ELSEVIER

Journal of Chromatography A, 903 (2000) 245–254

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Development and evaluation of a linear regression method for the prediction of maximal chiral separation of basic drug racemates by cyclodextrin-mediated capillary zone electrophoresis

Ann Van Eeckhaut, Suchitra Boonkerd, Marc Robert Detaevernier, Yvette Michotte*

Department of Pharmaceutical Chemistry and Drug Analysis, Pharmaceutical Institute, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium

Received 6 June 2000; received in revised form 22 August 2000; accepted 28 August 2000

Abstract

An important step in method development of chiral separations with neutral cyclodextrins (CDs) as chiral selectors is the estimation of the CD concentration that gives the highest degree of separation. From the equation $[S]_{opt}=1/(K_1K_2)^{1/2}$ this optimal CD concentration can be calculated if any knowledge is available about the binding constants K_1 and K_2 of both enantiomer complexes. These values can be obtained by measuring the effective velocities of each enantiomer as a function of the selector concentration and fitting these profiles by non-linear least-square regression. An alternative approach has been developed which makes it possible to predict the optimal CD concentration from a few experiments performed at low CD concentrations. The model is developed using some antimycotic imidazole derivatives (econazole, miconazole and isoconazole) as test substances and hydroxypropyl-β-CD as chiral selector. The results obtained by this method are in good agreement with those from non-linear least-square regression.

Keywords: Enantiomer separation; Binding constants; Pharmaceutical analysis; Regression analysis; Cyclodextrins; Econazole; Miconazole; Isoconazole; Imidazoles

1. Introduction

Many drugs are marketed as racemic mixtures, although the individual enantiomers frequently differ in both their pharmacodynamic and pharmacokinetic profiles [1]. The exploration of powerful enantioselective separation methods in pharmaceutical analysis is thus of great importance. For this purpose capillary electrophoresis (CE) is still proving to be a highly effective tool [2].

In order to achieve enantioselective separations in CE, several approaches are available. The most frequently used selectors are the cyclodextrins (CDs). CDs are oligosaccharides consisting of six (α), seven (β) or eight (γ) glucopyranose units. The shape is comparable to a truncated cone with a hydrophobic cavity and a hydrophilic surface. CDs are considered as host molecules in which individual guest molecules (say: enantiomers) can be entrapped leading to so-called inclusion complexes with specific affinities. The complexation is based on the inclusion of the hydrophobic part of a molecule into the cavity. Additionally secondary groups on the rim of the CD, stabilise this complex [3–6]. In capillary

*Corresponding author. Tel.: +32-2-4774-429; fax: +32-2-4774-113.

E-mail address: ymichot@minf.vub.ac.be (Y. Michotte).

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00897-9

zone electrophoresis (CZE) the neutral CD will migrate with the velocity of the electroosmotic flow (EOF). When a cation is included in the CD cavity the complex will acquire a positive electrophoretic mobility that, of course, is smaller than that of the free ionic species.

Important in method development of chiral separations using cyclodextrins as chiral selector is firstly the choice of an adequate CD derivative, and secondly, or even concomitantly, an estimation of its concentration that would give the largest difference in mobility or resolution.

Wren and Rowe [7–9] have developed a theoretical model relating mobility to the concentration of a CD selector. They have found that the difference in mobility depends on the concentration of the chiral selector, and that there is an optimal concentration. The size of this optimum depends inversely on the affinity of the enantiomers for the chiral selector. Penn et al. [10] have shown how the binding constants could be derived using this method. Another way of determining the binding constants is proposed by Gareil et al. [11] who has transformed the equation into a bilogarithmic function. The binding constant K can be determined at the inflection point. From another point of view Rawjee and co-workers [12–14] and Biggin et al. [15] have developed a multiple-equilibria-based model to account for the effects of pH and CD concentration of the buffer for both chiral weak acids and weak bases. Surapaneni et al. [16] have proposed a theoretical model for the separation of enantiomers of neutral species by employing a combination of charged and neutral cyclodextrins. The model extends the treatment of simultaneous multiple-equilibria, developed previously for charged analytes, to neutral analytes:

$$[S]_{opt} = \frac{1}{\sqrt{K_1 K_2}} \quad (1)$$

Eq. (1), derived from the model of Wren and Rowe [7], makes it possible to determine the optimal CD concentration if any knowledge is available about the binding constants K_1 and K_2 of both enantiomer complexes. These values can be obtained by measuring the effective velocity of each of the enantiomers as a function of the selector concentration and fitting these profiles by non-linear least-square regression. For an accurate estimation of the binding constants by non-linear regression there are at least two requisites: the need for data points of effective velocity measured over a large concentration range and some correction of these data according to the increase in viscosity when increasing amounts of selector are added to the buffer. Corrections of effective velocities are performed by comparing either the viscosity [10,17,18] or the developed current [7,19] between the buffer containing CD and the CD-free buffer.

