Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography A, 993 (2003) 17–28
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Enantioselective liquid chromatography of C_3-chiral 2,3-dihydro-1,2,5-benzothiadiazepin-4(5*H*)-one and thione 1,1-dioxides on polyacrylamide- and polysaccharide-based chiral stationary phases

R. Cirilli[a,*], R. Costi[b], R. Di Santo[b], M. Artico[b], A. Roux[b], B. Gallinella[a], L. Zanitti[a], F. La Torre[a]

[a]*Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*
[b]*Dipartimento di Studi Farmaceutici, Istituto Pasteur-Fondazione Cenci-Bolognetti, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, I-00185 Rome, Italy*

Received 11 June 2002; received in revised form 4 February 2003; accepted 4 February 2003

Abstract

Optically active synthetic and semisynthetic polymers were utilized as chiral stationary phases (CSPs) for the direct chromatographic enantioseparation of a series of 8-chloro-2,3-dihydro-3-methyl-1,2,5-benzothiadiazepin-4(5*H*)-one and thione 1,1-dioxide. Evaluation of stereochemical integrity of chiral analytes was assessed by enantioselective temperature and flow-dependent HPLC. A stopped-flow high-performance liquid chromatography (sfHPLC) procedure was developed for the determination of the rate constants and free energy barriers of enantiomerization of enantiomers of 8-chloro-2-(3-methyl-but-2-enyl)-2,3-dihydro-3-methyl-1,2,5-benzothiadiazepin-4(5*H*)-thione 1,1-dioxide (compound **2**) in the presence of Chiraspher and Chiralcel OD CSPs. In order to study the chiroptical properties of the individual enantiomers of analytes investigated, semipreparative chromatographic resolutions were performed. The assignment of the absolute configuration was empirically established by comparing the CD spectra of the separated enantiomers with those obtained from structural analogues.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Chiral stationary phases, LC; 2,3-Dihydro-1,2,5-benzothiadiazepin-4(5*H*)-one; Thione 1,1-dioxides

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is a pandemic disease whose primary etiological agent is human immunodeficiency virus type-1 (HIV-1). In the life cycle of this retrovirus the reverse transcriptase (RT) is a key multifunctional enzyme, which has been studied as a major target for the development of antiretroviral agents.

Currently two different classes of anti-AIDS chemotherapeutic agents that target this enzyme have been developed: nucleoside analogues (NRTIs) and NNRT (non-nucleoside reverse trascriptase) inhibitors [1]. Of the former, AZT, ddI, ddC and d4T are the compounds so far approved for the treatment of HIV-1 infection. Regarding the latter, drugs that passed the tests for clinical usage are nevirapine,

*Corresponding author. Fax: +39-6-4938-7100.
E-mail address: rcirilli@iss.it (R. Cirilli).

0021-9673/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0021-9673(03)00321-2

pyridones, BHAP, and tetrahydroimidazobenzodiazepinones and thiones (TIBO) [2–5].

TIBO derivatives bind to the biological target with different strengths depending on the configuration of the tetrahydroimidazolethione C_5 atom [2–4]. In fact, all $C_5(S)$ enantiomers showed higher potency against HIV-1 replication compared to $C_5(R)$ counterparts. Following our 10 years of work in the field of new anti-AIDS agents development [6–10], we designed new anti-HIV-1 agents related to TIBOs [11] and synthesized a number of 8-chloro-2,3-dihydro-3-methyl-1,2,5-benzothiadiazepin-4(5*H*)-ones (seco-TIBO) [12]. The main features of these new derivatives are: (i) the removal of the imidazolethione ring from TIBO structure; (ii) the presence of sulfone moiety replacing the CH_2 group in the 7 position of the tetrahydroimidazobenzodiazepinethione ring; (iii) the retaining of the chiral center at the carbon atom substituted with a methyl group.

Taking into account that stereochemistry is an important modulator of biological activity in the TIBO series, we decided to resolve a series of closely related racemic seco-TIBOs by chiral chromatography.

The resolution of C_3-chiral benzothiadiazepinones and thiones **1**–**12** was carried out by enantioselective HPLC on commercially available polymeric-based CSPs [13], on an analytical and semipreparative scale. With respect to the increased interest shown in the last few years by national regulatory committees in configurational stability of stereoisomers [14], opportune analytical procedures were applied for the evaluation of the stereochemical integrity of the chiral compounds investigated [15]. Interconversion of one enantiomer into another can lead to characteristic peak broadening and plateau formation, depending on the conditions of the chromatographic process [16].

In order to avoid the influence of the chiral selector on the on-column interconversion process and estimate such influence on the configurationally stability of the samples, effective high-performance liquid chromatography (HPLC) techniques, such as dynamic HPLC (DHPLC) [17] and stopped-flow HPLC (sfHPLC) [18], were developed.

Finally, the absolute configurations of the benzothiadiazepinones and thiones **1**–**12** were determined by comparing the chiroptical data of individual enantiomers with those of structurally related analytes.

The experimental data and their analysis reported here are a substantial step toward the complete stereochemical characterization of enantiomers of seco-TIBOs and the interpretation of their effective ability to selectively interact with binding sites of the biological target. Additional studies in order to provide further details of such interaction are currently in progress.

2. Experimental

2.1. Materials

Stainless-steel Chiralcel OD (250×4.6-mm I.D.), Chiralpak AD (250×4.6-mm I.D. and 250×10-mm I.D.), Chiralpak AS (250×4.6-mm I.D.) (Daicel Chemical Industries, Tokyo, Japan), Chiraspher (250×4.0-mm I.D.) and Chiraspher NT (250×10-mm I.D.) (Merck, Darmstadt, Germany) columns were used.

HPLC-grade solvents were supplied by Carlo Erba (Milan, Italy).

Analytes **1**–**12** (Fig. 1) were synthesized by chemical pathway reported elsewhere [12].

2.2. Apparatus

Chromatography was performed using a Waters (Milford, MA, USA) 510 pump equipped with a Rheodyne (Cotati, CA, USA) injector, a 1-ml sample loop, a HPLC (Perkin-Elmer, Norwalk, CT, USA) oven, a Waters Model 996 diode array detector (DAD) and a Jasco (Ishikawa-cho, Hachioji City, Tokyo, Japan) Model CD 995 UV/CD detector. The signal was acquired and processed by Millenium 2010 software.

Low-temperature chromatography was performed by placing the column in a proper guard of a MGW Lauda Cryostat (Messgerate-Werk Lauda, Germany) and employing a 1-m connecting capillary placed in the ethylene glycol cooling bath, to ensure thermal equilibration of the mobile phase [19].

