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HPLC quantitation of the four stereoisomers of benzoxathiepin derivatives with cellulose phenyl type chiral stationary phase and circular dichroism detection

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Abstract

The chiral separation of a new antianginal agent has been investigated on a chiral cellulose column with UV and circular dichroism (CD) detection. This benzoxathiepin derivative under development has two stereogenic centers whose (*R*,*S*) stereoisomer shows an interesting antianginal activity. After optimisation of the mobile phase composition, a baseline-resolved separation of the four stereoisomers was achieved on a Chiralcel OJ-H chiral column by using methanol–ethanol–diethylamine (25:75:0.1, v/v/v) as mobile phase. The CD detection system allowed quantitation and a linear response was observed within a 10–200 $\mu g\,mL^{-1}$ concentration range ($r^2 = 0.9966$) and limit of quantification down to 2 $\mu g\,mL^{-1}$ was achieved.

Keywords: Circular dichroism; HPLC; Benzoxathiepins; Derivatised cellulose; Basic drug; Chiral separation

1. Introduction

Most of the time, the optical isomers of bioactive chiral compounds exhibit differences in terms of pharmacodynamics (therapeutic action, toxicity) and in terms of pharmacokinetics (for example, plasmatic half-life, diffusion or metabolic rate). Regulatory authorities therefore recommend that new chiral drugs should ideally be marketed only in the form of pure enantiomers.

A novel antianginal agent, part of the chemical family of benzoxathiepins and patent protected [1], owns two chiral centers, resulting in four stereoisomers, namely (*R*,*S*), (*S*,*R*), (*R*,*R*) and (*S*,*S*). As with most chiral bioactive compounds, only one stereoisomer (*R*,*S*) shows an interesting antianginal activity and is currently undergoing drug development.

Sugihara and co-workers developed several benzoxathiepins showing an antagonistic activity towards serotoninergic receptors [2,3] and also published patents about their cardiovascular activity [4]. Other pharmacological issues of benzoxathiepins were reported in patents concerning glaucoma treatment [5] and antiplatelet aggregation [6].

Such differences in pharmacological activity necessitate analytical methods for the determination of the enantiomeric purity of drugs during chemical and pharmaceutical developments. Among various analytical techniques (HPLC, CE, GC) suitable for the separation of chiral drugs, the most widely used is HPLC despite the cost of chiral stationary phases (CSPs). This is due to the many interesting features of chiral HPLC: (i) applications to numerous chiral compounds through a wide range of available CSPs, (ii) reliability, and (iii) possibility to transfer analytical HPLC methods to preparative scale.

Polysaccharides are widely used as chiral stationary phases (CSPs) in LC [7]. Among these CSPs, cellulose tris(4-methylbenzoate) (Chiralcel OJ-H) has proven to be useful for the enantioseparation of drugs. This CSP was used successfully by Chankvetadze et al. to separate basic drugs in the polar organic mode [8]. Also ligands for melatonin receptors containing two chiral centers and cardiovascular drugs such as isradipine, formoterol and verapamil were analysed with a Chiralcel OJ-H column [9–10].

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Circular dichroism (CD) is of particular interest when dealing with chiral compounds, mostly due to its selectivity. In fact, only molecules showing chiroptical properties can be detected [11]. Furthermore, CD has a high selectivity because the CD signal can be observed in the UV region where an electronic transition occurs, giving rise to UV absorption phenomenon. The principle of CD is based on absorption differences between left and right circularly polarized lights. Nowadays, CD spectroscopy is gaining increasing importance in pharmaceutical analysis due to the commercially available CD detectors in liquid chromatography. Several HPLC-CD applications have been reported concerning metabolism studies [12,13] and determination of absolute configuration [14–16]. Several authors have previously shown the interest of using CD detection in HPLC for the resolution of compounds having two [17] or three [18] pairs of enantiomers. Another promising application concerns the simultaneous recording of UV and CD chromatograms at the same wavelength for the determination of enantiomeric excess on achiral HPLC stationary phases [19,20]. The CD signal depends only on the enantiomeric composition of the chiral molecule whereas the UV absorption signal is related to the analyte concentration. The dimensionless anisotropy factor ($g = \Delta\varepsilon/\varepsilon$) is independent of the concentration of the analyte but is linearly related to the enantiomeric purity.

