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Enantiomeric resolution on Chiral-AGP with the aid of experimental design
Unusual effects of mobile phase pH and column temperature

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Abstract

Baseline separation of the enantiomers of two structurally related dihydro-pyranoimidazopyridines was performed within 15 min on Chiral-AGP by using experimental design. The retention times of the enantiomers were found to have an optimum at a mobile phase pH of around 7. However, the enantioselectivity increased with increasing mobile phase pH. Besides the interesting effect of pH, only a minor decrease in the enantioselectivity was obtained by increasing the column temperature. The variables, column temperature, mobile phase pH and concentration of acetonitrile, influenced all four stereoisomers in the same way. However, the statistical model separated the loadings i.e., the influence of the descriptor variables on the capacity factors of the first and last eluted enantiomers of the two solutes investigated, which indicated differences in retention mechanisms.

Keywords: Enantiomer separation; Experimental design; Chemometrics; pH effects; Temperature effects; Mobile phase composition; Dihydropyranoimidazopyridines

1. Introduction

The most used technique for simultaneously separating enantiomers and determining enantiomeric purity is liquid chromatography. The enantiomers can be separated with or without a prior derivatization step of the two enantiomers. In the indirect separation mode, the two enantiomers react with an optically pure reagent and covalently bonded diastereoisomers are formed. These diastereoisomers can often be separated using an a non-chiral chromatographic system [1]. Using the direct separation technique, the enantiomers are injected into a chromatographic system where a chiral selector is present. The chiral selector can be dissolved in the mobile phase [2–4] and enantiomeric separation is performed due to differences in stability constants between the diastereomeric complexes in the mobile phase and/or differences in adsorption properties of the diastereomeric complexes to a non-chiral stationary phase. The chiral selector can also be immobilized on the stationary phase and enantioselective recognition is, in this case, performed based on differences in binding properties between the two enantiomers to the chiral selector. Macromolecules as proteins [5–7] and also smaller molecules [8,9] have been used as chiral selectors immobilized on silica gel.

The aim of this study was to separate the enantiomers of two compounds, 3-hydroxymethyl-2-methyl-9-phenyl-7H-8,9-dihydropyrano[2,3-*c*]-imidazo[1,2-*a*]pyridine (solute No. 1) and 3-methylethylether-2-methyl-9-phenyl-7H-8,9-dihydropyrano[2,3-*c*]-imidazo[1,2-*a*]pyridine (solute No. 2), on a Chiral-AGP

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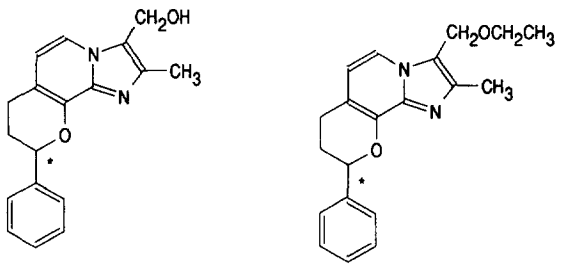

Solute No. 1 Solute No. 2

Fig. 1. Structures of the solutes.

column by using experimental design (structures in Fig. 1). Previous work has shown that enantioselective retention using immobilized α_1-acid glycoprotein as the chiral selector was influenced by the pH of the mobile phase, the column temperature as well as by the type and concentration of the organic modifier [10]. These three chromatographic parameters were chosen as variables in the experimental design and selectivity factors and the logarithm of the capacity factors were the modelled responses. A strategy for optimization of the evaluation of the statistical model using the software Modde 2.1 is described, as is the predictability of the final statistical model. Unique effects of the pH of the mobile phase on retention as well as the influence of the column temperature on the enantioselectivity were observed. An optimal chromatographic system was chosen from the statistical model and this system was used to determine the enantiomeric purity of the two enantiomers of solute 1.

2. Experimental

2.1. Apparatus

The chromatographic system consisted of an LKB 2150 LC pump (Pharmacia, Sollentuna, Sweden), an SP 8880 autosampler (Spectra-Physics, San Jose, CA, USA) with a 50-μl loop and a Linear UVIS 200 detector (Linear Instruments, NV, USA). The column used in the study was Chiral-AGP, 5 μm, 100×4.0 mm (ChromTech, Hägersten, Sweden). The temperature of the column and the mobile phase reservoir were maintained with a Julabo 12 B water bath (Julabo Labortechnik, Seelbach, Germany). The pH was measured with a PHM83 pH meter (Radiometer, Copenhagen, Denmark). The analytes were injected as a single injection and all chromatographic results reported are the mean of duplicate injections. All analytes were detected at 232 nm.