Optical rotations of enantiomers of **1**–**3**, dissolved in dichloromethane, were measured at a wavelength

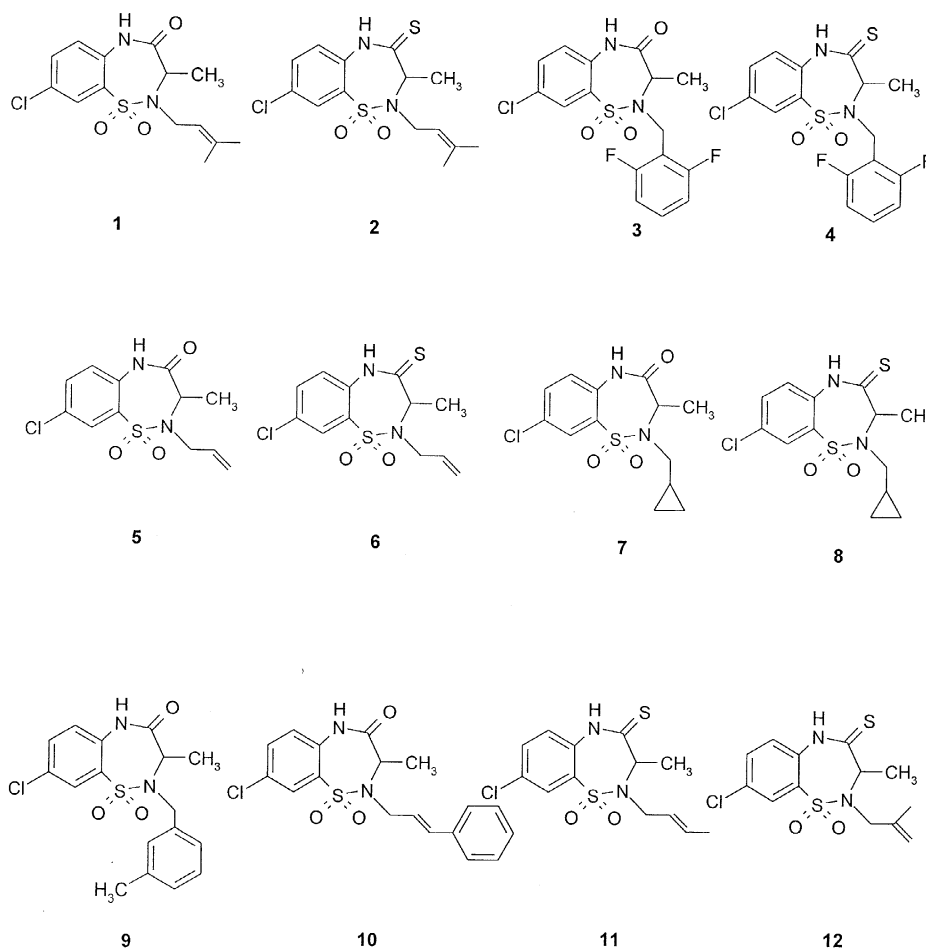

Fig. 1. Structures of the chiral analytes **1**–**12**.

of 589 nm by a Perkin-Elmer polarimeter model 241 equipped with an Na lamp. The volume of the measuring cell was 1 ml and the optical path was 10 cm. The system was kept at a constant temperature of 23 °C.

The circular dichroism (CD) spectra of enantiomers of **1**–**3** in dichloromethane, in a quartz cell (1-cm path length) at room temperature, were measured using a Jasco Model J-700 spectropolarimeter.

Off-column racemization reaction was performed by placing a glass vessel containing a solution of enantiomerically pure **2** in a Julabo (Julabo Labortechnik, Seelbach, Germany) Model HP-4 thermostat.

2.3. Operating conditions

The eluents for the chromatographic separations were prepared by mixing an alcohol modifier (ethanol or 2-propanol) with *n*-hexane at defined ratios or, in the case of HPLC of **2** on Chiraspher CSP, three different solvents (*n*-hexane, chloroform and 2-propanol). The mobile phases were filtered and degassed by sonication immediately before use. Flow rates of 0.5 or 1.0 ml min^{-1} for analytical separations, and 2.5 or 4.0 ml min^{-1} for semipreparative separations were used. UV detector was set up at 254 and 310 nm for analytical and semipreparative separations, respectively.

All analytical separations were performed at a constant temperature of 25 °C, except those designed for the evaluation of temperature effects. They were performed using standard solutions prepared by dissolving 2–5 mg of each analyte with 25 ml of a mixture *n*-hexane–ethanol (50:50, v/v). The injection volume was 20 μl. In the semipreparative run, 24 and 10 mg of racemate **1** and **3**, respectively, dissolved in 500 μl of ethanol, were injected onto a 10-mm I.D. AD CSP at 25 °C. A 500-μl volume containing 10 mg of racemate **2** was applied to the 10-mm I.D. Chiraspher NT CSP at 10 °C. After semipreparative separations, the collected fractions were analyzed by chiral analytical columns to determine their enantiomeric excess (e.e.).

Off-column racemization reaction was carried out on 0.20 mg of (+)-**2** enantiomer dissolved in 0.7 ml of a *n*-hexane–ethanol mixture (70:30, v/v) at a temperature of 50 °C. Progress of the racemization was traced by HPLC on Chiraspher CSP at 10 °C (*n*-hexane–chloroform–2-propanol, 50:50:2, v/v/v, as the eluent) by taking up an aliquot of the solution at appropriate intervals of time. The enantiomeric excess was monitored as a function of time and the racemization rate constant and free energy barrier were calculated from the graph ln e.e. versus time and by the Eyring equation [20], respectively.

3. Results and discussion

3.1. Analytical enantioseparations of ***1–12***

Optically active polymers have been largely used as chiral stationary phases for the direct chromatographic enantioseparation of racemic compounds. Several types of CSPs with a totally synthetic structure were designed to obtain chiral recognitions in the fields of synthetic and medicinal chemistry [21]. Chiraspher is a chiral HPLC phase synthesized by Blaschke et al. [22] by covalent binding of a poly(*N*-acryloyl-*S*-phenylalanine ethyl ester) on silica gel. The driving force of the enantiodiscrimination process is mainly due to π–π interactions and hydrogen bonds between enantiomers and chiral polymer. The polyacrylamide derivative support was selected to resolve analytes **1–12** because of its well-known ability to separate C3-chiral 1,4-benzodiazepin-2-ones enantiomers [23], which have chemical structure similar to compounds investigated in the present paper (Fig. 1). Baseline resolution were readily obtained at room temperature for all compounds using a mobile phase composed of a mixture of *n*-hexane–2-propanol (80:20, v/v). Chromatographic data reported in Table 1 show that substitution of the carbonyl oxygen with a less electronegative sulfur (**1** vs. **2**, **3** vs. **4**, **5** vs. **6**, **7** vs. **8**), did not give rise to a significant variation in retention but higher enantioselectivity factors, α, were obtained instead. Fig. 2 shows typical chromatograms illustrating the resolution of 3-methyl-2,3-dihydro-1,2,5-benzothiadiazepin-4(5*H*)-one 1,1-dioxide **5** and corresponding 3-methyl-2,3-dihydro-1,2,5-benzothiadiazepin-4(5*H*)-thione 1,1-dioxide **6** on the Chiraspher CSP. These results appear to be consistent with the assumption that the acceptor hydrogen bonding carbonyl oxygen does not play a dominant role in determining both enantioselectivity and retention.