An alternative approach has been developed in order to reduce the amount of experiments and associated (expensive) CDs. Measuring in the low CD concentration range further makes these corrections of the viscosity superfluous. This alternative approach is investigated for the enantiomers of the weak basic imidazole compounds econazole, isoconazole and miconazole (Fig. 1). These imidazole derivatives are used in the treatment and prophylaxis of mycoses. Chankvetadze et al. [20] have studied the enantioseparation of pharmaceutical compounds containing the imidazole moiety by CE using different CDs. They have found that, for these compounds, hydroxypropyl-β-CD (HPCD) has better chiral recognition abilities than the native CDs. The chiral separation is influenced not only by the CD type but also by the chiral selector concentration and by the presence of an organic modifier.

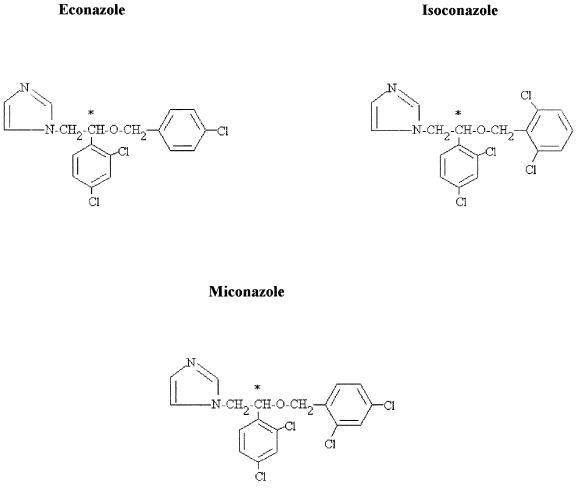


Fig. 1. Structures of econazole, isoconazole and miconazole.

2. Experimental

The separations are performed on a Beckman (Palo Alto, CA, USA) P/ACE 2100 System using a fused-silica capillary (Beckman) of 37 cm×75 μm I.D. with a detection window at 7 cm from the capillary outlet. The integration of the electropherograms is achieved by the chromatography software System Gold 7.11 (Beckman).

Isoconazole is a gift from Schering (Diegem, Belgium). Econazole nitrate is obtained from Certa (Braine-l'Alleud, Belgium) and miconazole nitrate from Alpha Pharma (Zwevegem, Belgium). HPCD is purchased from Aldrich (Gillingham, UK) and has an average molecular mass of 1380. Methanol (analytical-reagent grade) is purchased from Merck (Darmstadt, Germany) and isopropanol (analytical-reagent grade) from Carlo Erba (Milan, Italy).

The water used for preparing solutions is obtained from a Seralpur Pro 90 CN purification system (Seral, Germany). The separation buffer consists of 0.1 *M* orthophosphoric acid (85%) (Merck), adjusted to pH 3.0 with triethanolamine (TEA) (Fluka, Buchs, Switzerland) as proposed by Bechet et al. [21] for the separation of basic compounds. The buffer is filtered through a 0.2 μm membrane and organic modifier is added to the buffer at the concentration indicated. The appropriate amount of HPCD is dissolved in the buffer solution.

Stock solutions of each racemic test substance are prepared in methanol at a concentration of 500 μg/ml. The samples are diluted with water to a concentration of 25 μg/ml and degassed by sonication before use. Samples are introduced by pressure for 3 s. The applied voltage is 20 kV and UV detection is performed at 214 nm. The capillary is temperature controlled at 20°C by liquid cooling. In between runs, the capillary is flushed for 2 min with water and for 3 min with buffer. All samples are analysed in duplicate at the different HPCD concentrations to obtain an average value for the apparent velocity of each enantiomer.

3. Linear regression model

For a completely ionised compound, the total effective velocity of an enantiomer is given as:

$$v_{tot} = \frac{1}{1+K[S]} \cdot v_E + \frac{K[S]}{1+K[S]} \cdot v_{ES} \quad (2)$$

and for the two enantiomers E_1 and E_2, the velocity difference between E_1 and E_2 is given as:

$$\Delta v = \frac{(v_E - v_{ES})[S](K_2 - K_1)}{1+(K_1+K_2)[S]+K_1K_2[S]^2} \quad (3)$$

Since throughout the experiments the pH of the different run buffer solutions is kept at a constant value of 3±0.1 the analytes are always completely ionised. In TEA–phosphate buffer a weak anodic EOF is present. The velocity of this EOF is very small compared to the electrophoretic velocity of the compound. Therefore, the EOF can be neglected.