2.2. Chemicals

Acetonitrile (ACN, HPLC-grade) was obtained from Rathburn (Walkerburn, UK). Methanol (MeOH, HPLC-grade) was obtained from Fisons (Loughborough, UK). 2-Propanol (gradient grade), sodium dihydrogenphosphate (analytical-reagent grade) and disodiumhydrogenphosphate (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany). The analytes (*R*)-, (*S*)- and (*R,S*)-3-hydroxymethyl-2-methyl-9 -phenyl-7H-8,9 - dihydropyrano[2,3-*c*]-imidazo[1,2-*a*]pyridine (solute 1) and (*R,S*)-3-methylethylether-2-methyl-9 -phenyl-7H-8,9-dihydropyrano[2,3-*c*]-imidazo[1,2-*a*]pyridine (solute 2) were synthesized at Astra Hässle (Sweden); structures are given in Fig. 1.

2.3. Chromatographic outputs

The capacity factor (k') was calculated using the equation, $k'=(t_R/t_0)-1$, where t_R is the retention time for the solute and t_0 is the retention time for the first disturbance of the baseline after injection of pure water. The selectivity factor (α) was calculated using the equation, $\alpha=k'2/k'1$ where $k'2$ is the capacity factor for the last eluted enantiomer. The column efficiency, expressed as the number of theoretical plates (N), was calculated using the equation $N=16t_R^2/w_t^2$, where w_t is the bandwidth measured at the baseline. The resolution factor (R_s) is given as,

$$R_s = \frac{N^{1/2}k'_2(\alpha - 1)}{4(k'_2 - 1)\alpha}$$

2.4. Statistical methods

A 2^3 full factorial design with centerpoints was used to examine the influence of the descriptor

variables on the chromatographic responses for the two analytes. With three variables, the experimental design included eight experiments and, in addition, centerpoint experiments were replicated three times. All experiments were performed in random order, except for the centerpoint replicates, which were run at the beginning, in the middle and at the end of the experiments.

Design and evaluation of the statistical model were performed using the software Modde version 2.1, (Umetri, Sweden) using partial least squares (PLS) as the regression method. PLS is a multivariate regression method [11,12] that provides an overview of large data sets. It is most useful for data derived from experimentation according to factorial design, since such a combination guarantees accurate estimates of the coefficients in the regression equation [13] and, thus, inherently illuminating and reliable loading structures projected to the individual principal components (PCs) [12], provided they are significant [14]. The rationale of employing PLS can be to use the regression equation for predictions [11], but interpretations from plots derived from the various estimates from the individual PCs can also be informative [11,15].

Validation of the chemometric model was made with cross-validation [14]. As a measure of the suitability of the model, the fraction of the variation of the response explained by the model and the fraction of the variation of the response that can be predicted by the model were calculated. In addition, a test experiment was performed and its outcome was compared to predicted data.

The parameters used in this paper R^2 (the fraction of variation of the responses explained by the model) and Q^2 (the fraction of variation of the responses that can be predicted by the model) are given by:

$$R_2 = \mathrm{SS}_{\mathrm{REG}}/\mathrm{SS} \quad (1)$$

$$Q^2 = 1 - \mathrm{PRESS}/\mathrm{SS} \quad (2)$$

where $\mathrm{SS}_{\mathrm{REG}}$ is the sum of squares of Y corrected for the mean, explained by the model, SS is the total sum of squares of Y corrected for the mean and PRESS is the prediction residuals sum of squares. $R^2$and Q^2 are used as indicative criteria of the model fit.

3. Results and discussion

The enantiomers of solute 1 and a structurally related compound, 2, were separated on Chiral-AGP using factorial design. Type and concentration of an organic modifier, the pH of the mobile phase and the column temperature were the variables tested in this study. As a screening experiment, three different organic modifiers were tested, separately, in a mobile phase consisting of phosphate buffer (pH 7.6, ionic strength $I=0.01$) at a column temperature of 20°C (Table 1). Different concentrations of the three organic modifiers were used in order to obtain retention times of the same magnitude.