In addition to synthetic polymeric Chiraspher CSP, a second class of CSPs represented by commercial biopolymers obtained by modification of amylose or cellulose adsorbed on macroporous silica gel [24] was tested.

Among polysaccharide-based CSPs employed, amylose 3,5-dimethylphenyl-carbamate (Chiralpak AD) and amylose (*S*)-1-methylbenzyl-carbamate (Chiralpak AS) [25] exhibited good enantioselectivity for analytes **1–12**. The resolution of racemates was optimized utilizing normal-phase eluents, consisting of a mixture of *n*-hexane with ethanol or 2-propanol as organic modifier. Table 1 shows the results obtained after optimization, in terms of enantioselectivity and resolution, of the various sets of conditions tested.

Unlike the Chiraspher case, in both AD and AS CSPs, enantioselectivity factor values dropped sharply when thiocarbonyl group was present in place of carbonyl group. For example, the high enantioselectivity factor (2.8) observed for compound **1** on AD CSP and using 20% of ethanol (80:20, v/v) as mobile phase, became 1.0 for the corresponding thiocarbonyl compound **2**. On the same CSP, the compounds **4**, **6**, **8**, **11**, **12** were poorly resolved ($\alpha<1.17$) employing a mobile phase consisting of *n*-hexane–2-propanol (80:20, v/v) and the resolving

Table 1
HPLC of compounds **1–12**

Compound	Chiralpak AS[a]			Chiralpak AD			Chiraspher[b]		
	k_1[d]	α[e]	Rs[f]	k_1	α	Rs	k_1	α	Rs
1	1.55	2.15	11.74	1.82[c]	2.89	18.88	2.45	1.19	2.44
2	1.39	1.33	2.49	1.31[b]	1.30	2.89	2.75	1.30	3.22
3	3.68	1.95	10.94	3.58[b]	1.62	9.83	5.13	1.23	3.62
4	3.47	1.11	1.15	2.77	1.16	1.16	6.59	1.55	7.95
5	1.92	1.44	6.15	3.35[c]	1.55	9.13	3.79	1.20	3.04
6	1.60	1.06	0.80	1.75[c]	1.06	0.45	3.85	1.42	4.57
7	1.96	1.72	8.46	2.50[c]	3.03	18.62	3.84	1.19	2.94
8	1.30	1.27	3.20	1.64[b]	1.05	0.45	3.59	1.39	4.73
9	2.08	1.73	8.53	3.15[c]	2.34	16.43	4.87	1.22	3.18
10	2.46	1.71	8.17	5.37[c]	1.65	10.17	5.47	1.21	3.20
11	1.58	1.16	1.92	1.46[b]	1.17	1.40	3.20	1.33	3.44
12	1.17	1.20	2.08	1.66[b]	1.04	0.47	3.13	1.38	4.12

Columns, Chiralpak AD (250×4.6-mm I.D.), Chiralpak AS (250×4.6-mm I.D.) and Chiraspher (250×4.0-mm I.D.); eluent, *n*-hexane–alcohol modifier (ethanol or 2-propanol); flow-rate, 0.5 and 1.0 ml min^{-1} for polysaccharide-based and Chiraspher CSPs, respectively; temperature, 25 °C; UV detector, 254 nm.
[a] Eluent: *n*-hexane–ethanol (70:30).
[b] Eluent: *n*-hexane–2-propanol (80:20).
[c] Eluent: *n*-hexane–ethanol (80:20).
[d] The retention factor.
[e] The enantioselectivity factor.
[f] The resolution factor.

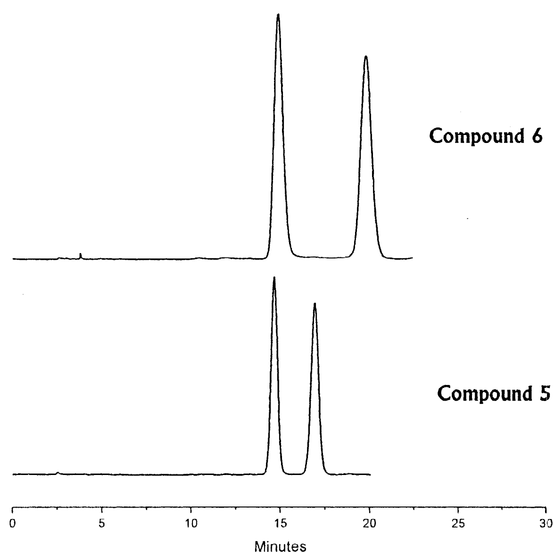

Fig. 2. HPLC resolution of compounds **5** and **6**. Column, Chiraspher (250×4.0-mm I.D.); eluent, *n*-hexane–2-propanol (80:20, v/v); flow-rate, 1.0 ml min^{-1}; detection wavelength, 254 nm; column temperature, 25 °C.

ability of AD CSP towards these compounds was lost when ethanol was used as organic modifier. According to these observations, we suggest that the carbonyl group of C_3-chiral 1,2,5-benzothiadiazepin-4-ones investigated is an important site for chiral discrimination on AS and AD CSPs because of its ability to interact by hydrogen bond with NH functionalities of carbamate groups of chiral polymers [26].

3.2. Dynamic high-performance liquid chromatography

A third polysaccharide-based CSP, namely Chiralcel OD [24], which contains the same phenylcarbamate moiety as Chiralpak AD but bound to the cellulose backbone instead of amylose, exhibited less chiral recognition ability than the aforementioned CSPs. Indeed, of six carbonyl analytes tested four were not resolved at all (compounds **1**, **3**, **5**, **7**) and enantioselectivity factors of 1.36 and 1.10 for **9** and **10**, respectively, were observed. The enantioselective HPLC was carried out at 25 °C and flow-rate of 0.5 ml min^{-1} with a mobile phase

consisting of *n*-hexane–ethanol (70:30, v/v). With these parameters, enantiomers were eluted within 25 min. At the same temperature and using a mixture of *n*-hexane–ethanol (85:15, v/v) as eluent, enantiomeric species of corresponding thiocarbonyl compounds gave two broad peaks with different ratios, the most abundant being the second eluted one.

In Fig. 3 a chromatogram for resolution of racemic **2** on OD CSP, at 25 °C is shown.

Using a HPLC apparatus equipped with a simultaneous UV and CD detection, it was clear that the two peaks did not correspond to the enantiomers of **2**. The first UV signal (solid trace) did not end up in a significant CD detection, whereas the second UV peak yielded a chiroptical trace (dotted trace) of opposite. Furthermore, the UV spectra of two eluted peaks, acquired during the chromatographic separation, were different. In order to study possible dynamic processes occurring inside the column and to evaluate the stereointegrity of chiral analytes a combination of temperature- and flow-dependent HPLC (dynamic HPLC, DHPLC) [27–33] was performed.