The general procedure of the model is demonstrated using isoconazole as test substance in a buffer containing 30% methanol. The first step of the analysis is the experimental data acquisition, i.e., the velocities of both enantiomers and the corresponding velocity differences (v_{tot1}, v_{tot2} and Δv), measured in the concentration range of selector between 0 and 4 m*M* with intervals of 0.5 m*M*. The different steps of the procedure are explained through the experimental data in Table 1.

When the concentration of the selector in the buffer is low and assuming that the velocity of the inclusion complex is considerably small compared to the velocity of the free ion, then the second term in

Table 1
Velocities and velocity differences of the two enantiomers of isoconazole[a]

[S] (m*M*)	v_{tot1} (cm/min)	v_{tot2} (cm/min)	Δv (cm/min)
0	3.633	3.633	0.000
0.5	3.393	3.323	0.070
1	3.297	3.174	0.123
1.5	3.096	2.932	0.164
2	3.036	2.845	0.191
2.5	2.923	2.717	0.206
3	2.866	2.642	0.224
3.5	2.733	2.506	0.227
4	2.681	2.448	0.233

[a] Experimental conditions: capillary: 37 cm (30 cm effective length)×75 μm I.D.; separation solution: 100 m*M* triethanolamine–phosphate buffer, pH 3.0+30% methanol+0–4 m*M* HPCD; detection: 214 nm; applied voltage: 20 kV; injection: 3 s (pressure); temperature: 20°C.

Eq. (2) can temporarily be neglected, leading to Eq. (4):

$$v_{tot} = \frac{v_E}{1 + K[S]} \quad (4)$$

The reciprocal of this equation yields a linear relationship between $1/v_{tot}$ and [S], from which the binding constant K can be determined:

$$\frac{1}{v_{tot}} = \frac{1}{v_E} + \frac{K[S]}{v_E} \quad (5)$$

$$K = \frac{\text{slope of the regression } (K/v_E)}{\text{intercept at the } y\text{-axis } (1/v_E)}$$

In Fig. 2 it can be observed that the point at zero HPCD concentration, corresponding to the reciprocal of the velocity of the free enantiomers, is situated below the regression line. This indicates that the velocity of the inclusion complex cannot be neglected. With increasing concentrations of chiral selector in the buffer the effect of the velocity of the complexed fraction on the apparent velocity is becoming significant. In other words, when the second term in Eq. (2) is omitted, then too much weight is given to the velocity of the free enantiomers. Reversal of these velocities thus yields smaller values then expected. Consequently the slopes of the regressions are smaller than the real ones, which leads to an underestimation of the K values. Therefore, a correction of the v_{tot} values has to be introduced.

We know that the velocity of the enantiomer–CD complex certainly lies between the velocity of the CD (which is equal to the EOF) and the velocity of the free enantiomer (v_E). This means that v_{ES} can be considered as a fraction of v_E or $v_{ES} = v_E/n$. For the further development of the model the assumption is made that $n = 3$. Other values for n have also been tested, but the results for $n = 3$ are in most cases best in agreement with the results of least-square non-linear regression (NLR). The velocity of the complexed fraction can then be estimated by using the supposed velocity and the underestimated K values derived from the original regression. Corrected values of v_{tot} (v_{totc}) are obtained by subtraction of the assumed velocities of the complexed fractions from the original v_{tot} values:

$$v_{totc} = v_{tot} - \frac{v_E}{3} \cdot \frac{K_u[S]}{1 + K_u[S]}$$

where K_u is the underestimated K value.

The regression of the reciprocal of these corrected velocities as a function of HPCD concentration should yield a linear relationship, which passes through the point where the concentration of HPCD is zero. The K values obtained from this correction are considered good estimates of the real values.

