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Original Paper

Enantiomeric resolution of bifonazole by supercritical fluid chromatography

The enantiomeric separation of bifonazole by supercritical fluid chromatography on Chiralpak AD has been studied. The effect of different modifiers (methanol, ethanol, 2-propanol, and acetonitrile) was examined. Enantioseparation was possible with all of them, but the best results were provided by the alcohol-type ones. The resolution was higher than 5 in all cases. The isoelution temperatures T_{iso} were calculated from the study of the temperature effect for the different organic modifiers. The value of T_{iso} was below the working temperature range on using methanol, but above it with ethanol or 2-propanol.

Keywords: Bifonazole / Chiral separation / Chiralpak AD / Modifier effect / Temperature effect

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1 Introduction

Chiral separations have gained great importance in the pharmaceutical industry because of the large number of examples of pairs of enantiomers that show differences in their bioactivity [1, 2]. This possibility that the enantiomers of a new chiral drug may show different behaviour has compelled the regulatory authorities to demand full documentation of the pharmacokinetic and pharmacological profiles of each enantiomer, as well as the racemic mixture, before the drug can be commercialised.

Therefore, numerous studies have been devoted to enantioselective separation methods [3 – 5]. Although many analytical techniques are used, the direct resolution of the enantiomers by chromatography on a packed chiral stationary phase (CSP) is the one most widely employed [3]. Traditionally, HPLC has been the choice for most of the laboratories in the pharmaceutical industry [6 – 8], but sub- and supercritical fluid chromatography (subFC and SFC) has been a field of growing interest during recent decades and has meanwhile become a popular technique for chiral separations [9 – 13].

Interest in this technique arises from the characteristics of supercritical fluids, which have densities and diffusivities similar to liquids but viscosities comparable to gases. Thus it is possible to obtain high resolutions in short analysis times. Carbon dioxide is the most commonly used supercritical fluid because of its low critical pressure and temperature, non-toxicity, and easy decompressibility.

A wide variety of CSPs are used for the direct separation of enantiomers, but among the most popular are the commercial polysaccharide-based CSPs. They show good chiral recognition ability towards a wide number of different racemic compounds both in HPLC and in SFC [13 – 16].

In this paper, we report the enantioseparation of bifonazole (Fig. 1), an antifungal drug that works by inhibiting the production of ergosterol, which is an essential component of fungal cell membranes. The separation was carried out under subcritical conditions using the polysaccharide-based CSP Chiralpak AD.

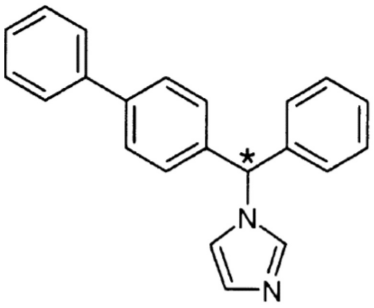

Figure 1. Structure of bifonazole.

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Abbreviations: CSP, chiral stationary phase; **DAD,** diode array detector; **SFC,** supercritical fluid chromatography; **subFC,** subcritical fluid chromatography

2 Experimental

2.1 Reagents

Bifonazole was purchased from Sigma-Aldrich (Madrid, Spain) in its racemic form. The stock solution was prepared in methanol at a concentration of 0.5 mg/mL.

The organic solvents methanol, absolute ethanol, and acetonitrile were purchased from Scharlau (Madrid, Spain) and 2-propanol from Lab-Scan (Deslian, Ireland). All the solvents were of HPLC grade. Carbon dioxide was of SFC grade and obtained from Carburos Metálicos (Barcelona, Spain).

2.2 Instrumentation

The supercritical fluid chromatograph used was an HP 1205A model from Hewlett Packard (Wilmington, DE, USA) equipped with a diode array detector (DAD) and a Rheodyne 7410 injector of 20 μL loop volume (Cotati, CA, USA). It was operated in the downstream mode at a flow-rate of 2 mL/min and a backpressure of 20 MPa. The detection wavelength was set at 225 nm. The system was controlled from the HP-SFC ChemStation Rev.A.01.02.

The column used was Chiralpak AD 250×4.6 mm, packed with the 3,5-dimethylphenylcarbamate derivative of amylose, coated on 10-mm silica-gel support, and obtained from J. T. Baker (Deventer, Holland).