The temperature-dependent chromatographic behavior of racemate **2** on OD CSP in the range −20 to +40 °C is illustrated in Fig. 4. At a temperature of −20 °C the chromatographic pattern showed two peaks corresponding to the enantiomers of **2** separated by an intermediate interconversion zone near the zero base line. The presence in the elution pattern of a plateau between the peaks of two separated enantiomers indicates that an interconversion phenomenon takes place within the time scale of chromatographic enantiomer separation [34]. By raising the temperature from −20 to +10 °C, the extent of enantiomerization increased and the relative area for the peak corresponding to the first enantiomer decreased accordingly to the second enantiomer. In addition, a new achiral species appeared before the first eluted enantiomer. In the temperature range +20 to +40 °C the two peaks due to the enantiomers of **2** coalesce yielding a broad single peak. At the higher temperature investigated (+40 °C) the CD signal (dotted trace) of the first enantiomer almost disappeared and two peaks bridged by plateau were observed by UV monitoring (solid trace). When the same chromatographic separation with elution at different flow-rates (1.3, 0.7 and 0.25 ml min^{-1}) and at a temperature of +10 °C was carried out, the extent of interconversion increased accordingly with the increased chromatographic time scale, as shown in Fig. 5.

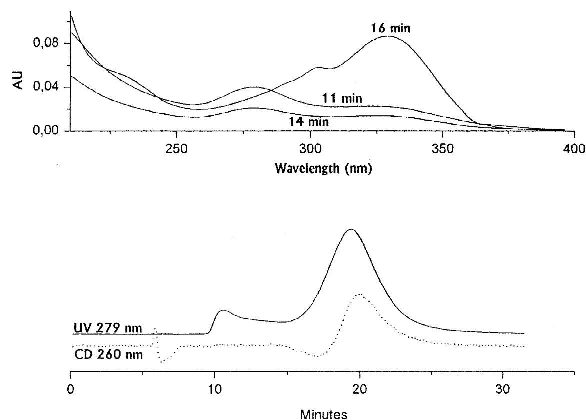

Fig. 3. Top: UV spectra acquired at run time of 11, 14 and 16 min during HPLC separation of **2**. Bottom: chromatogram of racemic **2** with UV (279 nm) (solid trace) and CD (260 nm) (dotted trace) detection. Column, Chiralcel OD (250×4.6-mm I.D.); eluent, *n*-hexane–ethanol (85:15, v/v); flow-rate, 0.5 ml min^{-1}; column temperature, 25 °C.

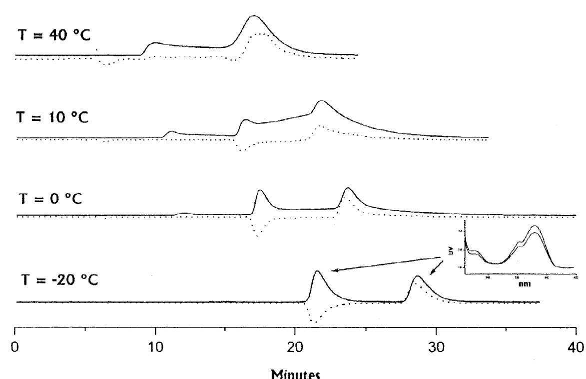

Fig. 4. UV (279 nm) (solid trace) and CD (260 nm) (dotted trace) detected variable-temperature HPLC of racemic **2** on Chiralcel OD CSP. Inset: UV spectra acquired during HPLC of **2** at −20 °C. Eluent, *n*-hexane–ethanol (85:15, v/v); flow-rate, 0.5 ml min^{-1}; column temperature, from top to bottom, +40, +10, 0, −20 °C.

The separated stereoisomers of 3-methyl-1,2,5-benzothiadiazepin-4-ones yielded symmetrical peaks in each experimental condition of enantioseparation (CSP, temperature, flow-rate) and no interconversion profile was detected. In contrast, the elution profiles obtained by enantioselective HPLC of chiral 3-

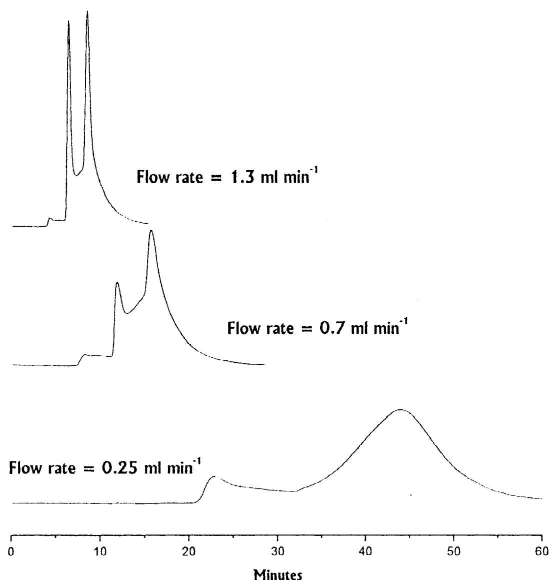

Fig. 5. Variable-flow HPLC of racemic **2**. Column, Chiralcel OD (250×4.6-mm I.D.); eluent, *n*-hexane–ethanol (85:15, v/v); detection wavelength, 279 nm; flow-rate, from top to bottom, 1.3, 0.7, 0.25 ml min^{-1}; column temperature, +10 °C.

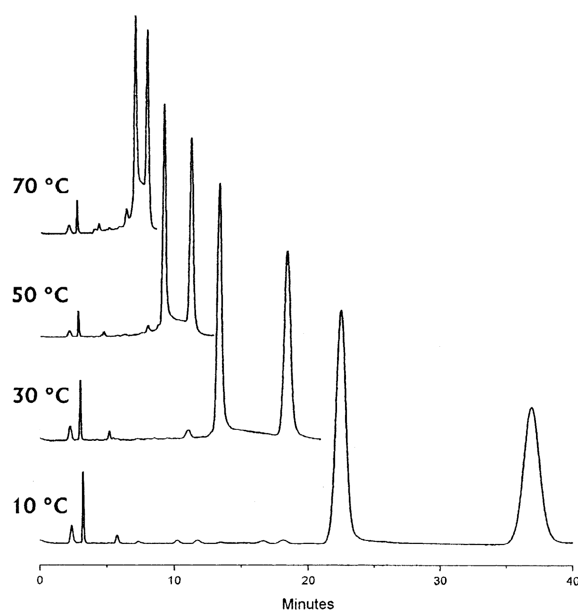

Fig. 6. Dynamic HPLC of racemic **4**. Column, Chiraspher (250×4.0-mm I.D.); eluent, *n*-hexane–2-propanol (70:30, v/v); flow-rate, 1.0 ml min^{-1}; detection wavelength, 254 nm; column temperature, from top to bottom, 70, 50, 30, 10 °C.

methyl-1,2,5-benzothiadiazepin-4-thiones were strictly dependent on which CSP was used. At room temperature and similar running time in column, peaks resulting from enantioseparation of benzothiadiazepinthiones on AS, AD (in the case of the resolved analyte **2**) and Chiraspher CSPs, exhibited a symmetric shape and slight broadening and the interconversion zone was considerably less pronounced compared to the one observed on OD CSP. The effect of temperature on shape elution profiles is illustrated in Fig. 6 by the resolution of **4** on Chiraspher CSP. The extent of on-column enantiomerization increased while increasing the temperature from 10 to 70 °C, without obtaining complete coalescence of two peaks at higher temperature investigated. In Fig. 7 peaks resulting from enantioseparation of **8** on AS CSP, between 10 and 40 °C are shown. In both of the Chiraspher and AS CSPs, the raise in temperature produced progressively sharper peaks and 50:50 peak areas were observed at each temperature investigated.

