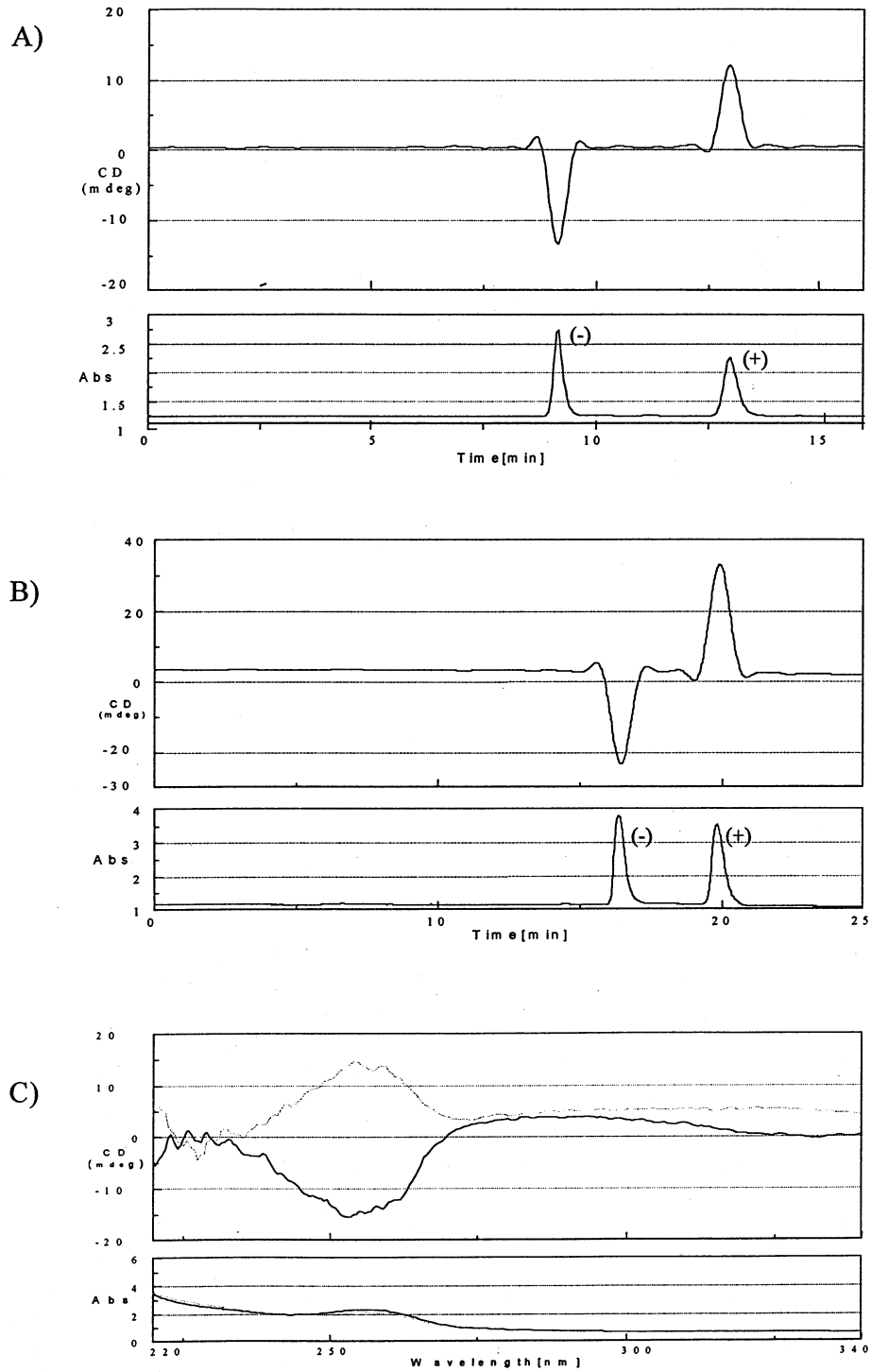

Fig. 3. (A) Chromatographic resolution of enantiomers of 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±) IDRA21) on Chiralcel OD column monitored by absorption (below) and circular dichroism (CD) detection (above). Eluent: hexane:2-propanol (70:30 v/v); Flow rate: 0.5 ml min^{-1}; $\alpha = 1.92$; $R_s = 5$. (B) Chromatographic resolution of enantiomers of (±) IDRA21 on Chiralcel OD-R column monitored by absorption (Below) and CD detection (above). Eluent, water:acetonitrile (60:40 v/v); flow rate, 0.5 ml min^{-1}; $\alpha = 1.26$; $R_s = 2.92$. (C) CD (above) and UV (below) spectra of enantiomers of IDRA21 registered in water:acetonitrile (70:30 v/v).