The experimental data show that methanol (25%, v/v) and acetonitrile (12%, v/v) gave approximately the same retention for the analyte but shorter retention times were observed when 2-propanol (12%, v/v) was used as the organic modifier. The separation factor decreased in the order acetonitrile > methanol > 2-propanol (Table 1). Because of these data, acetonitrile was used as the uncharged modifier throughout the experimental design. A further screening of the variables [acetonitrile concentration (Ac), mobile phase pH and column temperature (te)] gave the ranges given in Table 2.

The modelling of the responses using the default values in the software gave a high explanation (R^2) and over 90% of the variation of the experimental data was explained (Table 3). However, the predictability of the statistical model (Q^2) was rather low, between 50 and 60% (Table 3). The loading plot obtained from this modelling shows that there was only a minor influence on retention or selectivity of the interaction terms (pH·te, pH·Ac and Ac·te) as

Table 1
Influence of organic modifier added to the mobile phase on enantioselective retention

Organic modifier	Concentration (%, v/v)	k'_{R}	$\alpha_{\mathrm{S/R}}$
Acetonitrile	12	4.9	1.6
Methanol	25	4.9	1.5
2-Propanol	12	3.6	1.1

Solid phase, Chiral-AGP. Mobile phasem phosphate buffer (pH 7.6, $I=0.01$). Column temperature, 20°C. Solute, 1.

Table 2
Experimental design

Experiment number	Experiment name	Run order	pH	Temperature (°C)	Acetonitrile	k1	k2	k3	k4	a1	a2
1	N1	7	5.5	20	10	5.29	6.01	6.23	7.74	1.14	1.24
2	N2	8	7.5	20	10	10.2	16.2	10.5	20.8	1.59	1.97
3	N3	3	5.5	40	10	3.52	4.03	4.41	5.52	1.14	1.25
4	N4	10	7.5	40	10	4.73	7.13	5.54	10.1	1.51	1.82
5	N5	5	5.5	20	15	3.17	3.17	3.12	3.54	1.00	1.14
6	N6	11	7.5	20	15	3.78	5.17	3.30	5.56	1.37	1.69
7	N7	1	5.5	40	15	2.02	2.13	2.13	2.43	1.06	1.14
8	N8	6	7.5	40	15	1.57	2.18	1.62	2.72	1.40	1.68
9	N9	2	6.5	30	12.5	5.15	6.44	5.22	7.53	1.25	1.44
10	N10	9	6.5	30	12.5	5.09	6.35	5.24	7.55	1.25	1.44
11	N11	4	6.5	30	12.5	5.1	6.36	5.21	7.52	1.25	1.44

Abbreviations: k1 = k'_R – solute 1, k2 = k'_S – solute 1, k3 = k'_1 – solute 2, k4 = k'_2 – solute 2, a1 = $\alpha_{(S/R)}$ – solute 1 and a2 = $\alpha_{(2/1)}$ – solute 2.

they were oriented near the origin (Fig. 2). The two interaction terms nearest the origin were withdrawn from the model and replaced by the term pH, as results in the worksheet indicated a quadratic term of the mobile phase pH on the retention of the four enantiomers. A further discussion regarding loading plots are given later in this paper. This new model fitted the experimental data well (Table 3). Both the explained variation of the experimental data (>95%) and the predictory abilities of the model (up to 90%) increased for the responses, i.e., the logarithm of the capacity factors and the selectivity factors.

Table 3
Optimization of the statistical model. Fitting of the experimental data (R^2) and predictability of the model (Q^2)

Model	Terms	R^2 total (range)[a]	Q^2 total (range)[a]
Default	pH Ac te pH[a]·Ac pH[a]·te Ac[a]·te	0.948 (0.90–0.99)	0587 (0.43–0.63)
Optimized	pH Ac te pH[a]·pH pH[a]·Ac	0.974 (0.96–0.99)	0.889 (0.77–0.89)

[a] Range for individual responses (k1–k4 and a1–a2)

3.1. Influence of the variables on the responses

3.1.1. Capacity factors (k')

The loading plot of the optimized model is given in Fig. 3. All the four capacity factors are located at a high positive value in the first PLS component (*X*-axis) and at a negative value in the second PLS component (*Y*-axis). This means that all of the capacity factors are influenced by the variables in the same way. However, the two last-eluted enantiomers of the respective racemate are located as a pair that differ in absolute position from the pair consisting of the two first-eluted enantiomers.