2.3 Chromatographic method

Based on our previous experience, the pressure was set at 20 MPa and the flow rate at 2 mL/min. After changing the organic modifier, an equilibration time of 12 min was allowed and the baseline was monitored to confirm the shift.

All the data given in this work are the mean of three consecutive injections. Capacity factors were calculated from $(t_r - t_0)/t_0$. Retention times were measured at peak maximum, and the dead time (t_0) was estimated from the earliest baseline perturbation. The resolution values given by the software were calculated according to the mathematical expression:

$$R_s = 2\,(t_{r2} - t_{r1})/(\omega_1 + \omega_2) \quad (1)$$

with t_r being the retention time of the compound and ω the peak width at the base of the peak.

The column temperature was varied between 30 and 45°C. Higher temperatures were not examined to prevent damage to the chiral stationary phase and lower temperatures were not achievable by the equipment.

3 Results and discussion

On the basis of our previous experience in chiral SFC, the greatest influence on enantioseparations on a specific chiral stationary phase is exerted by the composition of the mobile phase, but temperature has also an important effect on the selectivity. These two parameters were varied to study the enantiomeric separation of bifonazole on Chiralpak AD. Although other chromatographic conditions such as flow rate and pressure also affect the elution of enantiomers by SFC, the resolution remains practically unaffected. As can be seen in Fig. 2, on increasing the pressure, the retention decreased as a consequence of the increase in density and solvating power of the mobile phase, but changes in the resolution were very small. The same effect on retention was observed on increasing the flow rate, whereupon the resolution also decreased but slightly. Taking into account these results and our previous experience [17,18] a pressure of 20 MPa and a flow rate of 2 mL/min were selected to carry out the study.

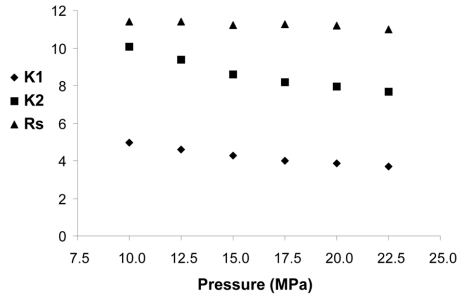

Figure 2. Effect of the pressure on the retention and resolution.

3.1 Effect of modifier

Bifonazole enantiomers were strongly retained on the chiral stationary phase, and the use of an organic modifier was necessary in order to obtain acceptable analysis times. The influence of four different modifiers, methanol, ethanol, 2-propanol, and acetonitrile, was studied to achieve the enantioresolution and to obtain reasonable analysis times. The percentages were varied from 15 to 40% at a temperature of 35°C.

All the modifiers assayed gave high resolutions (Table 1). As can be seen, the results obtained were better with the alcohol-type modifiers. Acetonitrile gave a good resolution, but the second enantiomer eluted was strongly retained, with the analysis time being higher than 50 min with 40% of the modifier (Fig. 3). When alcohol modifiers were used, the retention times were shorter, even if the percentage of alcohol in the mobile phase was more than twice as low. The resolutions obtained were always greatly enhanced, particularly with methanol. This modifier provided resolutions higher than 11, due to the great retention of the second eluted enantiomer.

The use of chiral stationary phases based on polysaccharide phenylcarbamates is widespread in enantiomeric separations, and although the chiral recognition mechan-

Table 1. Effect of the organic modifier in the enantiomeric separation of bifonazole. Chromatographic conditions: 20 MPa, 2 mL/min, 35°C.

Modifier	t_1 (min)	t_2 (min)	k_1	k_2	α	R_s
Methanol						
15%	18.85	40.33	11.57	25.89	2.24	13.51
20%	11.97	23.89	6.98	14.93	2.14	12.51
25%	8.96	17.34	4.97	10.56	2.12	11.95
30%	7.24	13.52	3.83	8.01	2.09	11.36
Ethanol						
15%	14.44	23.20	8.63	14.47	1.68	10.28
20%	9.59	14.86	5.39	8.91	1.65	9.29
25%	7.22	10.87	3.81	6.25	1.64	8.46
30%	5.38	7.86	2.59	4.24	1.64	7.39
2-Propanol						
15%	26.94	37.56	16.96	24.04	1.42	7.46
20%	15.18	20.91	9.12	12.94	1.42	6.67
25%	9.87	13.11	5.58	7.74	1.39	5.98
30%	7.08	9.21	3.72	5.14	1.38	5.21
Acetonitrile						
40%	16.42	46.33	9.95	29.89	3.00	6.40

ism has been extensively studied it has not been completely elucidated. It has been assumed that the most important adsorbing site for chiral discrimination is the polar carbamate group. These groups are located inside the polymer chain and the hydrophobic aromatic groups outside. Enantiomers can interact with the polar carbamate groups via hydrogen bonding or dipole–dipole interactions, but besides these polar interactions, π–π interactions between the aromatic groups of the CSP and a phenyl group of the solute could play some role in chiral discrimination [19–21].