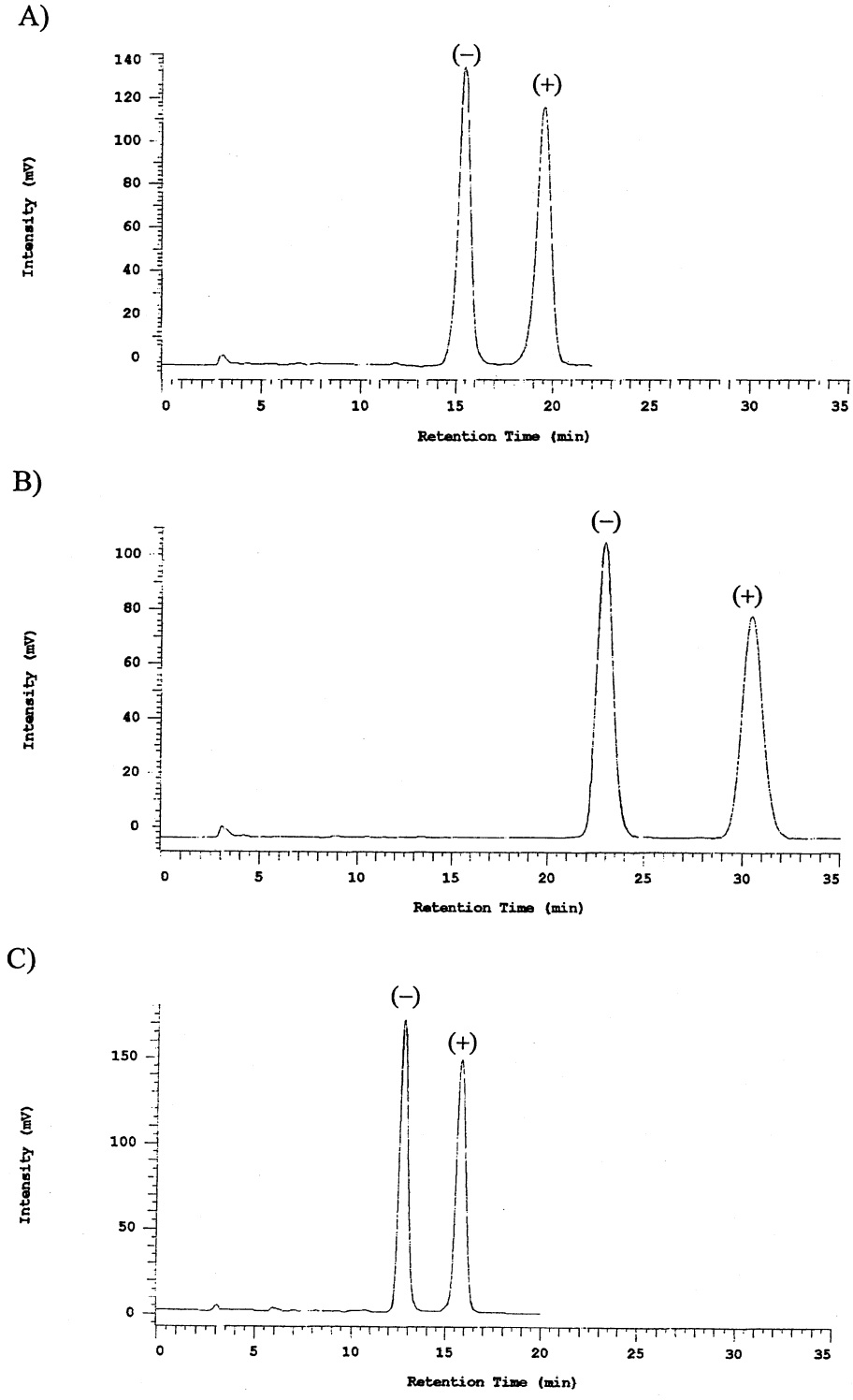

Fig. 4. Chromatographic resolution of enantiomers of 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±) IDRA21) on Chiralcel OD-R column. (A) Eluent: water:acetonitrile 60:40 (v/v), Flow rate: 0.5 ml min^{-1}; $T = 25°C$. (B) Eluent, water:acetonitrile 60:40 (v/v); flow rate, 0.5 ml min^{-1}; $T = 0°C$. (C) Eluent, water:acetonitrile 60:40 (v/v) and perchloric acid to pH 2.5; flow rate, 0.5 ml min^{-1}; $T = 25°C$.

effect of (±) IDRA21, which in experimental animals is more potent than that of aniracetam, suggests a potential applicability of this drug in the treatment of attention disorders in children and in senile dementias, including early stages of Alzheimer's disease [7].

(±) IDRA 21 posses a stereogenic carbon atom in the 3 position of benzothiadiazine ring, and as stereoisomers often show different pharmacological activities, it seemed advisable to resolve the racemic mixture to investigate the biological properties of each enantiomer. Liquid chromatography with chiral stationary phases (CSPs) has gained increasing importance to accomplish this task [8]. Few chromatographic separations of enantiomers of chiral benzothiadiazine structurally similar to IDRA21 have been reported in the literature. Blaschke et al. separeted the enantiomers of chiral benzothiadiazine diuretic drugs on cross-linked polyacrylamide CSPs [9]. Recently Uznov et al. [5] have been developed a new brush-type CSP employed for the optical resolution of (±) IDRA21 enantiomers, but enantioselectivity obtained ($\alpha = 1.21$) was not very high. In order to improve enantioselectivity and resolution, commercially available modified cellulose CSPs, known for the high resolving power of racemates [10] have now been investigated for their ability to resolve the enantiomers of (±) IDRA21.