Fig. 3 also shows that the intercepts of the

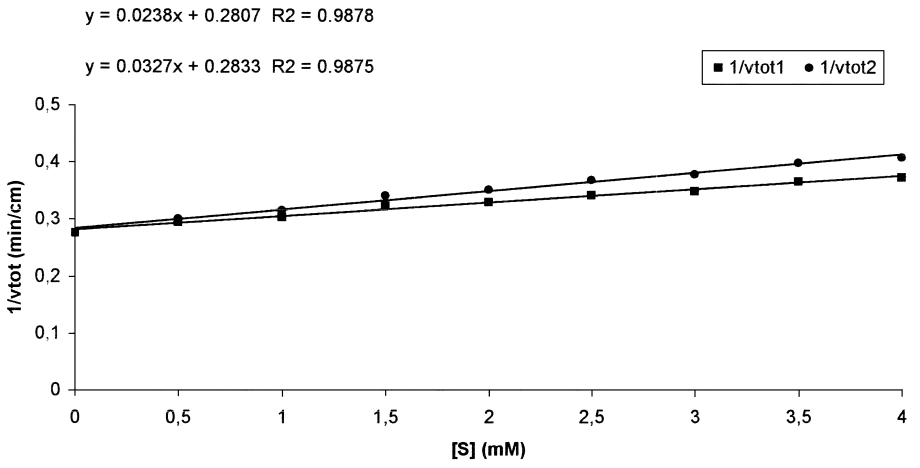


Fig. 2. Linear regression of the reciprocal of the velocities of the enantiomers of isoconazole as a function of the HPCD concentration. For experimental conditions, see Table 1.

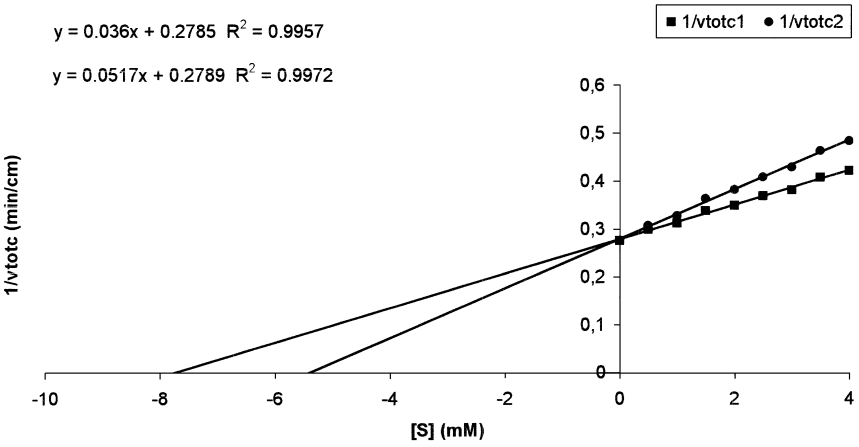


Fig. 3. Linear regression of the reciprocal of the corrected velocities of the enantiomers of isoconazole as a function of the HPCD concentration.

regression lines at the negative side of the *x*-axis ($1/v_{totc}=0$) correspond to the reciprocal of the *K* values ($[S]=1/K$). These two values limit the interval in which the optimum HPCD concentration is present.

The validity of the *K* values, determined by the second regression, could be tested using Eq. (3). The regression line obtained by plotting the velocity difference (Δv) of the two enantiomers as a function of $[S](K_2-K_1)/1+[S](K_1+K_2)+K_1K_2[S]^2$, should show a linear relationship with intercept equal to zero, giving a slope equal to ($v_E - v_{ES}$), providing an estimation of the velocity of the complex v_{ES}. The equation was calculated as $y=2.7872x+0.0042$ with $R^2=0.996$.

4. Results and discussion

The aim of this work is to evaluate the linear

Table 2
Comparison of *K*, $[S]_{opt}$ and v_{ES} values of econazole, miconazole and isoconazole enantiomers obtained by the linear regression method (LR) and by non-linear least square regression (NLR) without organic modifier[a]

Method	K_1 (mM^{-1})	K_2 (mM^{-1})	$[S]_{opt}$ (mM)	v_{ES} (cm/min)
Econazole				
LR	0.598	0.725	1.52	1.246
NLR	0.599±0.076	0.726±0.099	1.52	1.245±0.128[1] 1.258±0.125[2]
Miconazole				
LR	0.655	0.818	1.37	1.210
NLR	0.655±0.081	0.841±0.106	1.35	1.340±0.116[1] 1.364±0.106[2]
Isoconazole				
LR	1.280	2.044	0.62	1.513
NLR	1.211±0.113	1.874±0.177	0.66	1.234±0.074[1] 1.240±0.061[2]

[a] Experimental conditions: separation solution: 100 m*M* triethanolamine–phosphate buffer, pH 3.0+0–4 m*M* HPCD. Other conditions: see Table 1.

