Liquid chromatographic separation of the enantiomers of becliconazole and its potential impurities

R. Ferretti, B. Gallinella, F. La Torre*, L. Turchetto

Istituto Superiore di Sanità, Laboratorio di Chimica del Farmaco, Viale Regina Elena 299, 00161 Rome, Italy

Abstract

High-performance liquid chromatography (HPLC) was employed for the separation and determination of becliconazole, an imidazole derivative with antifungal activity containing a stereogenic centre, and its impurities. Chiral columns, containing carbamate of cellulose and amylose, were used. The effects of the organic modifiers, ethanol and 2-propanol, in the mobile phase, were studied. The HPLC method gave good performances from qualitative and quantitative standpoints, allowing the enantiomeric ratio of becliconazole and its impurities to be determined. The assay showed linearity over the range 400–4000 ng ml^{-1} for the impurities and 400–4000 µg ml^{-1} for becliconazole.

Keywords: Enantiomer separation; Beclinazole; Econazole; Miconazole

1. Introduction

Over the last decade, many analytical and preparative chromatographic and electrophoretic methods (mainly HPLC and capillary electrophoresis (CE)) have been developed to study the biological action and activity of enantiomeric drugs [1–6]. Among the methods currently used to achieve chiral separation of racemic mixtures, high resolution chromatographic systems based on chiral stationary phases (CSPs) (direct methods) are more rapid and suitable for resolution of racemic mixtures; as the drawbacks arising from optical impure reagents or different rates of formation of the diastereoisomers are avoided.

During the last three decades several new azole derivatives, containing a stereogenic centre, have

been developed; they revealed effectiveness for the treatment of fungal diseases, particularly in immuno-compromised patients such as patients undergoing organ transplants or anticancer chemotherapy and patients with acquired immunodeficiency syndrome [7,8].

The enantiomers of the azole antifungal agents are, in some cases, very different in their pharmacological activity. The (+)-form of tetraconazole was found to be the more fungitoxic than the (−)-enantiomer [9]. SCH 39304, a new triazole antifungal agent, is a racemic mixture of two enantiomers SCH 42427 and SCH 42426. SCH 42427 is two-fold more active in vitro than SCH 39304, while SCH 42426 is inactive [10].

The different pharmacological activity of the enantiomers of these products prompted the researchers to develop chromatographic methods able to resolve racemic mixtures of azole derivatives.

---

*Corresponding author.

Enantiomeric resolutions of some chiral pharmaceutical compounds, imidazole and triazole derivatives, were carried out using CE. Various native ciclodextrins and derivatized were used as chiral buffer modifiers [11].

Diniconazole and uniconazole enantiomers were separated on commercially available β and γ-cyclodextrin bonded columns [12,13], or using CPSs containing, as chiral selectors, some urea derivatives bearing two asymmetric carbon atoms attached to two nitrogen atoms of the urea group [14]. A new potent antifungal agent (2R,3R)-2-(2,4-difluorophenyl)-3-mercapto-1-(1H-1,2,4-triazol-1-yl)-2-butanol and its stereoisomers were prepared and their activity was tested in vitro and in vivo. The optical purity was assessed by HPLC using a chiral column after conversion to the corresponding thioacetate derivatives [15].

Becliconazole, 1-[(5-chloro-2-benzofuranyl)(2-chlorophenyl)methyl]1H-imidazole [16,17], a new imidazole derivative, under application for approval by Menarini, was proposed as racemic mixture; it shows effectiveness for the treatment of fungal infections. In this paper we describe an isocratic method for the simultaneous separation, identification and measurement of the enantiomeric ratio of becliconazole and its chiral and achiral impurities (Fig. 1) using columns containing amylose and cellulose derivatives, as stationary phases, absorbed on the silica surface.