Inspection of the data obtained by dynamic enantioselectivity chromatography of chiral compounds **1–12** reveals that by replacing the C=O by the C=S moiety an intermediate interconversion zone between the two separated peaks could be observed. The on-column enantiomerization and, consequently,

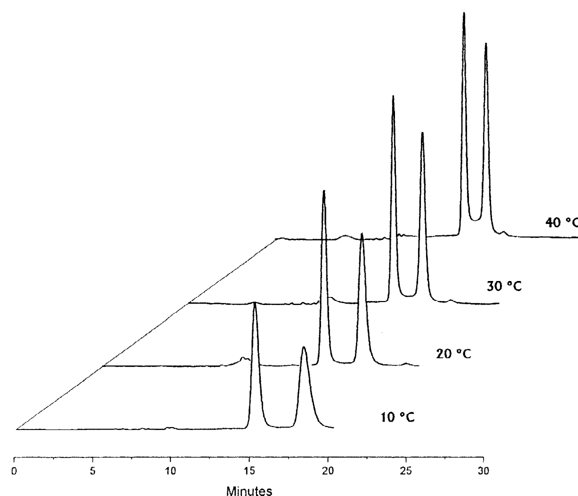

Fig. 7. Dynamic HPLC of racemic **8**. Column, Chiralpak (250×4.6-mm I.D.); eluent, *n*-hexane–ethanol (70:30, v/v); flow-rate, 0.5 ml min^{-1}; detection wavelength, 254 nm; column temperature, from top to bottom, 40, 30, 20, 10 °C.

peak deformations, were not detected on the chromatographic time scale when the rate of interconversion of exchanging species was made low enough, by varying column temperature and flow-rate, to be negligible with respect to physical enantioseparation process.

3.3. Stopped-flow high-performance liquid chromatography

In order to quantify the influence of the chiral selector on the enantiomerization process, a stopped-flow HPLC procedure was applied for determining the individual rate of interconversion and free energy barriers of stereolabile enantiomers of **2** during their flow through the Chiraspher and OD CSPs. The common mobile phase *n*-hexane–ethanol (70:30, v/v), compatible with both CSPs employed, was selected. Unfortunately, enantioselectivity of Chiraspher and OD CSPs was not sufficiently high to permit on-column kinetic investigations of racemic sample [18] and, consequently, preliminary semipreparative enantioseparation to isolate the individual enantiomers of **2** was performed. As a result of screening of some organic modifiers, it was found that using a ternary mixture *n*-hexane–chloroform–2-propanol (50:50:2, v/v/v) as eluent, the best resolution of **2** on Chiraspher CSP was achieved. Flow-rate of 1.0 ml min^{-1} and temperature of 10 °C yielded well-separated enantiomers, with an estimated enantioseparation factor (α) and resolution factor (*Rs*) of 1.55 and 5.71, respectively. The scale-up of analytical chromatographic parameters at semipreparative level permitted the enantiomeric fractionation of 10 mg of **2** for run on a 10-mm I.D. Chiraspher NT CSP within 20 min. Chromatographic and polarimetric analysis indicated that the first collected fraction showed an enantiomeric excess (e.e.) of 92.0% and specific rotation $[\alpha]_D^{23} + 160.0$ ($c = 0.254$, dichloromethane), while the recovered second fraction showed specific rotation $[\alpha]_D^{23} - 159.0$ ($c = 0.252$, dichloromethane) and similar enantiomeric excess (93.0%).

The single enantiomers were injected onto the chiral column (Chiraspher or Chiralcel OD) placed in a cryostat at temperature T_1. After a specific time (1.5 min) the eluent flow (flow-rate = 1.0 ml min^{-1} for Chiraspher CSP and 0.7 ml min^{-1} for OD CSP) was turned off and the column was brought in a thermostat set at higher temperature T_2. The enantiomer partially interconverted in the presence of the chiral medium for a period of time *t*. The enantiomerization process was stopped by cooling back the column to the previous temperature. Then, after 3 min the mobile phase flow was resumed and the areas of the resulting two peaks measured. At temperature T_2 (0 °C for Chiraspher CSP and −20 °C for OD CSP) the extent of on-column isomerization of **2** was negligible at HPLC time scale and peak coalescence was not recognizable. By changing the time *t* of permanence of enantiomer at the temperature T_2, the apparent enantiomerization rate constants, k^{app} (k^{app} represents a weighted mean of individual rate constants in the achiral mobile phase and in the chiral stationary phase [35]) could be calculated by plotting the ln e.e. of each enantiomer versus time [20]. On-column enantiomerization kinetics were recorded at temperatures of 25, 30, 35, 40, 45 and 50 °C in presence of Chiraspher CSP. The correlation coefficients of the regression lines were $r^2 > 0.99$. Similar reversible first-order kinetics were obtained during the permanence of the enantiomers of **2** on the OD CSP. In the latter case the stopped-flow HPLC experiments were performed at four different temperatures (6, 10, 15 and 20 °C). The enantiomerization process was too rapid at 25 °C for the exact rate constant to be determined. Fig. 8 represents a typical example of the decreasing e.e. with time in (−)-**2** at 20 and 25 °C during stopped-flow experiments on OD and Chiraspher CSPs, respectively. The initial enantiomeric excess of 92.0% gradually decreased to 14.0% after 10 min at constant temperature of 20 °C in presence of OD CSP, while the chiral environment of Chiraspher CSP produced a slower enantiomerization of (−)-**2** (the e.e. decreased to 39% after 120 min) at the higher temperature of 25 °C.