Table 3
Comparison of K, $[S]_{opt}$ and v_{ES} values of econazole, miconazole and isoconazole enantiomers obtained by the linear regression method (LR) and by non-linear least square regression (NLR) in the presence of 30% methanol[a]

Method	K_1 (mM^{-1})	K_2 (mM^{-1})	$[S]_{opt}$ (mM)	v_{ES} (cm/min)
Econazole				
LR	0.107	0.124	8.86	0.758
NLR	0.106±0.028	0.121±0.026	8.83	0.793±0.370[1]
				0.797±0.282[2]
Miconazole				
LR	0.099	0.119	9.21	0.858
NLR	0.107±0.026	0.129±0.029	8.51	0.919±0.305[1]
				0.944±0.260[2]
Isoconazole				
LR	0.129	0.185	6.47	0.846
NLR	0.125±0.031	0.195±0.040	6.41	0.843±0.313[1]
				0.960±0.210[2]

[a] Experimental conditions: see Table 1.

regression (LR) model in order to reduce the amount of experiments and CDs necessary. It is clear that such predictions should be beneficial in cases where highest velocity differences occur at CD concentrations beyond the highest concentration used in the model (for example here 4 mM). The imidazole derivatives used in this study have very high affinity for the chiral selector. Therefore, the optimal HPCD concentration is already reached in the low concentration range. Adding organic modifier to the CD containing buffer decreases the affinity of the enantiomers for the cyclodextrin and thus the optimal concentration is increased [22,23]. The organic modifier has two roles: it improves the solubility of the chiral substances and it decreases the affinity of chiral compounds for the hydrophobic cavity of chiral selector [20], thus decreasing the binding constants. Two different organic modifiers are used:

Table 4
Comparison of K, $[S]_{opt}$ and v_{ES} values of econazole, miconazole and isoconazole enantiomers obtained by the linear regression method (LR) and by non-linear least square regression (NLR) in the presence of 20% isopropanol[a]

Method	K_1 (mM^{-1})	K_2 (mM^{-1})	$[S]_{opt}$ (mM)	v_{ES} (cm/min)
Econazole				
LR	0.026	0.029	36.42	0.695
NLR	0.027±0.014	0.031±0.014	34.57	0.732±0.466[1]
				0.760±0.391[2]
Miconazole				
LR	0.041	0.045	23.28	1.070
NLR	0.036±0.017	0.041±0.017	26.03	0.967±0.318[1]
				0.972±0.267[2]
Isoconazole				
LR	0.047	0.061	18.68	0.741
NLR	0.027±0.013	0.038±0.014	31.22	0.523±0.526[1]
				0.613±0.322[2]

[a] Experimental conditions: separation solution: 100 mM triethanolamine–phosphate buffer, pH 3.0 + 20% isopropanol + 0–4 mM HPCD. Other conditions: see Table 1.

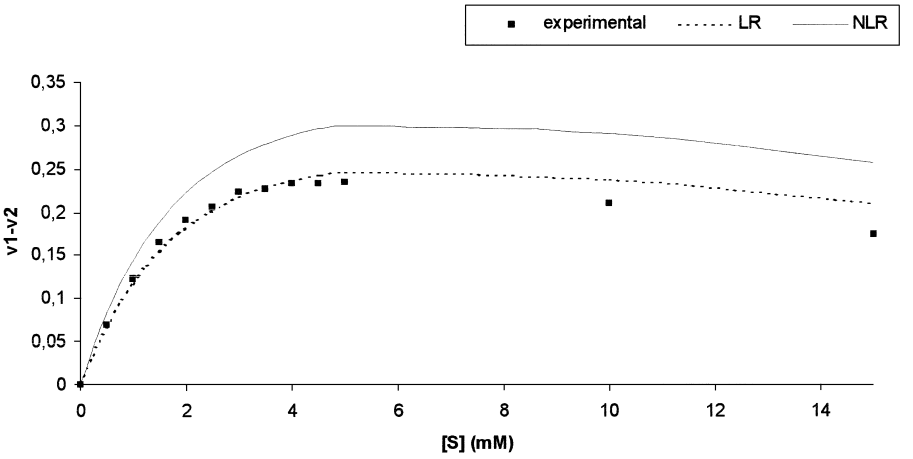


Fig. 4. Comparison of the experimentally obtained velocity differences of the enantiomers of isoconazole as a function of the HPCD concentration in buffer with 30% methanol and the velocity differences calculated with the values obtained by linear regression and those obtained by non-linear regression.

30% methanol added to the buffer increases the optimal concentration considerably, but with 20% isopropanol the optimal concentrations are even higher (Tables 2–4). It can also be observed that, when no chiral selector is added, the migration times are prolonged by the addition of organic modifier, and also affected by the nature of this modifier [24].

When the optimal concentration is already reached in the low concentration range (below 4 m*M*), then it is possible to use the LR model if only the concentrations below the maximum are used. In Table 2 the results for the three derivatives are shown. Another possibility is that in the low concentration range there are points where no velocity difference is observed. The values with $\Delta v = 0$ (except where $[S] = 0$) are discarded before use of the model. If no separation is seen between 0 and 4 m*M*, this means that the optimal concentration will be very high or that no separation will occur. In these cases the type of cyclodextrin seems inadequate.