2. Experimental

2.1. Materials

Stainless-steel Chiralpak AD, Chiralpak AS, Chiralcel OD, (250×4.6 mm I.D.) (Daicel Chemical Industries, Tokyo, Japan) and Chiral Phase DNB leucine (250×4.6 mm I.D.) (J.T. Baker, Phillipsburg, NJ, USA) columns were used. HPLC-grade solvents were purchased from Carlo Erba (Milan, Italy). Diethylamine (DEA) was obtained from Fluka Chemie (Buchs, Switzerland). Becliconazole and its chiral and achiral impurities were from Menarini (Florence, Italy). Econazole and miconazole were obtained from commercial samples.
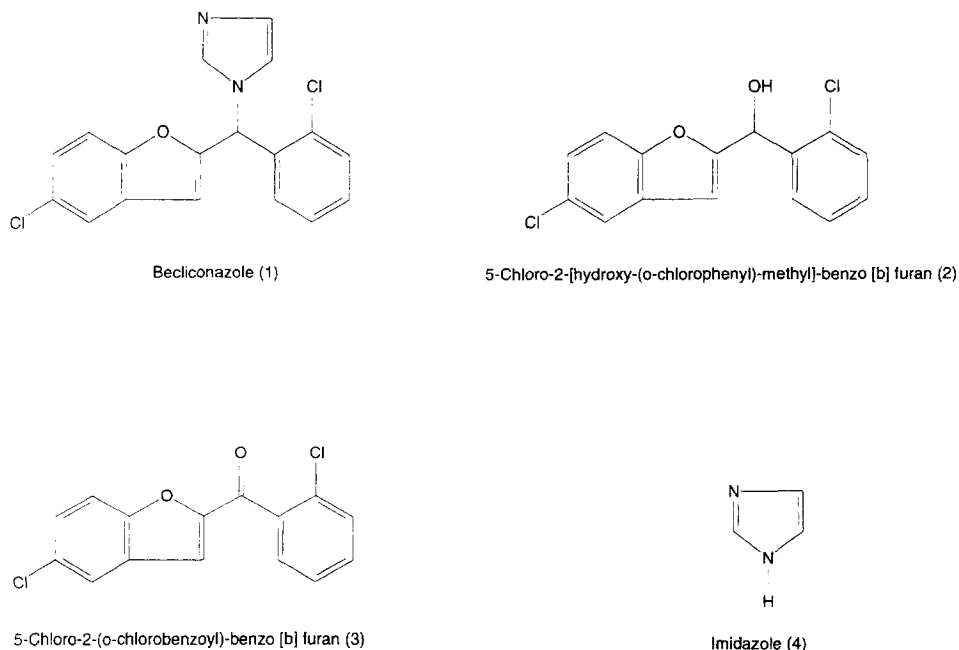

Becliconazole (1)

5-Chloro-2-[hydroxy-(o-chlorophenyl)-methyl]-benzo [b] furan (2)

5-Chloro-2-(o-chlorobenzoyl)-benzo [b] furan (3)

Imidazole (4)

Fig. 1. Structures of becliconazole and its impurities.

2.2. Apparatus

Chromatography was performed using a Waters Model 600 MS pump, a Waters Model U6K injector and a Waters Model 996 programmable multi-wavelength diode array detector (Waters, Milford, MA, USA).

2.3. Operating conditions

The following chromatographic conditions were used; with Chiralpak AD: mobile phase, n-hexane–ethanol–DEA (95:5:0.1, v/v), degassed with an ultrasonic bath before use; flow-rate, 0.5 ml min^{-1}; with Chiralcel OD: mobile phase, n-hexane–2-propanol–DEA (95:5:0.1, v/v), degassed with an ultrasonic bath before use; flow-rate, 0.5 ml min^{-1}; with Chiralpak AS: mobile phase, n-hexane–ethanol (97.5:2.5, v/v); with DNB leucine: mobile phase, n-hexane–methylene chloride–2-propanol (69:29:2, v/v); column temperature, 20°C; volume injected, 20 μl; detector wavelengths, 254 nm and 220 nm. Chiralpak AD and Chiralcel OD columns were easily regenerated at the end of every day by washing with ca. 100 ml of n-hexane–2-propanol (90:10, v/v) at a flow-rate of 0.2 ml min^{-1}. In this way, no efficiency loss was observed throughout our work. After the cleaning, the columns were reconditioned with ca. 100 ml of the mobile phase.