To calculate the apparent free energy barriers ($\Delta G^{\ddagger\ app}$) of the reversible microscopic process, the apparent rate constants of enantiomerization (k^{app}) were fit into the Eyring equation [20]. Kinetic (k^{app}) and thermodynamic ($\Delta G^{\ddagger\ app}$) data related to on-column enantiomerization of **2** are summarized in Table 2. The first eluted (−)-**2** enantiomer on OD CSP was found to enantiomerize more rapidly than the second eluted (+)-enantiomer at each tempera-

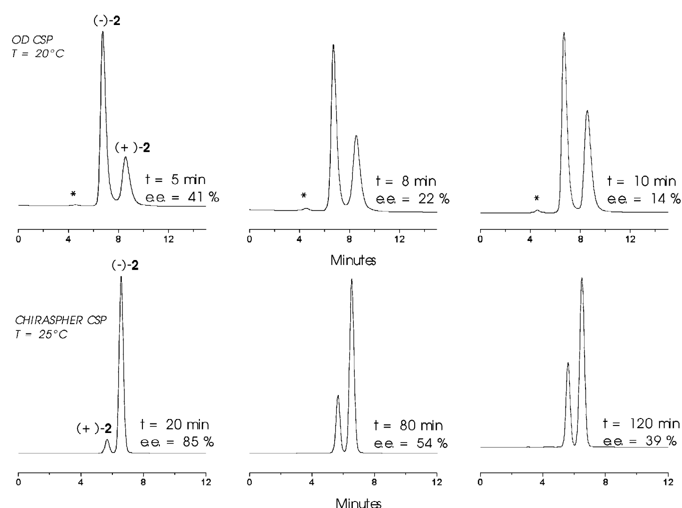

Fig. 8. Elution patterns of (−)-**2** obtained by stopped-flow HPLC. Top: OD CSP; time interval for on-column enantiomerization, 5, 8 and 10 min; temperature, 20 °C. Bottom: Chiraspher CSP; time interval for on-column enantiomerization, 20, 80 and 120 min; temperature, 25 °C; detection wavelength, 329 nm. Asterisk denotes an unknown species. See text for details.

ture investigated (entries 13–20). At a temperature of 20 °C the enantiomerization of (−)-**2** enantiomer was about 2-fold faster than (+)-**2** enantiomer (entries 19 and 20). On the contrary, the chiral environment of Chiraspher displays a small discriminating effect on the individual rates of stereoisomerization (entries

Table 2
Apparent enantiomerization rate constants (k^{app}) and apparent free energy barriers ($\Delta G^{\ddagger\ app}$) for the on-column enantiomerization of the enantiomers of **2** from stopped-flow experiments at various temperatures

Entry	Enantiomer	CSP	T (°C)	$\Delta G^{\ddagger\ app}$ (kJ mol^{-1})	k^{app} (s^{-1})
1	(+)-**2**	Chiraspher	25	96.8±0.0	$6.86\times10^{-5}\pm0.2\times10^{-5}$
2	(−)-**2**	Chiraspher	25	97.0±0.1	$6.26\times10^{-5}\pm0.33\times10^{-5}$
3	(+)-**2**	Chiraspher	30	97.4±0.1	$1.05\times10^{-4}\pm0.06\times10^{-4}$
4	(−)-**2**	Chiraspher	30	97.6±0.0	$9.53\times10^{-5}\pm0.21\times10^{-5}$
5	(+)-**2**	Chiraspher	35	98.3±0.1	$1.38\times10^{-4}\pm0.07\times10^{-4}$
6	(−)-**2**	Chiraspher	35	98.4±0.1	$1.36\times10^{-4}\pm0.05\times10^{-4}$
7	(+)-**2**	Chiraspher	40	98.7±0.1	$2.21\times10^{-4}\pm0.09\times10^{-4}$
8	(−)-**2**	Chiraspher	40	99.1±0.1	$1.89\times10^{-4}\pm0.05\times10^{-4}$
9	(+)-**2**	Chiraspher	45	99.6±0.1	$2.96\times10^{-4}\pm0.17\times10^{-4}$
10	(−)-**2**	Chiraspher	45	99.8±0.0	$2.72\times10^{-4}\pm0.04\times10^{-4}$
11	(+)-**2**	Chiraspher	50	99.8±0.1	$4.95\times10^{-4}\pm0.26\times10^{-4}$
12	(−)-**2**	Chiraspher	50	100.5±0.0	$3.78\times10^{-4}\pm0.06\times10^{-4}$
13	(+)-**2**	OD	6	87.0±0.1	$3.07\times10^{-4}\pm0.16\times10^{-4}$
14	(−)-**2**	OD	6	85.3±0.0	$6.28\times10^{-4}\pm0.20\times10^{-4}$
15	(+)-**2**	OD	10	87.5±0.1	$4.21\times10^{-4}\pm0.23\times10^{-4}$
16	(−)-**2**	OD	10	86.0±0.1	$7.94\times10^{-4}\pm0.41\times10^{-4}$
17	(+)-**2**	OD	15	88.4±0.1	$5.62\times10^{-4}\pm0.35\times10^{-4}$
18	(−)-**2**	OD	15	86.7±0.2	$1.16\times10^{-3}\pm0.08\times10^{-3}$
19	(+)-**2**	OD	20	89.4±0.1	$7.03\times10^{-4}\pm0{,}41\times10^{-4}$
20	(−)-**2**	OD	20	87.6±0.1	$1.50\times10^{-3}\pm0.07\times10^{-3}$

See text for operational details.

1–12). At the temperature of 50 °C the apparent rate constants were $4.95\times10^{-4}\pm0.26\times10^{-4}$ and $3.78\times10^{-4}\pm0.06\times10^{-4}$ s^{-1} for (+)-**2** (first eluted enantiomer) and (−)-**2** (second eluted enantiomer), respectively.

Single enantiomers of **2** were also subjected to off-column thermal racemization at 50 °C. The same mixture *n*-hexane–ethanol used for the on-column enantiomerization investigations was employed as reaction solvent. Similarly to the enantiomerization process, the off-column racemization also followed first-order reaction kinetics with a rate constant, *k*, of 2.5×10^{-5} s^{-1} at 50 °C. After conversion of the racemization rate constant to the enantiomerization constant [36] a value of free energy barrier, $\Delta G^{\ddagger}$, of 109.7 kJ mol^{-1} was obtained.

Analysis of kinetic and thermodynamic parameters indicates that the enantiomerization of **2** was strongly perturbed by the presence of the chiral selector being slower in free solution than in the presence of Chiraspher and OD CSPs. Moreover, under the influence of OD CSP substantial differences in the rate of interconversion of the two enantiomers of **2** were produced. As a rule of thumb, when a chiral analyte is introduced as racemate in a chiral HPLC column, the two peaks of enantiomers should appear in the dynamically modified plots with the same area. A shift of the original 1:1 ratio of stereolabile enantiomers after their passage through a chiral column cannot be realized under a usual chromatographic time scale [37]. We do not at the moment have any definitive interpretation for the above-mentioned data. A tentative explanation might be proposed if one takes into account that an achiral compound during the HPLC of **2** and off-column experiments was generated. The structure of such achiral species is unknown. We can suppose that a second process, such as a decomposition reaction in addition to enantiomerization, may occur. The occurrence of decomposition reaction on the chiral environment of the selector might lead to a stereospecific subtraction of enantiomers of **2** from reversible enantiomerization process.