The values obtained by the linear regression method are compared to those obtained by non-linear least square regression (Tables 2–4). For an accurate estimation by non-linear regression more data points have to be collected. For the experiments without organic modifier and for those with methanol, velocities are measured up to 15 m*M* HPCD, with isopropanol up to 60 m*M*. The data are corrected for viscosity by comparing the current of the CD containing buffer with the CD free buffer.

The $[S]_{opt}$ of the LR method are quite in agreement with those obtained with NLR taken as a reference, except for isoconazole in buffer with 20% isopropanol where a high deviated value is observed. The optimal concentration of HPCD in the buffer, which should provide the largest difference in velocity between the enantiomers, corresponds to the experimentally observed optimal concentration, which is shown in Fig. 4 for isoconazole in buffer containing 30% methanol. The separation of isoconazole enantiomers achieved at various concentrations of HPCD is shown in Fig. 5.

Table 5
Concentration that provides maximal velocity difference ($[S]_{opt}$) and concentration where maximal resolution ($[S]_{R_S}$) is seen for the three tested derivatives[a]

	$[S]_{opt}$ (m*M*)	$[S]_{R_S}$ (m*M*)
Econazole	1.5	3.0
Miconazole	1.4	2.5–3.0
Isoconazole	0.6	1.5

[a] Experimental conditions: separation solution: 100 m*M* triethanolamine–phosphate buffer, pH 3.0 + 0–4 m*M* HPCD. Other conditions: see Table 1.

Although these three derivatives only differ by one chlorine atom or the position of a chlorine atom (Fig. 1), the binding constants show some difference. This demonstrates that little difference in the structure of molecules can lead to differences in binding affinity of these molecules for a specific CD derivative. Isoconazole seems to have a higher affinity for HPCD then miconazole and econazole. In isoconazole the chlorine atoms are 2,6 bonded to the phenyl ring, whereas in miconazole they are 2,4 bonded and in econazole there is only one chlorine atom.

In Table 5 the optimal concentration is compared with the concentration that provides the highest resolution for the different derivatives in buffer without organic modifier. Maximal resolution will not occur at the exact same concentration as maximal velocity difference (Fig. 6). Resolution is more

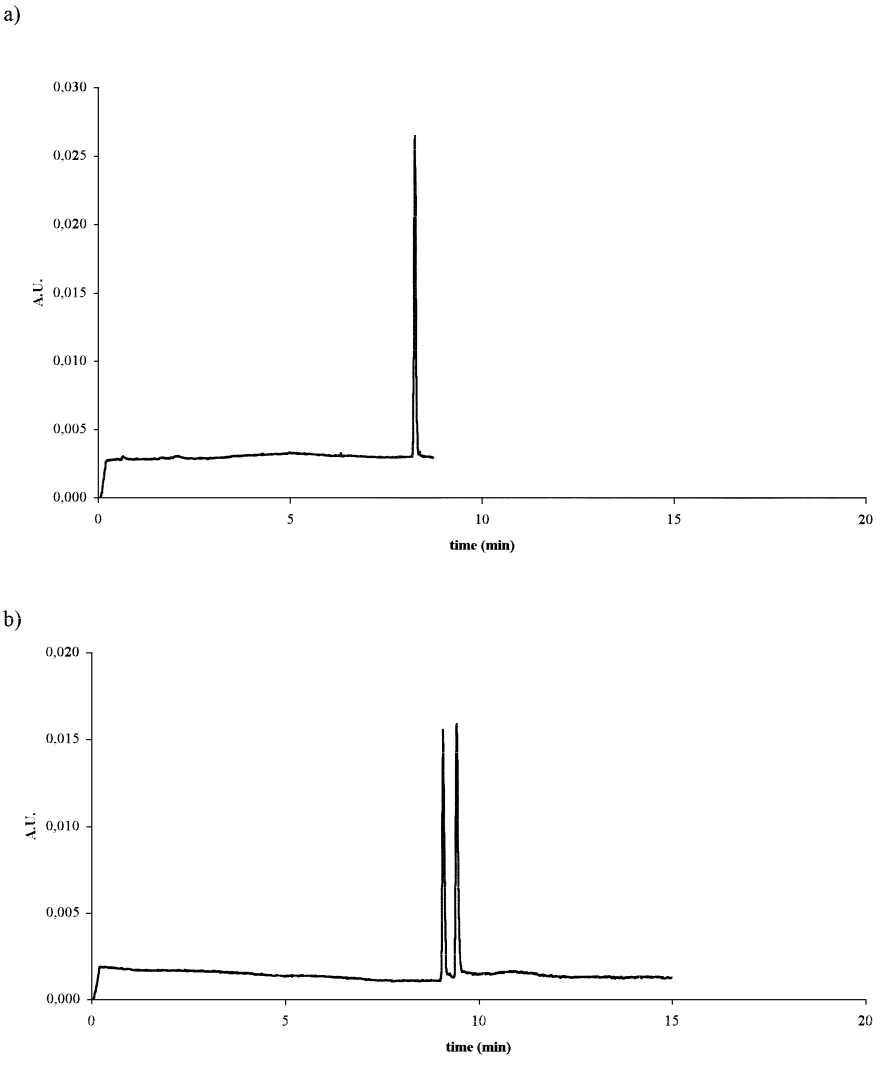
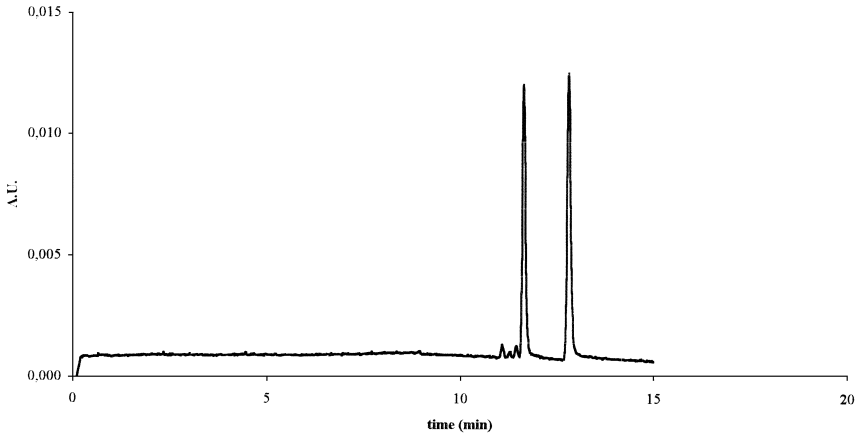