2.4. Linearity of detector response vs. standards concentration

Calibration graphs were obtained by injecting the impurities (compounds 2–4) in a concentration range of 400–4000 ng ml^{-1} and becliconazole in a concentration range of 400–4000 μg ml^{-1}. The limits of detection were 5 ng for becliconazole and compounds 2 and 3; 10 ng for compound 4, at 220 nm, calculated on a response of three times the noise level.

3. Results and discussion

The aim of this work is the quantification of the enantiomeric ratio of becliconazole and compound 2 together with the determination of compounds 3 and 4, in bulk products.

Chiral recognition mechanism in CSPs phases, such as cellulose and amilose esters derivatives, is, in most cases, unpredictable, as the discriminating sites are not clearly identified, in contrast to CSPs in which the chiral selector, bound to the silica gel, is well defined [18–20].

Nevertheless attempts to separate the enantiomers of becliconazole using a DNB leucine column did not succeed.

Enantiomeric inclusion in chiral cavities which might be multiple and competitive in cellulose- and amilose-based CSPs seems to be responsible for the chiral discrimination [21].

Amylose and cellulose-based columns (Chiralpak AD, AS and Chiralcel OD) were used throughout our work. Amylose is a well known polysaccharide; the monomer unit of amylose is D-glucose, which is the same as that of cellulose. However, amylose is said to have a helix structure based on the α-linkage of D-glucose units. Hence the chiral discrimination ability of amylose derivatives is very different from that of cellulose [22–29].

In preliminary trials, we tried to separate four enantiomers (compounds 1, 2).

All the four compounds were baseline resolved by Chiralpak AD and Chiralcel OD columns.

Cellulose based column (Chiralcel OD) was more effective than the amylose based column (Chiralpak AD) in terms of enantioselectivity toward the four enantiomers (Tables 1 and 2); the use of ethanol as organic modifier enhanced the differences of enantioselectivity and resolution between Chiralcel OD and Chiralpak AD columns.

Surprisingly the regioselectivity factors, between compounds 1 and 2, obtained with Chiralpak AD column were higher than those obtained with Chiralcel OD column, using ethanol as organic modifier in the eluent. Chiralpak AS column showed poor enantioselectivity and resolution values, but gave the highest regioselctivity factors either with 2-propanol or ethanol in the mobile phase (Tables 1 and 2). It was not possible to determine the order of elution of enantiomers of compounds 1 and 2 for lack of single enantiomers.

However, when all chiral (compounds 1, 2) and achiral (compounds 3, 4) compounds were com-

Table 1
Chromatographic data for compounds 1 and 2 on Chiralcel OD, Chiralpak AD and AS columns with organic modifier 2-propanol

Compound	Column	k_1' [a]	α [b]	R_s [c]	α [d]	Eluent [e]
1	Chiralpak AD	3.73	1.13	2.19	1.10	A
		4.97	1.13	2.18	1.15	B
		7.11	1.12	2.21	1.20	C
		10.11	1.12	1.59	1.22	D
	Chiralcel OD	5.90	1.18	1.35	1.36	B
		10.01	1.26	1.88	1.43	D
	Chiralpak AS	6.54	1.00	No res.[f]	3.34	E
2	Chiralpak AD	2.16	1.57	8.22		A
		2.82	1.54	8.65		B
		3.82	1.55	8.90		C
		5.39	1.54	9.24		D
	Chiralcel OD	2.42	1.78	7.08		B
		3.72	1.88	8.55		D
	Chiralpak AS	1.86	1.05	0.71		E

[a] The capacity factor of the first eluted enantiomer.
[b] The enantioselectivity factor.
[c] The resolution factor.
[d] Regioselectivity factor calculated as: k' (first eluted enantiomer of Becliconazole)/k' (second eluted enantiomer of compound 2).
[e] Eluents employed were: (A) n-hexane–2-propanol–diethylamine (87.5:12.5:0.1, v/v/v); (B) n-hexane–2-propanol–diethylamine (90:10:0.1, v/v/v); (C) n-hexane–2-propanol–diethylamine (93:7:0.1, v/v/v); (D) n-hexane–2-propanol–diethylamine (95:5:0.1, v/v/v); (E) n-hexane–2-propanol (90:10, v/v).
[f] No resolution.