The enantiomeric separation of (±) IDRA21 by normal and reversed phase of cellulose tris(3,5-dimethylphenyl)carbammate CSPs on silica gel (Chiralcel OD and Chiralcel OD-R, respectively) and effects of temperature on racemization rates of enantiomers of (+) and (−) IDRA21 were investigated. Furthemore the isolation of optical isomers of (±) IDRA21 was perfomed using a semipreparative column packed with the same cellulose OD CSPs.

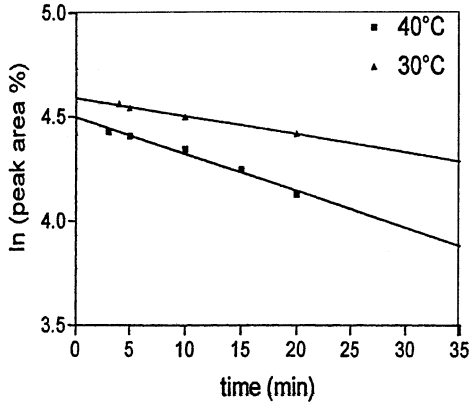

Fig. 5. Racemization kinetics of (+) enantiomer of IDRA21. The decreasing ln peak area % was recorded at 30 and 40°C. The correlation coefficients of the regression lines were $r^2 > 0.99$. (See text for details.)

2. Experimental

2.1. Istrumentation

The chromatographic apparatus consisted of a Jasco PU-980 intelligent Pump, a 7125 Reodhyne manual injector equipped with a 20 or 200 μl sample loop for analytical and semipreparative scale, respectively. A model Jasco J-710 dichrograph (Dipartimento di Chimica, Università di Modena e Reggio Emilia, Italy) or a UV a Jasco 875-UV was used as detector. Chromatograms were recorded with J-700 system program or a Spectra Phisics Integrator. A 7000 Reodhyne valve post column permits to isolate the cell of the spectropolarimeter detector for the acquisition of circular dichroism (CD) and UV spectra. Column temperature regulation was achieved by a termostated water bath Haake F3.

Normal phase chromatography was carried out on a Chiralcel OD column (tris(3,5-dimethylphenyl)carbammate; 250 × 4.6 mm I.D.; 10 μm), and on a Chiralcel OD semipreparative column (tris(3,5-dimethylphenyl)carbammate; 250 × 10 mm I.D.; 10 μm). Reversed phase chromatography was carried out on a Chiralcel OD-R column (tris(3,5-dimethylphenyl)carbammate; 250 × 4.6 mm I.D.; 10 μm).