Fig. 5. Separation of isoconazole enantiomers with different concentrations HPCD. Buffer: 100 m*M* triethanolamine–phosphate, pH 3.0 + 30% methanol + (a) 0 m*M* HPCD, (b) 1 m*M* HPCD, (c) 5 m*M* HPCD, (d) 15 m*M* HPCD.

c)



d)

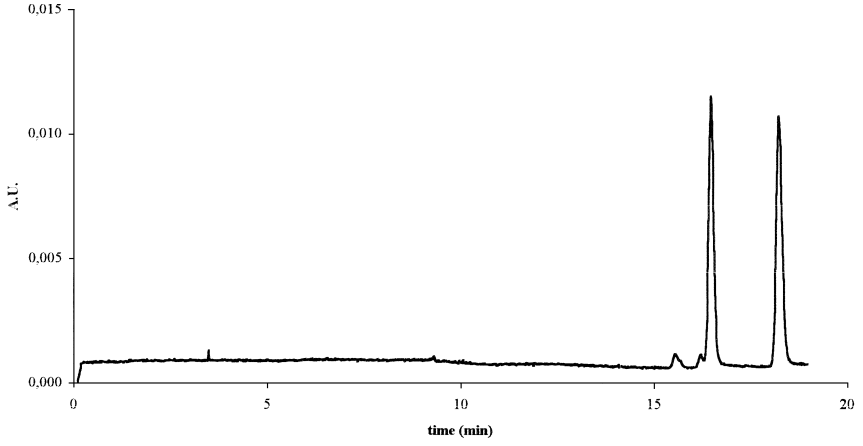


Fig. 5. (*continued*)

complex because it must also consider electroosmotic mobility, band broadening due to diffusion and other factors such as injection and detector path length. For the three derivatives maximal resolution is observed at higher concentration than maximal velocity difference (Table 5). This difference can be attributed to the influence of μ_{ep} in the denominator of the resolution equation: when the concentration of chiral selector increases, then μ_{ep} becomes smaller [8].