It should the noted that bifonazole does not have any hydrogen-bonding site, apart from the imidazole group, to interact with the stationary phase, and in this case π–π interactions acquire special importance. The high resolution achieved with the four modifiers could be caused by π–π interactions between the phenyl group of the chiral stationary phase and the aromatic groups of bifonazole, which have effects on the chiral recognition. Polar interactions could also be possible, like those between the polar carbamate groups of the CSP and the imidazole group of bifonazole.

The type of organic modifier also has an influence on the retention times of the analytes, and the effect is different for each enantiomer. The lowest retention, for both enantiomers, was achieved using ethanol but the behaviour was different when methanol or 2-propanol were used. The retention of the first eluted enantiomer increased in the order ethanol < methanol < 2-propanol, except when the percentage used is 30%, where the retention with 2-propanol is somewhat lower than with methanol. On the other hand, the retention of the second eluted enantiomer increased in the order ethanol < 2-propanol < methanol, and as a consequence the highest resolutions were achieved using methanol.

Figure 4 shows the chromatograms obtained on using the different modifiers.

3.2 Effect of temperature

Temperature is one of the most important variables affecting selectivity and the data obtained are useful for improving the separation. The effect of temperature was studied with the alcohol-type modifiers because of the high retention observed with acetonitrile. The temperature was varied between 30 and 45°C. The results are given in Table 2 and, as can be seen, in all the cases the retention decreased when the temperature increased.

The selectivity is also influenced by the temperature according to a relationship which can be expressed in terms of the van't Hoff equation:

$$\ln k = -\Delta H/RT + \Delta S/R + \ln \phi \quad (2)$$

$$\ln \alpha = \ln (k_2/k_1) = -\Delta(\Delta H)/RT + \Delta(\Delta S)/R \quad (3)$$

$$\ln \alpha = -\Delta(\Delta G)/RT \quad (4)$$

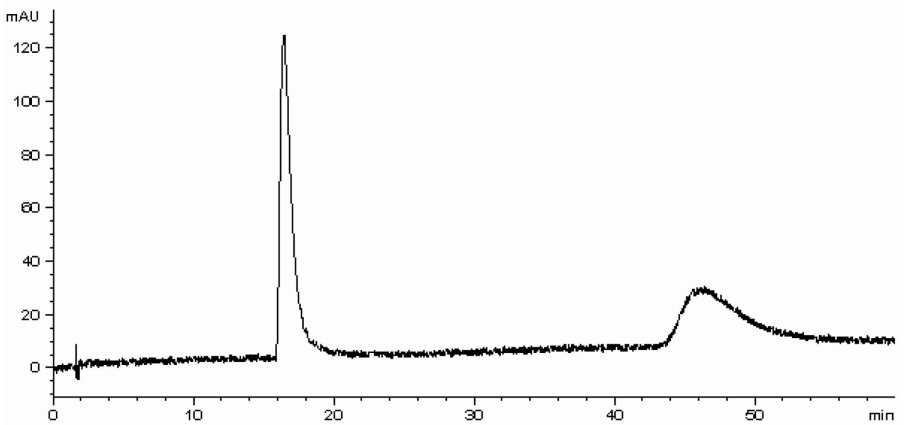

Figure 3. Enantiomeric separation of bifonazole. Chromatographic conditions: 20 MPa, 2 mL/min, 35°C, 40% acetonitrile.