In this study, we investigate the stereo-specific analysis of the four stereoisomers of this basic drug by chiral HPLC with CD detection.

2. Experimental

2.1. Equipment

HPLC experiments were carried out on a Varian (Walnut Creek, CA, USA) model 9010 ternary pump equipped with a Rheodyne (Cotati, CA, USA) model 7125 injection valve fitted with 20 μL (or 200 μL) loop. The chiral separations were achieved under isocratic conditions using a Chiralcel OJ-H column (250 mm × 4.6 mm i.d., particle size 5 μm), preceded by a guard cartridge (10 mm × 4 mm i.d., same CSP as column) purchased from Daicel (Tokyo, Japan). This chiral column is made with a spherical silica support onto which a chiral selector cellulose tris(4-methylbenzoate) is physically coated. The column was maintained at 35 °C with the help of a Gecko-Cil oven (Cluzeau-Info-Labo, Sainte Foy La Grande, France). The compounds were eluted at a flow rate of 1 mL min^{-1} and detected by a Jasco CD-2095 circular dichroism detector (Jasco, Tokyo, Japan). The Jasco CD-2095 is a commercially available detector specifically designed for the on-line monitoring of circular dichroism at a fixed wavelength. The path-length and the volume of the detection cell were equal to 25 mm and 40 μL, respectively. Chromatographic data were collected and analyzed using EZChrom Elite software version 2.5 (Scientific Software, Pleasanton, CA, USA). The resolution R_S was calculated using the following expression: $R_S = 2\,(t_{R2} - t_{R1})/(w_{b1} + w_{b2})$, where t_R is the retention time and w_b is the width at the peak base. The CD spectra of the solutions were measured at ambient temperature with a 1 nm increment in the range 220–350 nm using a 1.0 mm path-length cell using a Jasco J-810 Spectropolarimeter. The dichroic signal is expressed in terms of molar circular dichroism, i.e. $\Delta\varepsilon$.

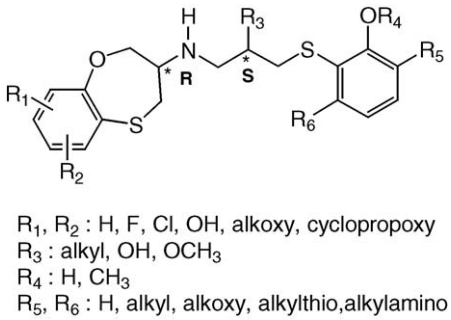

Fig. 1. General structure of benzoxathiepin derivatives.

2.2. Chemicals

Methanol and ethanol are HPLC grade from SDS (Peypin, France). Diethylamine (DEA) was purchased from Fluka (Saint-Quentin-Fallavier, France). The four stereoisomers of this drug were synthesised and purified at Pierre Fabre Research Center (Castres, France). The pK_a (7.4) and log P (5.45) values were measured by using D-PAS and GLp*K*a apparatus, respectively both from Sirius analytical instruments (Forest Row, UK).

2.3. Mobile phases and samples

The mobile phases for chiral separation were a mixture of methanol and ethanol containing diethylamine (0.1%, v/v) as a modifier and were degassed before use. All stock solutions (1 mg mL^{-1}) were prepared in methanol. Appropriate volumes of stock solutions of each stereoisomer were mixed to give required concentrations in a final solution containing methanol with 0.1% DEA. All samples were filtered with 0.22 μm pore-size PTFE membrane filter (Alltech, Templemars, France).

3. Results and discussion

The isomer of interest (*R*,*S*), and its three relative optical impurities (*R*,*R*), (*S*,*R*) and (*S*,*S*), belong to benzoxathiepin family and their chemical structure are given in Fig. 1. The positions assigned *R* and *S* of the isomer of interest are also given. This drug is a weakly basic compound owing to a secondary amine function (pK_a = 7.4).