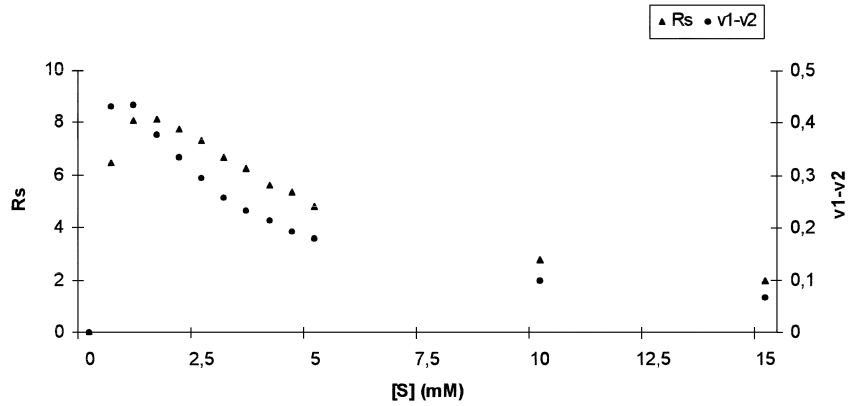


Fig. 6. Velocity difference and resolution for the enantiomers of isoconazole as a function of the HPCD concentration.

5. Conclusion

An alternative linear regression method has been developed to estimate the binding constants of analyte–CD complexes. This makes it possible to predict the CD concentration that gives the largest velocity difference from a few experiments performed at low concentrations.

The results obtained by this method are quite in agreement with the results obtained by non-linear regression. The optimal CD concentration in the buffer, which would provide the largest difference in velocity between the enantiomers, corresponds to the experimentally observed optimal concentration.

The antimycotic imidazole derivatives tested have very high affinities for the chiral selector HPCD. This implicates that the concentration that provides maximal velocity difference is very low (1–2 m*M*). With organic modifiers like methanol and isopropanol, this optimal concentration is considerably increased, emphasising the role of organic modifiers in this kind of analysis.

Further investigation of this model with other test compounds and different cyclodextrins is currently in progress.

Acknowledgements

The authors thank Dr. R. Hauspi for his critical considerations concerning the use of the NLR technique.

References

[1] A.J. Hutt, S.C. Tan, Drugs 52 (Suppl. 5) (1996) 1.
[2] S. Fanali, J. Chromatogr. A 735 (1996) 77.
[3] R. Kuhn, F. Stoecklin, F. Erni, Chromatographia 33 (1992) 32.
[4] R. Vespalec, P. Bocek, Electrophoresis 15 (1994) 755.
[5] G. Gübitz, M.G. Schmid, J. Chromatogr. A 792 (1997) 179.
[6] S. Fanali, J. Chromatogr. A 792 (1997) 227.
[7] S.A.C. Wren, R.C. Rowe, J. Chromatogr. 603 (1992) 235.
[8] S.A.C. Wren, J. Chromatogr. 636 (1993) 57.
[9] S.A.C. Wren, Electrophoresis 16 (1995) 2127.
[10] S.G. Penn, D.M. Goodall, J.S. Loran, J. Chromatogr. 636 (1993) 149.
[11] P. Gareil, D. Pernin, J.P. Gramond, F. Guyon, J. High Resolut. Chromatogr. 16 (1993) 195.
[12] Y.Y. Rawjee, D.U. Staerk, Gy. Vigh, J. Chromatogr. 635 (1993) 291.
[13] Y.Y. Rawjee, Gy. Vigh, Anal. Chem. 66 (1994) 619.
[14] Y.Y. Rawjee, R.L. Williams, L.A. Buckingham, Gy. Vigh, J. Chromatogr. A 688 (1994) 273.
[15] M.E. Biggin, R.L. Williams, Gy. Vigh, J. Chromatogr. A 692 (1995) 319.
[16] S. Surapaneni, K. Ruterbories, T. Lindstrom, J. Chromatogr. A 701 (1997) 249.
[17] S.G. Penn, E.T. Bergstrom, D.M. Goodall, J.S. Loran, Anal. Chem. 66 (1994) 2866.
[18] Baumy, Morin, M. Dreux, M.C. Viaud, S. Boye, G. Guillaumer, J. Chromatogr. A 707 (1995) 311.
[19] S.G. Penn, E.T. Bergstrom, I. Knights, G. Liu, A. Ruddick, D.M. Goodall, J. Phys. Chem. 99 (1995) 3875.
[20] B. Chankvetadze, G. Endresz, G. Blaschke, J. Chromatogr. A 700 (1995) 43.
[21] I. Bechet, P. Paques, M. Fillet, P. Hubert, J. Crommen, Electrophoresis 15 (1994) 818.
[22] S.A.C. Wren, R.C. Rowe, J. Chromatogr. 609 (1992) 363.
[23] E. Szökó, J. Gyimesi, L. Barcza, K. Magyar, J. Chromatogr. A 745 (1996) 181.
[24] G.M. Janini, K.C. Chan, J.A. Barnes, G.M. Muschik, H.J. Issaq, Chromatographia 35 (1993) 497.