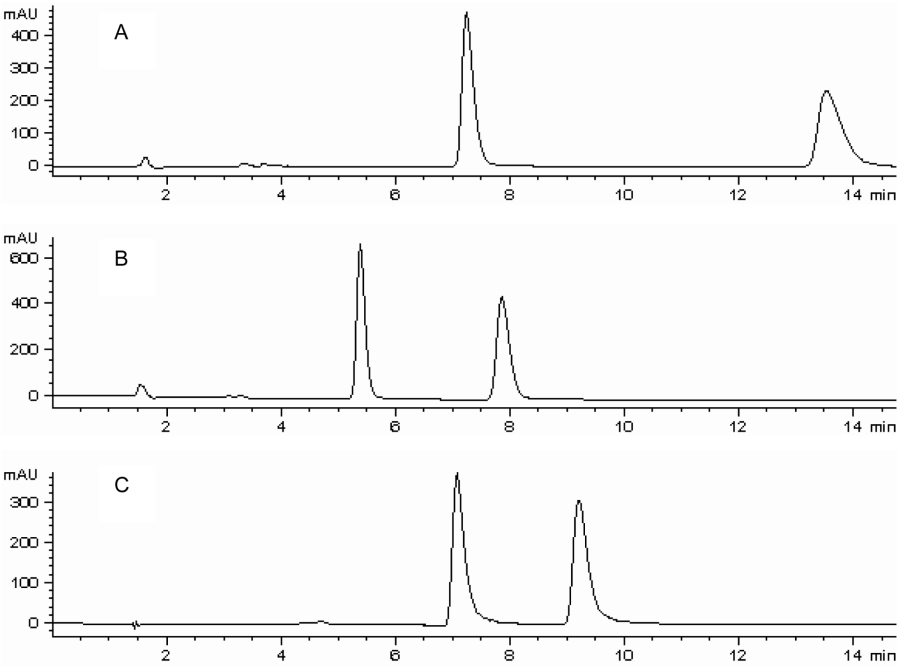

Figure 4. Enantiomeric separation of bifonazole. Chromatographic conditions: 20 MPa, 2 mL/min, 35°C; A) 30% methanol; B) 30% ethanol; C) 30% 2-propanol.

Table 2. Effect of temperature on the enantiomeric separation of bifonazole. Chromatographic conditions: 20 MPa, 2 mL/min.

Temperature	t_1 (min)	t_2 (min)	k_1	k_2	α	R_s
30% Methanol						
30°C	7.57	13.90	4.04	8.27	2.04	10.10
35°C	7.27	13.39	3.85	7.93	2.06	10.74
40°C	7.01	12.90	3.67	7.60	2.07	11.34
45°C	6.70	12.30	3.47	7.20	2.08	11.6
25% Ethanol						
30°C	7.47	11.37	3.98	6.58	1.65	8.59
35°C	7.27	10.94	3.84	6.29	1.64	8.45
40°C	7.04	10.46	3.69	5.98	1.62	8.25
45°C	6.76	9.92	3.51	5.61	1.60	8.01
30% 2-Propanol						
30°C	7.20	9.38	3.80	5.26	1.38	5.21
35°C	7.00	9.06	3.67	5.04	1.38	5.20
40°C	6.90	8.88	3.60	4.92	1.37	5.14
45°C	6.83	8.75	3.55	4.83	1.36	5.08

Where R stands for the ideal gas constant, T is the absolute temperature, ϕ is the phase volume ratio, and ΔH and ΔS represent the enthalpic and the entropic change of the enantiomer-stationary phase interactions, respectively.

Van't Hoff curves can be very informative and explain both the nature of the separation and the importance of temperature in achieving an adequate resolution. When a high positive value of $\Delta\Delta S$ is obtained, it means that the solute molecules are more restricted in the stationary phase and this loss of freedom is responsible for the separation. Because the change in entropy is the major contribution to the change in free energy, the separation, in thermodynamic terms, is said to be "entropy-driven". In contrast, large negative values of $\Delta\Delta H$ mean that molecular forces predominantly control the separation, and because the change in enthalpy is the major contribution to the change in free energy, the separation, in thermodynamic terms, is said to be "enthalpy-driven". Nevertheless chromatographic separations are not exclusively "enthalpy-driven" or "entropy-driven". In most cases the true mechanism has both enthalpic and entropic contributions and can change with the temperature.