Two of the variables, the concentration of acetonitrile and the column temperature, are located at a negative position in the first PLS component and at a positive position in the second PLS component. This means that these two variables have negative influences on the retention or, in other words, an increase in the acetonitrile concentration or the column temperature will decrease the retention of the four stereoisomers. A more complicated pattern was observed for the effect of the pH of the mobile phase on the retention. The effect of the mobile phase's pH itself was positive, but the effect of the quadratic term was negative. These two effects should result in a non-linear effect of the mobile phase's pH. This hypothesis was tested using four mobile phases that differ in the pH of the mobile phase (Fig. 4). A maximum in retention for the two enantiomers of solute 1 was observed at pH 6.5–7.0. A similar

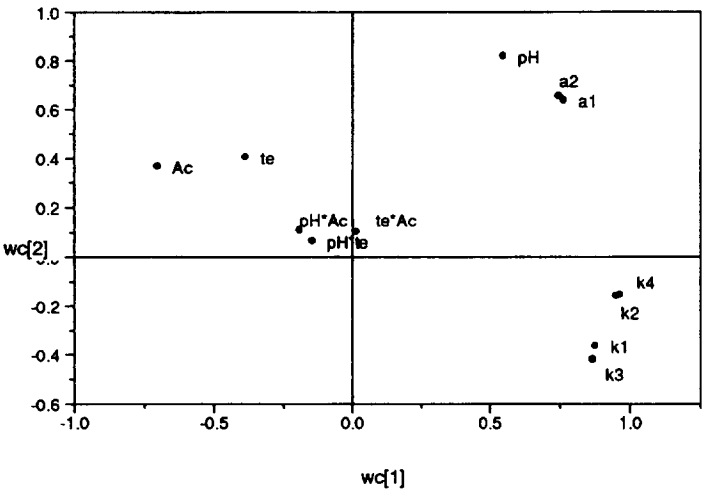

Fig. 2. Loading plot with the model parameters in the software, Modde, set as default. Abbreviations: Responses, k1–k4 and a1–a2, as in Table 2. Descriptor variables, Ac = content of acetonitrile; te = column temperature; pH = pH of the mobile phase.

effect of the pH of the mobile phase on enantioselective retention was previously observed using chiral imprinted polymers [16]. An explanation for this unusual effect of the mobile phase pH was probably that the solute changes its charge from +1 to 0 when the pH is changed from 5.5 to 7.6. In the whole pH range, the net negative charge increase for the immobilized protein increased the possibilities for the positively charged solute to interact by electrostatic interactions. When the pH in the mobile phase increased above the pK_a value of the solute (pK_a = 6.5; determined in pure water), the net charge will be 0 and its ability to interact with negatively charged groups on the protein will disappear. This resulted in decreased retention times for the two enantiomers of solute 1.

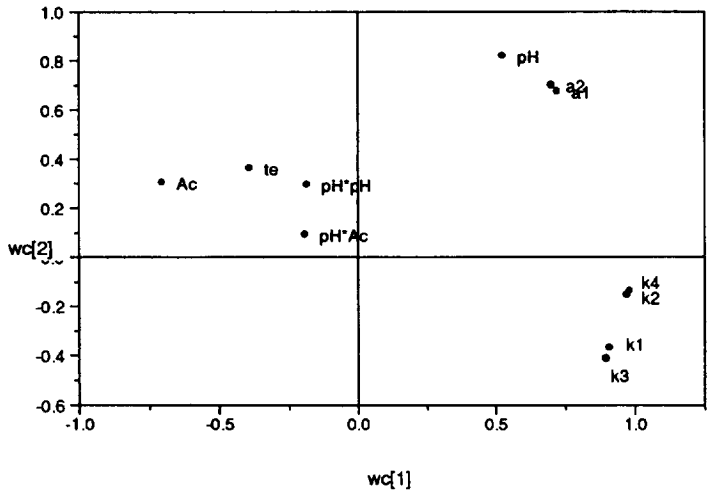

Fig. 3. Loading plot showing the influence of descriptor variables on the responses after optimization of the model parameters in Modde. Abbreviations as in Fig. 2.