Table 2
Chromatographic data for compounds 1 and 2 on Chiralcel OD, Chiralpak AD and AS columns with ethanol as organic modifier

Compound	Column	k_1' [a]	α [b]	R_s [c]	α [d]	Eluent [e]
1	Chiralpak AD	3.41	1.13	2.33	1.10	A
		4.31	1.11	2.36	1.46	B
		6.85	1.04	2.03	1.70	C
		10.04	1.08	1.65	1.73	D
	Chiralcel OD	2.55	1.35	2.85	1.24	B
		4.89	1.39	3.55	1.34	D
	Chiralpak AS	10.60	1.12	0.96	2.12	E
2	Chiralpak AD	2.05	1.18	3.15		A
		2.56	1.15	2.60		B
		3.68	1.09	2.01		C
		5.37	1.08	1.77		D
	Chiralcel OD	1.42	1.45	4.34		B
		2.63	1.39	4.02		D
	Chiralpak AS	4.31	1.16	1.32		E

[a] The capacity factor of the first eluted enantiomer.
[b] The enantioselectivity factor.
[c] The resolution factor.
[d] Regioselectivity factor calculated as: k' (first eluted enantiomer of Becliconazole)/k' (second eluted enantiomer of Compound 2).
[e] Eluents employed are: (A) n-hexane–ethanol–diethylamine (87.5:12.5:0.1, v/v/v); (B) n-hexane–ethanol–diethylamine (90:10:0.1, v/v/v); (C) n-hexane–ethanol–diethylamine (92.5:7.5:0.1, v/v/v); (D) n-hexane–ethanol–diethylamine (95:5:0.1, v/v/v)); (E) n-hexane–ethanol (97.5:2.5, v/v).

bined, the chromatographic requirements became more complicated. In fact, compound 4 showed a bad tailing on Chiralcel OD column using either 2-propanol or ethanol as modifier, the same pattern was observed with the Chiralpak AD column using 2-propanol as modifier. In addition the peak of compound 4, with Chiralcel OD column, partially overlapped with the first eluted enantiomer of compound 1 (Fig. 2b)

Compounds 3 and 4 were not tested with Chi-

ralpak AS column, as this stationary phase did not give any resolution of becliconazole enantiomers with mobile phases consisting of n-hexane–2-propanol.

Using ethanol in the mobile phase and Chiralcel OD column, compound 4 coeluted with the second eluted enantiomer of compound 2, whereas compound 4 partially overlapped with the first eluted enantiomer of compound 1 using Chiralpak AS column (Fig. 2c). For this reason we deemed the Chiralpak AD column more appropriate than the other two columns to solve the present problem (Fig. 2a).

A small quantity of DEA added to the mobile phase had a beneficial effect on the separation and resolution; however, with contents of DEA in the mobile phase higher than 0.1% the elution time of compound 4 was too long.

Econazole and miconazole enantiomers were separated using the same conditions as described for becliconazole in Table 2 (column: Chiralpak AD, mobile phase: B). The enantioselectivity and resolution factors were respectively: $\alpha = 1.23$, $R_s = 1.70$ for econazole and $\alpha = 1.30$, $R_s = 1.53$ for miconazole.

The method is capable of accurately determining 0.2% of each impurity in the bulk product and these limits appeared adequate for application to industrial products.