If $\Delta\Delta H$ and $\Delta\Delta S$ are independent of the temperature, $\ln\alpha$ *vs.* $1/T$ should be linear, and the isoelution temperature T_{iso} (where the enthalpic and entropic contributions to the selectivity are balanced and coelution of enantiomers occurs) can be calculated as the ratio between the molar differential enthalpy ($\Delta\Delta H$) and entropy ($\Delta\Delta S$) of enantioselective adsorption. The isoelution temperature may, or may not, be in the practical operating range of the chromatographic system, but it is clear that temperature determines the column selectivity for closely eluting peaks. When the isoelution temperature is below the working range, the selectivity increases with the temperature and the separation will be improved on working at high temperatures. On the contrary, if the isoelution temperature is above the working range, the selectivity increases when the temperature decreases and in

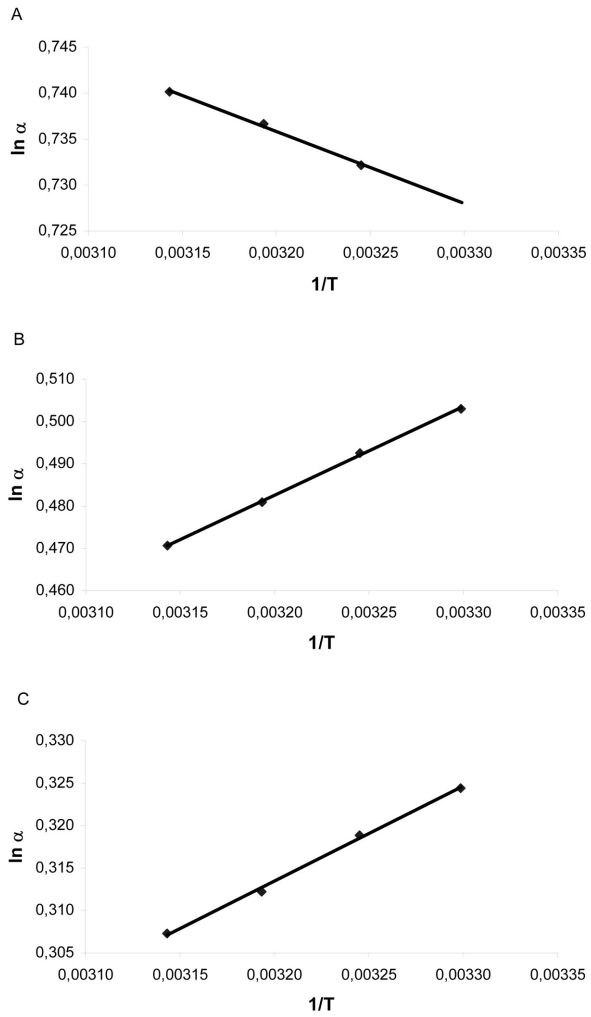

Figure 5. Temperature effect on the enantiomeric separation of bifonazole. Chromatographic conditions: 20 MPa, 2 mL/min, A) 30% methanol; B) 25% ethanol; C) 30% 2-propanol.

this case the separation will be improved on working at low temperatures.

As can be seen in Fig. 5, linear van't Hoff plots were obtained with the three modifiers. When methanol was used as modifier, the selectivity increased with the temperature, so the isoelution temperature was below the working temperature range. On the other hand, the opposite results were obtained when ethanol or 2-propanol were used.

The thermodynamic parameters were calculated from the van't Hoff plots (Table 3). It should be noted that the values of $\Delta\Delta H$ and $\Delta\Delta S$ are positive if methanol is used, which means that the separation is entropy-driven under the chromatographic conditions used. On the contrary, on using ethanol or 2-propanol, these values are negative and the separation is enthalpy-driven.

Table 3. Thermodynamic parameters for bifonazole enantiomers. Chromatographic conditions: 20 MPa, 2 mL/min.

Modifier	$\Delta\Delta H$ (cal · mol^{-1})	$\Delta\Delta S$ (cal · mol^{-1}K^{-1})
30% Methanol	155.7 ± 5.2	1.96 ± 0.02
25% Ethanol	−416.2 ± 3.8	−0.37 ± 0.01
30% 2-Propanol	−222.6 ± 3.9	−0.09 ± 0.01

4 Concluding remarks

The enantiomeric separation of bifonazole can be achieved using supercritical fluid chromatography on a Chiralpak AD column. The enantioseparation was possible with the four modifiers assayed, methanol, ethanol, 2-propanol, and acetonitrile, and always gave resolutions higher than 5. The best results were provided by the alcohol-type modifiers because a high retention was observed with acetonitrile. Using ethanol, the enantioseparation was achieved with a resolution higher than 7, and the analysis time was lower than 10 minutes. Study of the temperature showed that on using methanol T_{iso} is below the working temperature range and the separation is entropy-driven. On the other hand, when ethanol or 2-propanol were used, the separation is enthalpy-driven and T_{iso} is above the working temperature range.

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