2.2. Chemicals

(±) IDRA21 used in this study were synthetized as previously described [4]. HPLC-grade 2-propanol, hexane and acetonitrile were obtained

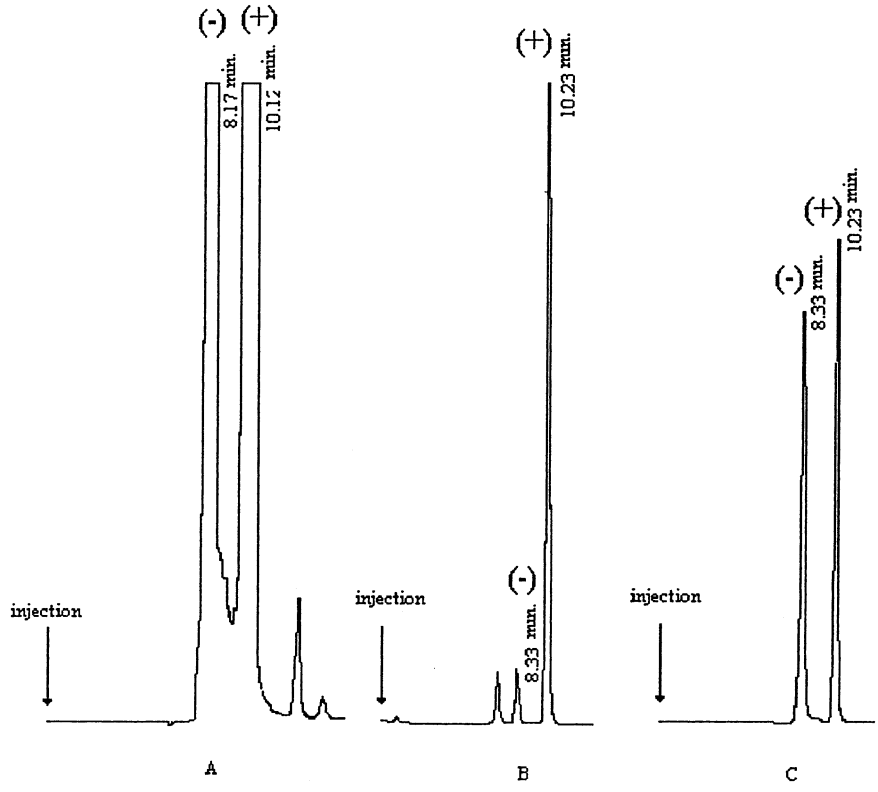

Fig. 6. (A) Semipreparative resolution of 0.2 mg of racemic 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±) IDRA21) on Chiralcel OD column (250 × 10 mm). Eluent, hexane:2-propanol (50:50 v/v); flow rate, 2 ml min^{-1}. (B) Determination of enantiomeric purity of last retained enantiomer of IDRA21 prior evaporation of mobile phase e.e. = 93%. Column, Chiralcel OD (250 × 10 mm). Eluent, hexane:2-propanol (50:50 v/v); flow rate, 2 ml min^{-1}. (C) Determination of enantiomeric purity of (+) IDRA21 after evaporation of mobile phase undre reduce pressure (e.e. = 7%). Column, Chiralcel OD (250 × 10 mm). Eluent, hexane:2-propanol (50:50 v/v); flow rate, 2 ml min^{-1}.

from Baker. 1,3,5-Tri-*tert*-butylbenzene, trifluoroacetic acid, perchloric acid and diethylamina were purchased by Fluka.

2.3. Chromatographic conditions

Analytical enantioseparations of (±) IDRA21 were carried out isocratically at room temperature. The mobile phase consited of hexane and 2-propanol for Chiralcel OD columns, water and acetonitrile for Chiralcel OD-R column. (±) IDRA21 was dissolved in 2-propanol or acetonitrile depending of the chromatographic mode and the samples were passed through a 0.45 μm filter prior to injection. The detectors were set at 254 nm.