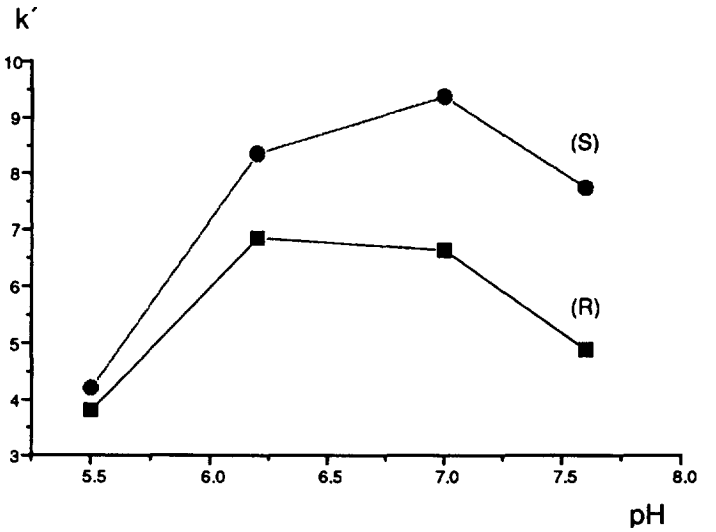

Fig. 4. Influence of the pH of the mobile phase on enantioselective retention. Solid phase, Chiral-AGP. Mobile phase, phosphate buffer ($I=0.01$)–acetonitrile (88:12, v/v). Column temperature, 20°C. Solute, 1.

3.2. Selectivity factors (α)

The influence of the variables on the selectivity are shown in the loading plot (Fig. 3). An increase of the mobile phase's pH resulted in increased selectivity factors, while the concentration of acetonitrile and column temperature gave the opposite effect. As mentioned earlier, the capacity factors decreased at a mobile phase pH above the pK_a of the solutes. An explanation for the further increase in the selectivity at higher pH values might be that an electrostatic interaction between the solutes and the protein surface is not essential for enantioselective retention and that the increased enantioselectivity is caused by hydrophobic effects. The chromatographic results indicated that the electrostatic interaction contributes to a non-chiral adsorption and, by decreasing the positive charge of the solutes, the selectivity increased. Another plausible explanation for the increase in enantioselectivity is the possiblity that the immobilized protein would change its conformation. The chiral centre of the solutes are rigid, as the carbon that binds four different substituents is located in a cyclic structure. The substituents that bind closely to this carbon atom could, besides the electrostatic attraction, interact with the protein surface by $\pi-\pi$ interaction, hydrogen bonding, hydrophobic attraction and also could exhibit steric hindrance.

Several authors have been studying the effect of column temperature on enantioselective retention [17–19]. Enantioselectivity most often decreases with increasing column temperature. However, in this study, the column temperature gave rise to only a minor decrease in the separation factor, i.e., the variable column temperature is located near the origin in the loading plot (Fig. 3). This result was further verified by varying the column temperature from 20 to 45°C (Fig. 5 and Table 4). These complementary data show a decrease in retention by a factor of two, while the selectivity factor only decreased from 1.55 to 1.46 when using a high mobile phase pH (pH=7.6), where the solutes, to a high extent, are uncharged. A stereoselective hydrophobic effect upon binding may be the main reason for the observed temperature dependence [20]. However, the same pattern was observed when using a lower mobile phase pH (pH=6.2), where the solutes are mainly positively charged. These results indicated that the minor effect of the column temperature on the separation factor was the same with or without the possibility that the enatiomers could

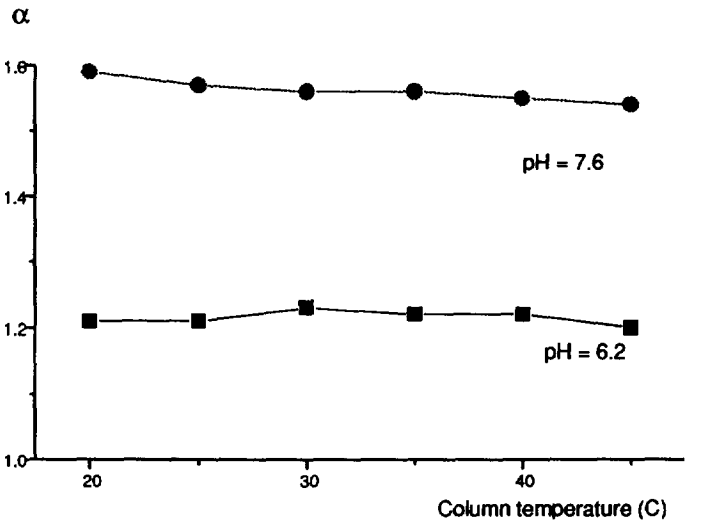

Fig. 5. Column temperature and enantioselectivity. Solid phase, Chiral-AGP. Mobile phase, phosphate buffer (I=0.01)–acetonitrile (88:12, v/v). Solute, 1.