The calibration graphs (six points for impurities and becliconazole) showed good linearity with a correlation coefficient of 0.9940 for the first eluted enantiomer of becliconazole $(c = 2.32 \times 10^{-4} A - 5582.2)$, 0.9969 for the second eluted enantiomer of becliconazole $(c = 2.16 \times 10^{-4} A - 4691.1)$, 0.9987 for the first eluted enantiomer of compound 2 $(c = 1.15 \times 10^{-4} A - 2.8597)$, 0.9989 for the second eluted enantiomer of compound 2 $(c = 1.15 \times 10^{-4} A - 3.0534)$, 0.9985 for compound 3 $(c = 1.59 \times 10^{-4} A - 3.7718)$, 0.9985 for the compound 4 $(c = 4.95 \times 10^{-4} A + 1.2647)$, $(c =$ sample concentration in ng ml^{-1}, $A =$ area counts). Table 3 shows the accuracy and precision data at each individual standard concentration.

The use of a linear photodiode array detector turned out to be useful in confirming that chiral separation of a pure racemic compound had, in fact, taken place. As enantiomers behave identically in symmetrical environments, they will absorb non-
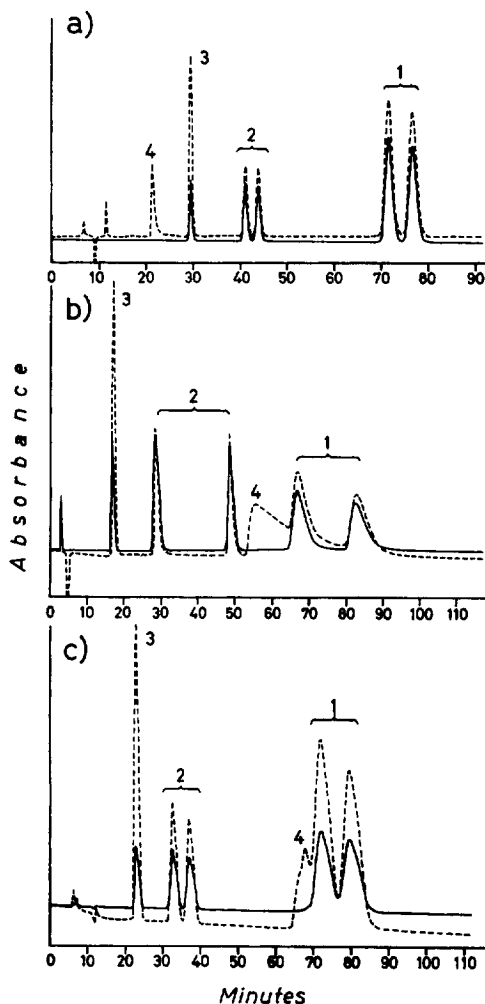
Fig. 2. HPLC of compounds in Fig. 1. (a) Eluent, n-hexane–ethanol–DEA (95:5:0.1, v/v); column Chiralpak AD. (b) Eluent, n-hexane–2-propanol–DEA (95:5:0.1, v/v); column Chiralcel OD. (c) Eluent, n-hexane–ethanol (97.5:2.5, v/v); column Chiralpak AS. Flow-rate 0.5 ml min^{-1}. (- - -) λ 220 nm, (——) λ 254 nm.

Table 3
Inter-day precision and accuracy of becliconazole and its relative impurities

Compound	Parameter[a]	Amounts of samples injected (μg/20 μl)					
		51.37	41.10	30.82	20.55	10.27	5.14
1	M	49.28	42.39	32.11	21.30	9.52	4.65
First eluted	R.S.D.(%)	1.58	0.12	0.34	0.23	3.05	2.36
enantiomer	R.E.(%)	−4.24	+3.04	+4.02	+3.52	−7.88	−10.54
1	M	49.98	41.99	31.76	20.99	9.60	4.94
Second eluted	R.S.D.(%)	1.52	0.81	0.41	0.71	3.96	2.23
enantiomer	R.E.(%)	−2.78	+2.12	+2.96	+2.10	−6.98	−4.05
		Amounts of samples injected (ng/20 μl)					
		103.37	82.70	62.02	41.35	20.67	10.34
2	M	101.69	84.43	62.18	41.43	22.09	9.27
First eluted	R.S.D.(%)	0.73	0.95	0.11	0.02	3.39	2.91
enantiomer	R.E.(%)	−1.65	+2.05	+0.26	+0.19	+6.43	−11.54
2	M	102.12	84.64	61.16	41.61	21.06	9.81
Second eluted	R.S.D.(%)	0.43	0.52	0.51	0.26	1.95	2.24
enantiomer	R.E.(%)	−1.22	+2.29	−1.41	+0.62	+1.85	−5.40
		118.35	94.68	71.01	47.34	23.67	11.83
3	M	118.01	95.43	70.02	46.63	25.85	10.40
	R.S.D.(%)	0.12	0.78	0.33	1.65	4.95	3.36
	R.E.(%)	−0.29	+0.78	−1.41	−1.52	+8.43	−13.75
		102.88	82.30	61.73	41.15	20.58	10.29
4	M	102.23	82.63	62.45	41.63	18.86	11.37
	R.S.D.(%)	1.91	1.12	1.84	1.10	2.07	1.32
	R.E.(%)	−0.63	+0.40	+1.15	+1.15	−9.12	+9.50