Mobile phases consisting of hexane:2-propanol in different proportion were used for semipreparative enantiomers resolution of (±) IDRA21. Flow rate was always 2 ml min^{-1}. Elution of the individual enantiomers was monitored with a UV detctor operating at 254 nm. The eluent fractions corresponding to the peaks of enantiomers were collected separately. The mobile phase in the respective fractions was evaporated under reduce pressure at room temperature.

The enantiomeric purity of the enantiomers collected was monitored on the semipreparative Chiralcel OD column by injections of 20 μl of each eluent fractions corresponding to the peaks of enantiomers.

2.4. Chromatographic parameters

The separation factor (α) was calculated as K'_2/K'_1 and capacity factors (K'_1 and K'_2) as $K'_1 = (t_1 - t_0)/t_0$ where t_1 and t_2 refer to the retention times of the first and second enantiomers respectively. The resolution factor (R_s) was calculated by the formula $R_s = 2(t_2 - t_1)/(w_1 + w_2)$ where w_1 and w_2 are the peak widths for the first and second eluting enantiomers peak respectively. The dead-time of the columns (t_0) was determined by injection of 1,3,5-tri-*tert*-butylbenzene.

2.5. Enantiomerization

To determine the racemization rates of (+) and (−) IDRA21, the on-column inversion of enantiomers via a stopped-flow method was investigated [11,12].

As show in Fig. 2 the racemic mixture of (±) IDRA21 was chromatographed on OD CSPs. After a specific time, the mobile phase flow was stopped and the column was kept at a set temperature for a set time to affect enantiomerization of partial separated enantiomers of (±) IDRA21. Afterwards, the column was cooled back to the