3.1. Circular dichroism spectra

Fig. 2 shows the CD spectra of four stereoisomers recorded on a spectropolarimeter. As expected, spectra showed intensity of opposite signs considering the couples of enantiomers *R*,*R*/*S*,*S* on one hand and *R*,*S*/*S*,*R* on the other hand. The availability of the CD spectrum enables the best CD wavelength in terms of sensitivity to be selected. As each stereoisomer has an optimum

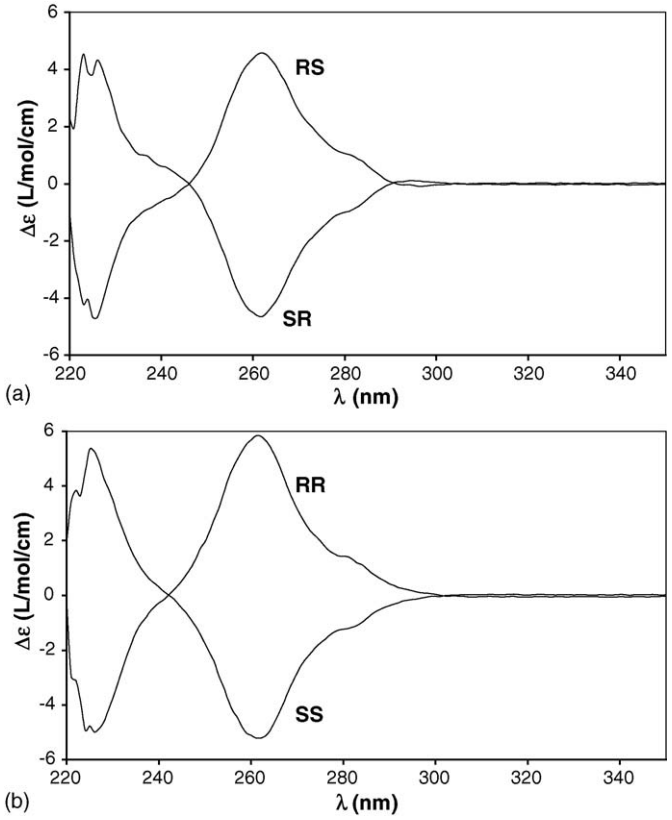

Fig. 2. CD spectra of the four benzoxathiepin stereoisomers recorded with a J-810 spectropolarimeter. Conditions: Section 2. Stereoisomer concentration: 333 μg mL^{-1} dissolved in HPLC mobile phase.

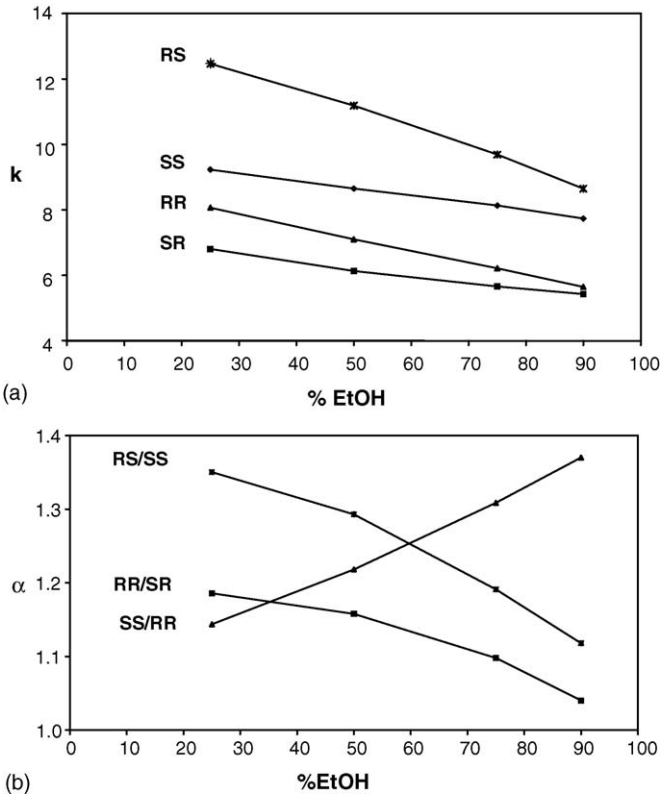

Fig. 3. Influence of ethanol fraction volume in the mobile phase upon (a) retention factor (b) selectivity on cellulose-based chiral column operating in the polar organic HPLC mode. Column: Chiralcel OJ-H; dimensions: 250 mm × 4.6 mm i.d., particle size 5 μm; mobile phase: methanol–ethanol (v/v) with 0.1% of DEA; flow rate: 1 mL min^{-1}; temperature: 35 °C; loop volume: 20 μL; detection wavelength: 262 nm; stereoisomer concentration: 25 μg mL^{-1}.

of intensity at 262 nm, all UV and CD detectors were set at this wavelength.