3.4. Elution order

In order to study the chiroptical properties of individual enantiomers of analytes investigated semipreparative chromatographic resolution of representative 3-methyl-1,2,5-benzothiadiazepin-4-ones **1** and **3** on a 10-mm I.D. AD CSP were performed. The injection of 24 mg of racemic compound **1** and 10 mg of racemic compound **3** yielded both enantiomers with a high degree of purity (e.e.>98%) and with a yield of 60 and 65%, respectively. Specific rotation values were determined by polarimetry. Specific rotation of the isolated enantiomers showed the following values: (+)-**1**, e.e.=98.1%, $[\alpha]_D^{23}$ +99.0 (c=0.25, dichloromethane); (−)-**1**, e.e.=98.3%, $[\alpha]_D^{23}$ −101.0 (c=0.20, dichloromethane); (+)-**3**, e.e.=98.0%, $[\alpha]_D^{23}$ +129.0 (c=0.21, dichloromethane); (−)-**3**, e.e.=99.1%, $[\alpha]_D^{23}$ −140.0 (c=0.17, dichloromethane). Fig. 9 shows typical chromatograms illustrating the semipreparative enantioseparation of **3** and the analytical investigations of the two collected fractions.

In both cases of **1** and **3**, the (−)-form was the second eluted enantiomer on all CSPs studied. The assignment of the absolute configuration of enantiomers of compounds **1** and **3**, could be empirically established according to their CD spectral properties. As shown in Fig. 10, the CD spectra of (−)-enantiomers of **1** and **3**, exhibited an intense negative and broad Cotton-effect around 260 nm and a sharper band, of opposite sign, near 240 nm. For the

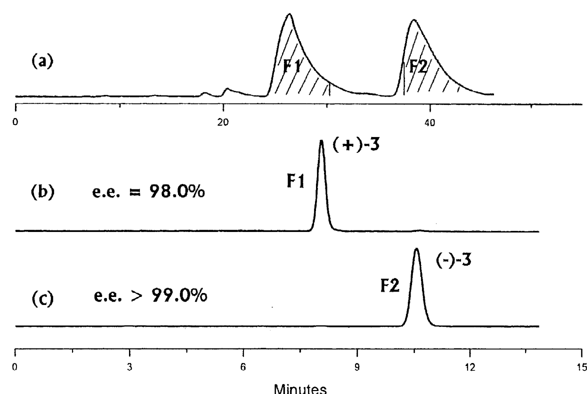

Fig. 9. Trace (a): semipreparative enantioseparation of 10 mg of **3**. Column, Chiralcel AD (250×10-mm I.D.); eluent, *n*-hexane–2-propanol (80:20, v/v); flow-rate, 2.5 ml min^{-1}; detection wavelength, 310 nm; column temperature, 25 °C. Traces (b,c): purity control of collected fractions F1 and F2, respectively. Column, Chiralpak AD (250×4.6-mm I.D.); eluent, *n*-hexane–2-propanol (70:30, v/v); flow-rate, 1.0 ml min^{-1}; detection wavelength, 254 nm; column temperature, 25 °C.

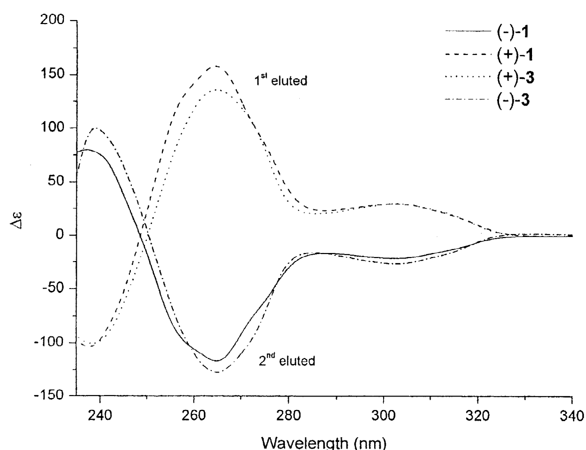

Fig. 10. Circular dichroism (CD) spectra of the enantiomers of **1** and **3** in dichloromethane at 25 °C. The elution order is attributed to all CSPs investigated in the present paper.

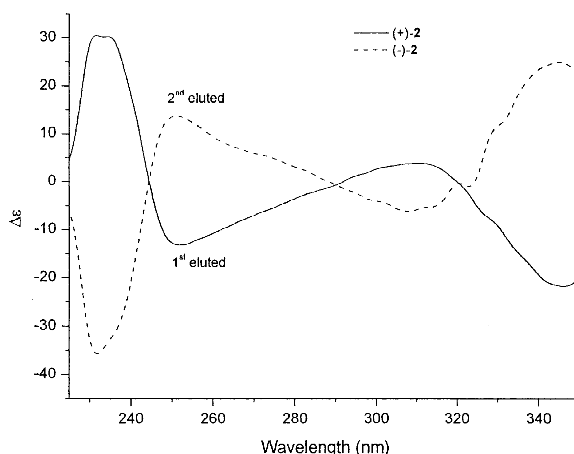

Fig. 11. Circular dichroism (CD) spectra of the enantiomers of **2** in dichloromethane at 25 °C. The resolution of the pair of the enantiomers was carried out by analytical HPLC on Chiraspher CSP: solid and dotted traces correspond, respectively, to the first and second eluted enantiomer.

corresponding (+)-enantiomers the CD spectra were inverted, as expected. Taking into account the CD profiles of related 1,4-benzodiazepin-2-ones reported in literature, we can assign the absolute configuration at C_3 of 3-methyl-1,2,5-benzothiadiazepine-4-ones on the basis of sign of the 260 nm CD band. The (*R*)-enantiomer of 3-substituted 1,4-benzodiazepinones has been found to be levorotatory [38–40]. The 260 nm CD band is ascribed to a ^1L_b transition of the aryl group and it is negative in the CD spectra of the (*R*)-enantiomers of 3-methyl-1,4-benzodiazepinones [41] and other 3-substituted benzodiazepinones [42,43]. Thus, with the assumption of a structural analogy between the two classes of compounds, the chiroptical assignment of dextrorotatory and levorotatory enantiomers of **1** and **3** as (+)-(*S*) and (−)-(*R*), respectively, is straightforward. The elution order for the C3-chiral 1,2,5-benzothiadiazepin-4-ones on polyacrylamide- and polysaccharide-based CSPs was (+)-(*S*)- before (−)-(*R*)-enantiomer. By structural analogy, it could be expected that the other 3-methyl-1,2,5-benzothiadiazepin-4-ones would show same sense chiral recognition mechanism with preferential retention of the (−)-(*R*)-enantiomer. The stereochemical relationship between **1** and **2** was determined by chiroptical characterization of the individual enantiomers of **2** obtained by semipreparative chiral chromatography (see Section 3.3). The CD spectrum of the less retained enantiomers of (+)-**2**, as shown in Fig. 11, exhibited negative Cotton effect at the benzenoid ^1L_b band. This data allowed the assignment of (*R*)-configuration to the first eluted enantiomer on Chiraspher CSP and confirmed that an inversion of the elution order between of the enantiomers of **1** and **2** on the polyacrylamide based CSP occurred (Fig. 8). On the contrary, the compound **2** showed the same elution order as that of **1** on the polysaccharides-based CSP studied, being the (+)-(*R*)-**2** the more retained enantiomer. The observed reversal elution order indicates that, despite of a simple visual examination of the obtained chromatographic data, the carbonyl moiety of analytes results critically implicated in the resolution mechanism on Chiraspher CSP. Indeed, by replacing C=O by C=S group, a different contribution of interactions, such as hydrogen bond and electrostatic interactions, with electrically complementary moieties present on the selector occurred. Consequently, higher affinity of (−)-(*S*)-enantiomer over (+)-(*R*)-enantiomer of **2** towards Chiraspher CSP was observed.