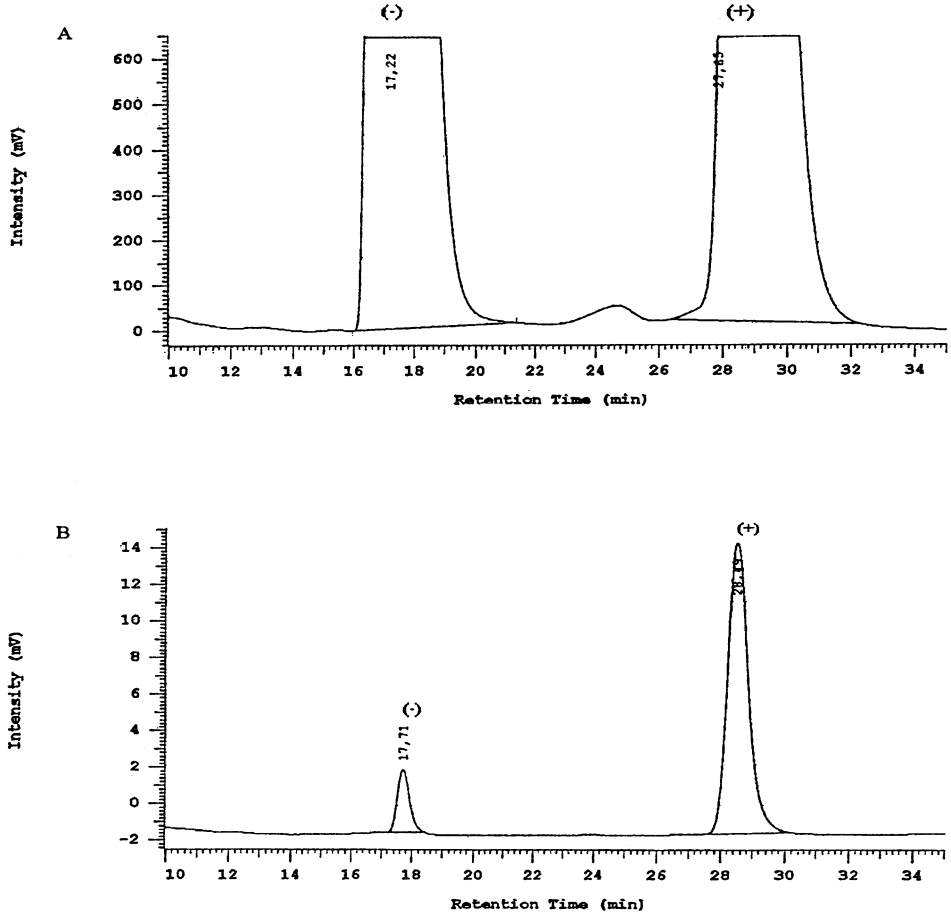

Fig. 7. (A) Semipreparative resolution of 0.2 mg of racemic 7-chloro-3-methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide ((±) IDRA21) on Chiralcel OD column (250 × 10 mm). Eluent, hexane:2-propanol (80:20 v/v); flow rate, 2 ml min^{-1}. (B) Determination of enantiomeric purity of last retained enantiomer of (±) IDRA21 after evaporation of mobile phase diluted with hexane (mobile phase:hexane (1:3 v/v)); e.e. = 79%. Column, Chiralcel OD (250 × 10 mm). Eluent, hexane:2-propanol (50:50 v/v); flow rate: 2 ml min^{-1}.

previous temperature and the original flow rate was reinforced.

3. Results and discussion

3.1. Analytical separation of enantiomers

Results of enantioseparation of (±) IDRA21 are showed in Fig. 3A,B.

Baseline resolution was obtained with Chiralcel OD column ($\alpha = 1.92$, $R_s = 5$) as well as Chiralcel OD-R column ($\alpha = 1.2$, $R_s = 2.92$). The retention times and resolutions were found to be stable for hundreds of injections. Small amount of diethylamine or trifluoroacetic acid were used as modifier to the hexane:2-propanol mobile phase but they did not influence the chromatographic parameters dramatically. Change of pH (pH 2.5 with perchloric acid) were made to the water:acetonitrile mobile phase but only small retentions time variations were observed (Fig. 4) [13]. Also the temperature of the column has been partly investigated and it was founded, as expected, that lower temperature increased the retention times with small changes in resolution on both Chiralcel OD and Chiralcel OD-R (Fig. 4) [14].

The UV and CD spectra of the two eluted peaks are showed in Fig. 3C: as expected the UV absorbance spectra were identically but the Cotton effects are opposite. The CD spectra of the IDRA21 enantiomers were as yet not known and may be useful to assign the absolute configuration of the resolved enantiomers by comparing the CD curve of the molecule under study with that of a similar compound having known absolute configuration [15,16].

3.2. Racemization kinetics

The racemization rates of (+) and (−) IDRA21 was determined by the on-column inversion of enantiomers via a stopped-flow method. As shown in Fig. 2 (±) IDRA21 were chromatographed on Chiralcel OD or OD-R. After a set time, the mobile phase flow was stopped. The sample was already resolved into the enantiomers (Fig. 2, step 1). The column was heated at 30 and 40°C for a set time and the separated enantiomers are left to interconvert for a certain period. The first (−) enantiomer was partially converted into the (+) enantiomer, conversely the (+) enantiomer was also trasformed into the (−) enantiomer at the same rate (Fig. 2, step 2). The column was then cooled to the original temperature and the previous mobile phase flow was reinforced leading to enantiomers separation (Fig. 2, step 3). The net result was the presence of 4 peaks: 1 and 4° peaks were the signals of the two enantiomers of IDRA21 while 2 and 3° peaks were the signals corresponding to the interconverted sample. Consequently enantiomerization rates were calculated from chromatographic peaks areas [11,12].

For the (±) IDRA21 enantiomers, no configurational changes at the chiral center were observed in exane:2-propanol 70:30 (v/v) on Chiralcel OD column at 20–40°C temperatures, but the enantiomers racemize in water:acetonitrile 70:30 (v/v) on Chiralcel OD-R column as a function of temperature and enantiomerization time. The decrease of peaks area for the enantiomers of (±) IDRA21 followed a first-order kinetics at 40 and 30°C (low racemization rate was observed at 20°C for 30 min of enantiomerization time). Fig. 5 shows, for example, the linear ln peak area versus time plot obtained for (+) enantiomer of IDRA21 at 30 and 40°C.

The racemization rates profile of (±) IDRA21 were in agreement with those of benzothiadiazine diuretics drugs wich have the same thiadiazine moiety [9].

It should also be kept in mind that the rate observed may not always rapresentative of that in bulk solution, because the racemization process may be catalysed by the surface of CSP with wich the solutes comes into contact in the column [17].