3.2. Chiral separation

Several compounds of the benzodiazepin family which have structural similarities with benzoxathiepins were previously resolved on cellulose CSPs. The four stereoisomers of the diltiazem were resolved in SFC using cellulose tris(3,5-dimethylcarbbamate) (Chiralcel OD) as sorbent and with CO_2–isopropanol as mobile phase [21]. Oxazepam was resolved on Chiralcel OJ with a mixture of *n*-hexane and isopropanol [22]. Baseline separation of some benzodiazepin derivatives was also obtained on the latter column with *n*-hexane–isoprapanol mobile phases [23].

The Chiralcel OJ-H column was used for the present study. It works under normal phase and polar organic mode and is made of derivatised cellulose adsorbed on a silica backbone. The derivative of this homochiral polymer consists in cellulose tris(4-methylbenzoate). Cellulose esters are linear polymers for which a superimposed helical structure has been proposed in the case of cellulose [24] and cellulose tris-benzoate [25]. The enantioseparation process involves different types of interaction on such derivatised cellulose phases: (i) hydrogen bonding, (ii) dipole–dipole interaction and (iii) hydrophobic interaction. Extra π–π interactions may occur between aromatic compounds and the phenyl moiety of the Chiralcel OJ-H column [8,26–28].

The separation of the four benzoxathiepin stereoisomers was performed at 35 °C in the polar organic mode using different mixtures of methanol–ethanol containing 0.1% DEA. Fig. 3A shows the influence of ethanol percentage on retention factors (*k*). When ethanol content rises from 20% to 90% (v/v), a general decrease in retention factor of the enantiomers is observed. This confirms here that the retention of stereoisomers is ruled more by hydrophobic interactions than by hydrogen bonding [27]. (*S*,*R*) stereoisomer eluted first before (*R*,*R*), (*S*,*S*) and (*R*,*S*) stereoisomers. Fig. 3b reports the variation of the stereoselectivity between three stereoisomer pairs versus ethanol percentage in the mobile phase. No general trend happens and the selected mobile phase composition ethanol–methanol–DEA (75/25/0.1, v/v/v) mixture is a compromise between resolution and analysis time. Baseline separation of the four stereoisomers was achieved in 30 min in these conditions (Fig. 4) and the separations parameters (selectivity, resolution) are given in Table 1. Resolutions between two adjacent peaks are sufficiently high: R_S (*S*,*R*–*R*,*R*) = 1.67, R_S (*R*,*R*/*S*,*S*) = 4.67 and R_S (*S*,*S*/*R*,*S*) = 3.26. The Chiralcel OJ-H column allows the separation of these benzoxathiepin stereoisomers. This is mainly due to a good match between the apolar nature of the analyte (log *P* = 5.45), the presence of two phenyl groups on the molecule and the specific retention properties of cellulose tris(4-methylbenzoate) phase.