interact with the protein surface by electrostatic attraction.

A useful option in the statistical program Modde 2.1 is the use of three-dimensional plots that show the predicted values of a chosen response when varying two of the variables. The predicted effect of the column temperature and acetonitrile concentration at high pH (pH=7.5) on the selectivity factor of solute 1 are given in Fig. 6. This three-dimensional plot illustrated the minor effect of the column temperature on enantioselectivity and also the effect of the concentration of acetonitrile.

3.3. Prediction of a useful chromatographic system

A chromatographic system that separated all four

Table 4
Column temperature and enantioselective resolution

Column temperature (°C)	System A			System B		
	k'_S	N	R_s	k'_S	N	R_s
20	8.71	1461	3.15	9.27	2323	1.89
25	7.13	1512	3.06	8.03	2631	1.98
30	5.89	1557	2.99	6.93	2521	2.05
35	4.78	1663	2.98	5.93	2672	1.99
40	3.92	1780	2.95	5.08	2818	2.00
45	3.18	1820	2.81	4.35	4160	2.19

Solid phase, Chiral-AGP. Mobile phase, (A) phosphate buffer (pH 7.6, I=0.01)–acetonitrile (88:12, v/v); (B) phosphate buffer (pH 6.2, I=0.01)–acetonitrile (88:12, v/v). Solute, 1.

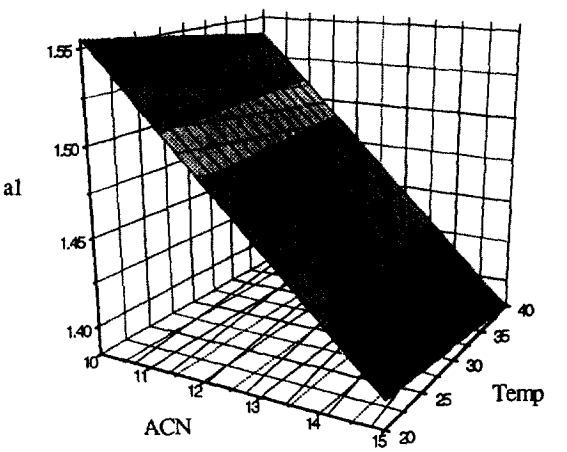

Fig. 6. Influence of the concentration of acetonitrile and the column temperature on the selectivity factor of solute 1 given by the statistical model. Abbreviations: al = selectivity factor; ACN = content of acetonitrile; Temp = column temperature.

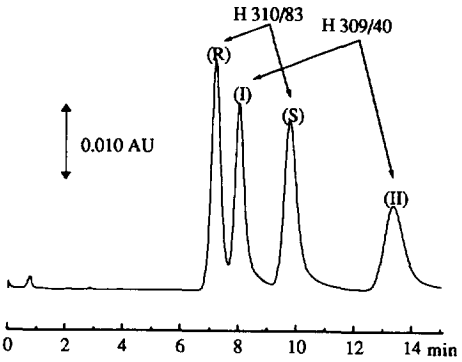

Fig. 7. Enantioselective separation of solutes 1 and 2. Solid phase, Chiral-AGP. Mobile phase, phosphate buffer (pH 7.5, $I=0.01$)–acetonitrile (9:1, v/v). Column temperature, 40°C.

stereoisomers was chosen from the statistical model and the enantiomers of solutes 1 and 2 could be simultaneously separated within 15 min (Fig. 7).