3.3. Semipreparative enantiomers resolution

Fig. 6A shows the enantiomeric resolution of 0.2 mg of (±) IDRA21 on Chiralcel OD semipreparative column. The e.e. of enatiomers collected was monitored prior and after storage for one month at −20°C of the collected peaks

fractions, and prior and after the evaporation under reduce pressure of mobile phase (Fig. 6B). No racemization occured when the collected peaks fractions were stored, while the two enantiomers racemized during evaporation of mobile phase (Fig. 6C). Same results were obtained by changes in mobile phase composition: hexane:2-propanol 70:30 (v/v), hexane:2-propanol 80:20 (v/v). A more favorable situation (e.e. of about 80% for both enantiomers) was achieved by dilution with hexane of eluents fractions corresponding to the peaks of enantiomers prior evaporation (Fig. 7). Since 2-propanol evaporated after hexane, the protic solvent could stimulate racemization of (±) IDRA21 enantiomers. The amounts of relative pure enantiomers obtained by semipreparative chromatography was sufficient to conduct preliminary biological tests.

4. Conclusions

The enantioseparation of (±) IDRA21 employing cellulose tris(3,5-dimethylphenylcarbammate) as chiral stationary phases known as Chiralcel OD and Chiralcel OD-R were studied, and both systems showed similar enantioselectivity suitable to determinate enantiomeric purity. Furthermore, the on-column inversion of (±) IDRA21 enantiomers via a stopped-flow method was investigated, and a first order racemization kinetics in aqueous solutions at 30 and 40°C were determined. Finally, the isolation of optical isomers of (±) IDRA21 was perfomed using a semipreparative column packed with the same cellulose OD CSP. However, it turned out that, during work-up of the collected fractions, partial racemization occurs which can be significantly suppressed by hexane dilutions. The enantioseparation method developed for reversed phase conditions seems appropriate to be used also for biological samples, since a transfer from an aqueous to a non-aqueous sample solvent is no longer necessary [18].

Acknowledgements

The authors thanks Professor Dr W. Lindner for the kind reviewing of this article and for the precious technical suggestion about on-column racemization. Moreover a grateful thanks goes Professor A. Forni (Dipartimento di Chimica, Università di Modena e Reggio Emilia, Italy) who conceded the use of dichrograph.

References

[1] L. Wyklicky, D.K. Patneau, M.L. Mayer, Neuron 7 (1991) 971–984.
[2] D.K. Patneau, L. Wyklicky, M.L. Mayer, J. Neurosci. 13 (1993) 3496–3509.
[3] K.A. Yamada, C.M. Tang, J. Neurosci. 13 (1993) 3904–3915.
[4] M. Bertolino, M. Baraldi, C. Parenti, D. Braghiroli, M. Di Bella, S. Vicini, E. Costa, Recept. Channels 1 (1993) 267–278.
[5] D.P. Uzunov, I. Zivkovich, W.H. Pirkle, E. Costa, A. Guidotti, J. Pharm. Sci. 84 (1995) 937–942.
[6] I. Zvkovic, D.M. Thompson, M. Bertolino, D. Uzunov, M. DiBella, E. Costa, A. Guidotti, J. Pharmacol. Exp. Ther. 272 (1995) 300–309.
[7] F. Impagniatiello, A. Oberto, P. Longone, E. Costa, A. Guidotti, Proc. Natl. Acad. Sci. 94 (1997) 7053–7058.
[8] H.Y. Aboul-Enein, Anal. Lett. 25 (1992) 231.
[9] G. Blaschke, J. Maiboun, J. Pharm. Sci. 74 (1985) 438–442.
[10] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403–419.
[11] V. Schurig, A. Glaush, M. Fluck, Tetrahedron:asymmetry 6 (9) (1995) 2161–2164.
[12] G. Weseloh, C. Wolf, W.A. König, Angew. Chem. 107 (1995) 1771–1772.
[13] A. Ishikawa, T. Shibata, J. Liq. Chromatogr. 16 (1993) 859–878.
[14] E. Yashima, P. Sahavattanapong, Y. Okamoto, Chirality 8 (1996) 446–451.
[15] G. Blaschke, Angew. Chem. 92 (1980) 14.
[16] P. Salvadori, C. Bertucci, C. Rosini, Chirality 3 (1991) 376–385.
[17] A. Mannschreck, Chirality 4 (1992) 163–169.
[18] M. Kummer, G. Werner, J. Chromatogr. A 825 (1998) 107–114.