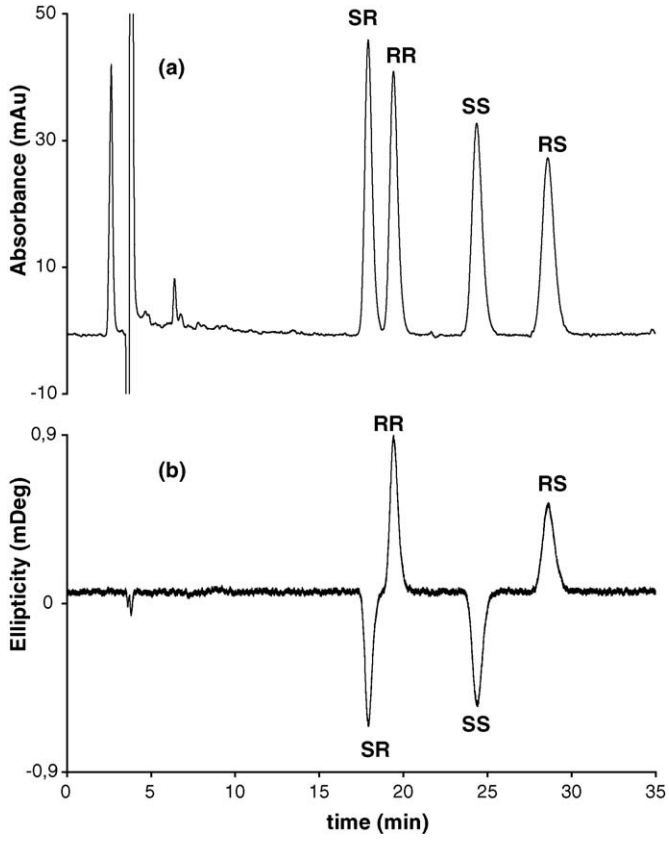

Fig. 4. UV (a) and CD (b) chromatograms during the chiral separation of the four benzoxathiepin stereoisomers on Chiralcel OJ-H column. Same experimental conditions as for Fig. 3, except the mobile phase composition (ethanol–methanol–DEA 75/25/0.1, v/v/v).

UV and CD traces were recorded simultaneously (Fig. 4). HPLC-CD has a higher selectivity than HPLC-UV, due to the fact that only chiral solutes are detected with the CD detector. The intensity of the dichroic signals is measured as a function of ellipticity (expressed in mDeg).

3.3. Characteristics of the circular dichroism signal

3.3.1. Linearity of CD signal

In such HPLC conditions, the linearity of the CD signal was tested for (*R*,*S*) stereoisomer over the 10–200 μg mL^{-1} concentration range. Thus, the linear relationship of CD peak area versus concentration (expressed in μg mL^{-1}) gave the following equation for (*R*,*S*) stereoisomer: $y = 9711.3x + 31413$. A correlation coefficient (r^2) greater than 0.9994 was obtained by least-squares regression. In this report, the limit of quantification (LOQ) was calculated by linear extrapolation of the signal-to-noise (S/N) ratio as a function of concentration. For (*R*,*S*) stereoisomer, S/N value of 10 was used to define LOQ, and was equal to 2.0 μg mL^{-1} with CD detection and 0.6 μg mL^{-1} with UV detection.

Table 1
Separation parameters corresponding to the resolution of the four benzoxathiepin stereoisomers

Stereoisomer	t_R (min)	k	α	R_S
(*S*,*R*)	17.88	5.82		
			1.10	1.67
(*R*,*R*)	19.39	6.40		
			1.30	4.67
(*S*,*S*)	24.35	8.29		
			1.19	3.26
(*R*,*S*)	28.54	9.89		

Same conditions as for Fig. 4.

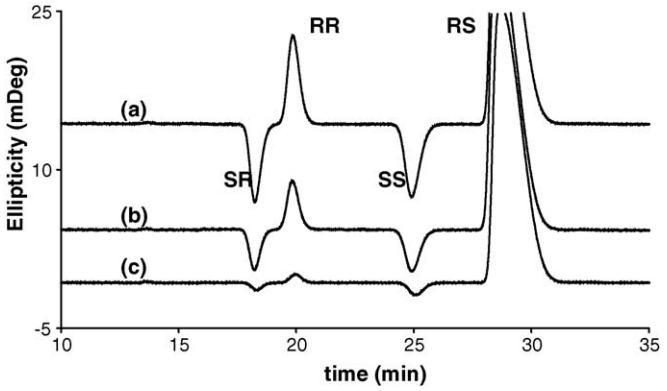

Fig. 5. Separation of benzoxathiepin stereoisomers by HPLC-CD at various stereoisomer concentration ratios. Mobile phase: ethanol–methanol–DEA (75/25/0.1, v/v/v); flow rate: 1 mL min^{-1}; temperature: 35 °C; loop volume: 200 μL; detection wavelength: 262 nm. *R*,*S* stereoisomer concentration: 200 μg mL^{-1}. *R*,*R*; *S*,*R*; *S*,*S* stereoisomer concentrations: (a) 20 μg mL^{-1}; (b) 10 μg mL^{-1}; (c) 2 μg mL^{-1} concentration ratios (*S*,*R*)/(*R*,*S*), (*R*,*R*)/(*R*,*S*), (*S*,*S*)/(*R*,*S*): (a) 10%; (b) 5%; (c) 1%.