A useful chromatographic system for enantioresolution of solute 1 was evaluated from the model presented above. The criteria were to use a pH of the mobile phase that was not too high (in order to increase the stability of the column), moderate retention times and, if possible, to use a column temperature that was close to ambient temperature. A mobile phase with a pH of 7 and 14% (v/v) acetonitrile and a column temperature of 20°C were the values of the variables that were chosen (see chromatogram in Fig. 8). The predicted and found values of the capacity and selectivity factors are given in Table 5. The data obtained correlated well with the predicted data and a difference of less than 1% was observed. The optimised chromatographic system was used for the estimation of enantiomeric purity of authentic samples of the enantiomers of solute 1 (Fig. 8). The enantiomeric purity, 97% for both of the enantiomers of solute 1, was estimated from the peak areas and standard curves. The standard curves were linear in the concentration range 0.1–100 mM, with correlation factors >0.999. The minimum detectable quantities (MDQ) expressed as peak height that was three times the noise level were calculated to be $0.50 \cdot 10^{-12}$ mol (first eluted enantiomer) and $0.40 \cdot 10^{-12}$ mol (second eluted enantiomer) for solute 1.

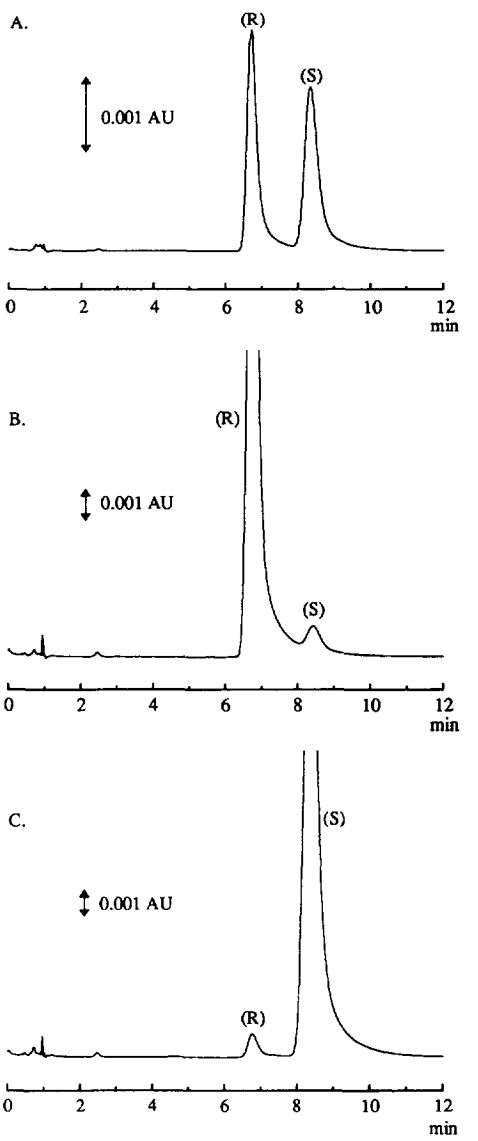

Fig. 8. Enantioselective separation of solute 1 in the optimized chromatographic system. (A)=(*R*,*S*)-solute 1; (B)=(*R*)-solute 1; (C) (*S*)-solute 1. Solid phase, Chiral-AGP. Mobile phase, phosphate buffer (pH 7.0, $I=0.01$)–acetonitrile (86:14, v/v). Column temperature, 20°C.

4. Conclusion

Experimental design was used to optimize the enantiomeric resolution of (R,S)-3-hydroxymethyl-2-

Table 5
Experimental and predicted chromatographic data

	Predicted	Found	Deviation (%)
k'_R	4.83	4.81	−0.4
k'_S	6.30	6.28	−0.3
$\alpha_{S/R}$	1.32	1.31	−0.8

Solid phase, Chiral-AGP. Mobile phase, phosphate buffer (pH 7.0, $I=0.01$)–acetonitrile (86:14, v/v). Column temperature, 20°C. Solute, 1.

methyl-9-phenyl-7H-8, 9-dihydropyrano[2, 3-*c*]-imidazo[1,2-*a*]pyridine (solute 1). The enantiomers of solute 1 and a structurally related compound, solute 2, were separated within 15 min. An optimum in retention was observed at a mobile phase pH that was close to the pK_a value of the solutes and, interestingly, the column temperature, 20–40°C, had only a minor effect on the enantioselective resolution. Retention data calculated from the statistical model correlated well with experimental data, with a deviation of less than 1%. The chromatographic system was highly stable and was used to determine the enantiomeric purity of the two enantiomers of solute 1.

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