[a] M = mean value; R.S.D. = relative standard deviation; R.E. = relative error; $n = 3$ in each instance.

polarised light in exactly the same way, giving identical spectra (Fig. 3).

4. Conclusions

The HPLC method, described for the determination of becliconazole and its impurities in bulk product and pharmaceutical formulations, appears to be easy to use, reproducible, sensitive and applicable to other diazole derivatives (econazole and miconazole)

Both Chiralpak AD and Chiralcel OD columns gave baseline separations of the enantiomers of compounds 1 and 2. Furthermore no derivatization of the compounds containing a stereogenic centre was required, and therefore, the drawbacks due to the racemization or different rates of reaction of the single enantiomer or side-reactions of the impurities which do not contain a stereogenic centre, with the derivatizing agent were avoided.

The Chiralpak AD column is preferable to the Chiralcel OD because of the higher regioselectivity shown toward compounds 1 and 2.

Becliconazole was proposed by the manufacturer as racemic mixture; nevertheless, it is well known, from the literature, that, in many cases, enantiomers of diazole and triazole antimycotic agents show different pharmacological activity.

The decision either to develop a drug as a single enantiomer or racemate should be based on the efficacy of each enantiomer, their relative rates of metabolism, pharmacological differences, and/or potential toxicological differences. All these factors
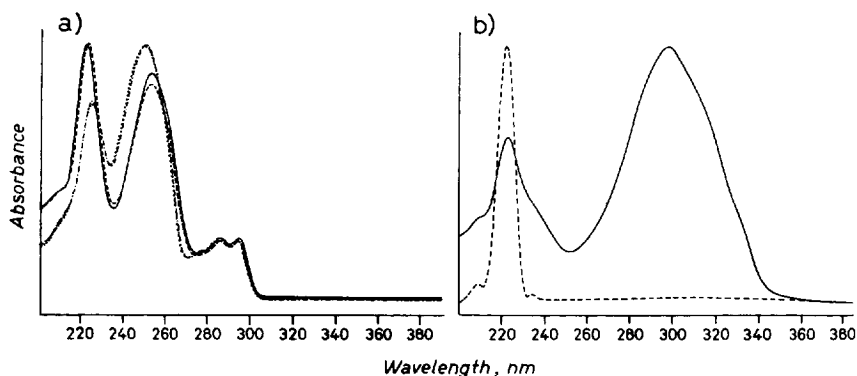
Fig. 3. UV spectra of compounds in Fig. 1.

need to be carefully evaluated, and a chromatographic method able to separate the enantiomers can be helpful.

In addition, with the increasing interest in enantiomerically pure drug formulations from both pharmaceutical industries [30] and regulatory authorities [31–35], a chromatographic method able to determine simultaneously chiral and achiral impurities is quite suitable for a quality control in bulk product and pharmaceutical formulations.

Acknowledgments

References

[1] G. Gübitz, Chromatographia, 30 (1990) 555.

[2] E. Francotte and A. Junker-Buchleit, J. Chromatogr., 576 (1992) 1.

[3] W.H. Pirkle and T.C. Pochapsky, Chem. Rev., 89 (1989) 347.