3.3.2. Linearity of peak area ratio versus concentration ratio

Linearity of CD signal was also investigated when the three optical impurities (*S*,*R*; *S*,*S*; *R*,*R*) were present at concentration ratios varying from 1 to 10% relative to the concentration of (*R*,*S*) stereoisomer. So, several standard solutions of (*R*,*S*) stereoisomer (concentration of 200 μg mL^{-1}) and of three other stereoisomers (2–20 μg mL^{-1} concentration range) were prepared. CD chromatograms of three calibrates, corresponding to concentration ratios equal to 1%, 5% and 10%, are shown in Fig. 5. Linear relationships were obtained for all three stereoisomers in the concentration range investigated (Fig. 6). A correlation coefficient (r^2) greater than 0.9996 was obtained by least-squares regression (Table 2). These results demonstrate that this mode of detection allows the control of enantiomeric purity at high levels [29].

Table 2
Parameters of CD linearity curves relative to the stereoisomer area ratios vs. their concentration ratios

Ratio	Slope	*y*-intercept	Correlation coefficient (r^2)
R,*R*/*R*,*S*	1.5302	−0.1995	0.9998
S,*S*/*R*,*S*	1.4413	0.9286	0.9996
S,*R*/*R*,*S*	1.2309	−0.1660	0.9997

Ratios, expressed in percentage for both concentration and area, are as follows: *R*,*R*/*R*,*S*; *S*,*S*/*R*,*S* and *S*,*R*/*R*,*S*. Same conditions as for Fig. 6.

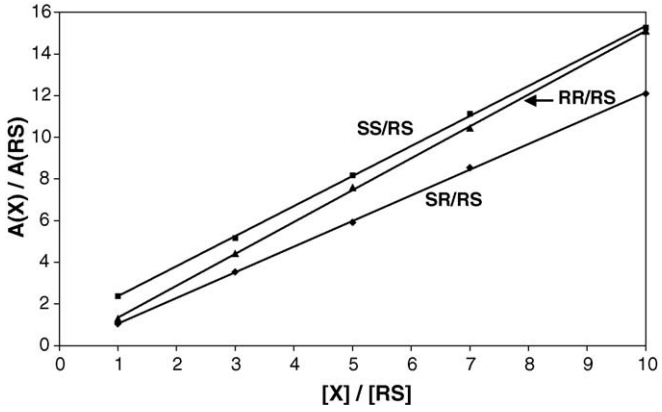

Fig. 6. CD calibration curves of stereoisomer area ratios vs. concentration ratios. Ratios, expressed in percentage for both concentration and area, are as follows: *R*,*R*/*R*,*S*; *S*,*S*/*R*,*S* and *S*,*R*/*R*,*S*. Same conditions as for Fig. 5.

3.3.3. Repeatability

The intra-day repeatability was assessed by injecting six times using a standard mixture of four stereoisomers at 25 μg mL^{-1} concentration. Repeatabilities, expressed as R.S.D.s on retention and peak areas, were found to range between 0.2–0.3% and 0.7–1.6%, respectively.

4. Conclusion

In this work, the four stereoisomers of a novel antianginal agent having two chiral centers were successfully resolved using a chiral HPLC method with circular dichroism detection. Limit of quantification (S/N = 10) down to 2 μg mL^{-1} was achieved for the stereoisomer of interest. In addition, the enantiomeric purity of *R*,*S* stereoisomer has been quantitatively determined between 1 and 10%. The method described could be further applied to study the pharmacokinetics of the enantiomers of this chiral drug in biological samples and compared with results obtained by capillary electrophoresis [30].

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