Acknowledgements

We are grateful to Ms. L. Turchetto for her helpful

collaboration. Thanks are due to the Italian MIUR (Cofin 2000) for partial financial support.

References

[1] M. Artico, Farmaco 51 (1996) 305.
[2] M.J. Kukla, H.J. Breslin, R. Pauwels, C.L. Fedde, M. Miranda, M.K. Scott, R.G. Sherrill, A. Raeymaekers, J. Van Gelder, K. Andries, M.A.C. Janssen, E. De Clerq, P.A.J. Janssen, J. Med. Chem. 34 (1991) 746.
[3] M.J. Kukla, H.J. Breslin, C.J. Diamond, P.P. Grous, C.H. Ho, M. Miranda, J.D.R. Rodgers, R.G. Sherrill, E. De Clerq, R. Pauwels, K. Andries, L.J. Moens, M.A.C. Janssen, P.A.J. Janssen, J. Med. Chem. 34 (1991) 3187.
[4] H.J. Breslin, M.J. Kukla, D.W. Ludovici, R. Mohrbacher, W. Ho, M. Miranda, J.D. Rodgers, T.K. Hitchens, G. Leo, D.A. Gauthier, C.H. Ho, M.K. Scott, E. De Clerq, R. Pauwels, K. Andries, M.A.C. Janssen, P.A.J. Janssen, J. Med. Chem. 38 (1995) 771.
[5] W. Ho, M.J. Kukla, H.J. Breslin, D.W. Ludovici, P.P. Grous, C.J. Diamond, M. Miranda, J.D. Rodgers, C.H. Ho, E. De Clerq, R. Pauwels, K. Andries, M.A.C. Janssen, P.A.J. Janssen, J. Med. Chem. 38 (1995) 794.
[6] S. Massa, R. Di Santo, R. Costi, M. Artico, A.G. Loi, M. Doa, P. Scano, P. La Colla, Med. Chem. Res. 4 (1994) 554.
[7] M. Artico, R. Di Santo, R. Costi, S. Massa, F. Scintu, A.G. Loi, A. De Montis, P. La Colla, Bioorg. Med. Chem. Lett. 7 (1997) 1931.
[8] R. Di Santo, R. Costi, M. Artico, S. Massa, M.E. Marongiu, A.G. Loi, M. Putzolu, P. La Colla, Chem. Chemother. 9 (1998) 127.
[9] M. Artico, R. Di Santo, R. Costi, E. Novellino, G. Greco, S. Massa, E. Tramontano, M.E. Marongiu, A. De Montis, P. La Colla, J. Med. Chem. 41 (1998) 3948.
[10] R. Costi, R. Di Santo, M. Artico, S. Massa, A. Lavecchia, T. Marceddu, L. Sanna, P. La Colla, M.E. Marongiu, Chem. Chemother. 11 (2000) 117.
[11] R. Costi, R. Di Santo, M. Artico, S. Massa, J. Heterocycl Chem. 39 (2002) 81.
[12] R. Costi, R. Di Santo, M. Artico (in press).
[13] G. Gubitz, Chromatographia 30 (1990) 555.
[14] Anonymous, Chirality 4 (1992) 338.
[15] K. Cabrera, M. Jung, M. Fluck, V. Schurig, J. Chromatogr. A 731 (1996) 315.
[16] W. Burkle, H. Karfunkel, V. Schurig, J. Chromatogr. 288 (1984) 1.
[17] O. Trapp, G. Schoetz, V. Schurig, Chirality 13 (2001) 403.
[18] E. Tobler, M. Lammerhofer, G. Mancini, W. Lindner, Chirality 13 (2001) 641.
[19] C. Villani, W.H. Pirkle, Tetrahedron Asymmetry 6 (1995) 1.
[20] K.P. Scharwachter, D.H. Hochmuth, H. Dittmann, W.A. Konig, Chirality 13 (2001) 679.
[21] T. Nakano, J. Chromatogr. A 906 (2001) 205.
[22] G. Blaschke, W. Broker, W. Fraenkel, Angew. Chem. Int. Ed. Engl. 25 (1986) 860.
[23] K. Krause, M. Girod, B. Chankvetadze, G. Blaschke, J. Chromatogr. A 837 (1999) 51.
[24] Y. Okamoto, E. Yashima, Angew. Chem. Int. Ed. 37 (1998) 1020.
[25] E. Yashima, C. Yamamoto, Y. Okamoto, Synlett April (issue 4) (1998) 344–360.
[26] Y. Okamoto, Y. Kaida, J. Chromatogr. A 666 (1994) 403.
[27] F. Gasparrini, I. D'Acquarica, M. Pierini, C. Villani, J. Sep. Sci. 24 (2001) 941.
[28] J. Veciana, M.I. Crespo, Angew. Chem. Int. Ed. Engl. 30 (1991) 74.
[29] K. Lorenz, E. Yashima, Y. Okamoto, Angew. Chem. Int. Ed. Engl. 37 (1998) 1922.
[30] J. Oxelbark, S. Allenmark, J. Org. Chem. 64 (1999) 1483.
[31] F. Gasparrini, D. Misiti, M. Pierini, C. Villani, J. Chromatogr. A 724 (1996) 79.
[32] O. Trapp, G. Schoetz, V. Schurig, Chirality 13 (2001) 403.
[33] R. Cirilli, C. Di Bugno, F. La Torre, Chromatographia 49 (1999) 628.
[34] A. Mannschreck, H. Zinner, N. Pustet, Chimia 43 (1989) 165.
[35] J. Oxelbark, S. Allenmark, J. Chem. Soc. Perkin Trans. 2 (1999) 1587.
[36] E.L. Eliel, S.H. Wilen, in: Stereochemistry of Organic Compounds, Wiley, New York, 1994, p. 426.
[37] F. Gasparrini, L. Lunazzi, D. Misiti, C. Villani, Acc. Chem. Res. 28 (1995) 163.
[38] J.F. Denissen, J. Chromatogr. 462 (1989) 454.
[39] S.K. Yang, X. Lin Lu, J. Pharm. Sci. 78 (1989) 789.
[40] H. Kanazawa, Y. Kunito, Y. Matsushima, S. Okubo, F. Mashige, J. Chromatogr. A 871 (2000) 181.
[41] T. Alebic-Kolbah, F. Kajfez, S. Rendic, V. Sunjic, A. Konowal, G. Snatzke, Biomed. Pharmacol. 28 (1979) 2457.
[42] S.K. Yang, X. Lin Lu, J. Pharm. Biomed. Anal. 11 (1993) 1189.
[43] P. Salvadori, C. Bertucci, E. Dominici, G. Giannaccini, J. Pharm. Biomed. Anal. 7 (1989) 1735.