[4] Y. Okamoto and Y. Kaida, J. Chromatogr. A, 666 (1994) 403.

[5] D.R. Taylor and K. Maher, J. Chromatogr. Sci., 30 (1992) 67.

[6] S. Terabe, K. Otsuka and H. Nishi, J. Chromatogr. A, 666 (1994) 295.

[7] F. Barchiesi, A. Giacometti, D. Arzeni, P. Branchesi, C. Crescenzi, F. Ancarani and G. Scalise, J. Chemother., 4 (1992) 381.

[8] M.S. Saag and W E. Dismukes, Antimicrobial Agents and Chemotherapy, 32 (1988) 1.

[9] D. Bianchi, P. Cesti, S. Spezia, C. Garavaglia and L. Mirenna, J. Agr. Food Chem., 39 (1991) 197.

[10] D. Loebenberg, A. Cacciapuoti, R. Parmegiani, E.W.L. Moss JR., F. Menzel, Jr., B. Antonacci, C. Norris, T. Yarosh-Tomaine, R.S. Hare and G.H. Miller, Antimicrob. Agents Chemother., 36 (1992) 498.

[11] B. Chankvetadze, G. Endresz and G. Blaschke, J. Chromatogr., 700 (1995) 43.

[12] R. Furuta and H. Nakazawa, J. Chromatogr., 625 (1992) 231.

[13] R. Furuta and H. Nakazawa, Chromatographia, 35 (1993) 555.

[14] N. Oi, H. Kitahara and R. Kira, J. Chromatogr., 535 (1990) 213.

[15] A. Tasaka, N. Tamura, Y. Matsushita, K. Teranishi, R. Hayashi, K. Okonogi and K. Itoh, Chem. Pharm. Bull., 41 (1993) 1035.

[16] V. Pestellini, D. Giannotti, A. Giolitti, N. Fantò, L. Riviera and M.G. Bellotti, Chemioterapia, VI (1987) 269.

[17] L. Riviera, M.G. Bellotti, V. Pestellini, D. Giannotti, A. Giolitti and N. Fantò, Chemioterapia, VI (1987) 272

[18] W.H. Pirkle, P.G. Murray and J.A. Burke, J. Chromatogr., 641 (1993) 21.

[19] K.B. Lipkowitz, J. Chromatogr. A, 666 (1994) 493.

[20] K.B. Lipkowitz, K.M. Green, J. Yang, G. Pearl and M.A. Peterson, Chirality, 5 (1993) 51.

[21] E. Francotte and R.M. Wolf, Chirality, 2 (1990) 16.

[22] Y. Okamoto, M. Kawashima and K. Hatada, J. Am. Chem. Soc., 106 (1984) 5357.

[23] S.A. Matlin, M.E. Tiritan, A.J. Crawford, Q.B. Cass and D.R. Boyd, Chirality, 6 (1994) 135.

[24] Y. Okamoto and Y. Kaida, J. Chromatogr., 666 (1994) 403.

[25] Y. Okamoto, M. Kawashima and K. Hatada, J. Chromatogr., 363 (1986) 173.

[26] Y. Okamoto, R. Aburatani, Y. Kaida and K. Hatada, Chem. Lett., 1125 (1988).

[27] Y. Okamoto, R. Aburatani, T. Fukumoto and K. Hatada, Chem. Lett., 1857 (1987).

[28] E. Yashima, M. Yamada, Y. Kaida and Y. Okamoto, J. Chromatogr. A, 694 (1995) 347.

[29] K. Oguni, H. Oda and A. Ichida, J. Chromatogr. A, 694 (1995) 91.

[30] M.N. Cayen, Chirality, 3 (1991) 94.

[31] H. Shindo and J. Caldwell, Chirality, 3 (1991) 191.

[32] A.J. Hutt, Chirality, 3 (1991) 161.

[33] W.H. De Camp, J. Pharm. Biomed. An., 11 (1993) 1167.

[34] A.G. Rauws and K. Groen, Chirality, 6 (1994) 72.

[35] H. Shindo and J. Caldwell, Chirality, 7 (1995) 349.