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Short communication

Chiral ion-pair chromatography on porous graphitized carbon using *N*-blocked dipeptides as counter ions

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Abstract

Two newly synthesized chiral di-anionic counter ions were tested for enantiomeric resolution of a set of amino alcohols on porous graphitized carbon, Hypercarb. *Z*-L-Aspartyl–L-proline dissolved in methanol baseline resolved nine of 12 tested racemates. One of its diastereoisomers, *Z*-L-aspartyl–D-proline was also tested but resulted in low separation factors, <1.1. Sodium hydroxide was added to the mobile phase in order to titrate the counter ion to its mono- or di-anionic form. Results show that the di-anionic form was found to be superior to the mono-anionic form regarding enantioselectivity. Increased content of the counter ion in the mobile phase, with constant ratio between counter ion and sodium hydroxide concentration, decreased retention but only slightly affected enantioselectivity. Increased retention and enantioselectivity were observed with decreased column temperature. Resolution factors >3 were obtained between the enantiomers in atenolol and metoprolol with a total retention time of less than 15 min. Further, all four stereoisomers of an analogue to metoprolol were separated using Hypercarb and a mobile phase of 5 m*M* *Z*-L-aspartyl–L-proline and 9 m*M* sodium hydroxide in methanol.

Keywords: Enantiomer separation; Porous graphitized carbon; Ion-pair chromatography; Mobile phase composition; Amino alcohols

1. Introduction

The importance to develop new chiral discrimination separation systems to determine enantiomers in bulk drug substance, drug formulations and in biological fluids increases. This because stereoisomers may have different pharmacological effects [1] and also show differences in pharmacokinetic and pharmacodynamic properties [2]. Chromatographic techniques have been shown to be useful for this purpose [3]. Several examples in the literature use chiral stationary phases (CSPs) to separate enantiomers [4]. Separation is performed due to differences in adsorption properties to the immobilized chiral selector. Macromolecules such as α_1-acid glycoprotein and teicoplanin [5,6] as well as smaller molecules such as *N*-acylated proline anilides [7] have been

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used to achieve appropriate chiral discrimination systems. Also exemplified in the literature are separation systems where the chiral selector has been dissolved in the mobile phase, chiral mobile phase additives (CMPAs) [8]. Enantioselective separations are in this case caused by differences in complex-formation constants in the mobile phase and/or differences in the adsorption constants of the formed diastereoisomeric complexes to a non-enantioselective stationary phase [9]. As CMPAs, macromolecules such as albumin [10] and smaller molecules such as cyclodextrins [11] have been used. One common type of CMPA is obtained by dissolving chiral protolytes such as quinine or *N*-blocked dipeptides in the mobile phase [12]. The counter ion forms diastereoisomeric ion-pairs with enantiomers of opposite charge and these may differ in adsorption properties to a non-enantioselective stationary phase. Enantiomers of amines, i.e., quinine [12,13] as well as acids, i.e., *N*-blocked dipeptides [12,14] have been used to separate enantiomers of acids and amines, respectively. This technique was first developed using silica supports in the straight phase mode [8]. However, 10 years ago a new stationary phase support, porous graphitized carbon (Hypercarb), was made commercially available. This support withstand all mobile phase pH values and is also inert to the most common organic modifiers used. The flat surface of the porous graphitized carbon material presents new and alternative adsorption properties in comparison to the traditional silica supports [15]. This support makes it possible to use chiral ion-pair chromatography together with polar solvents [16,17]. Several amines have been enantioresolved using mono-anionic (such as *Z*-glycyl–L-proline [17]) and di-anionic counter ions (such as *Z*-L-glutamyl–L-proline [18]).

In the present study two new di-anionic chiral counter ions were studied with regard to the enantioseparation of racemic amines. The two *N*-blocked peptides, *N*-L-aspartyl–L-proline and its diastereoisomer, *N*-L-aspartyl–D-proline, were examined to separate enantiomers of a set of structurally closely related amino alcohols. The effect of charge and concentration of the counter ion was examined. The influence of column temperature on enantioselectivity and retention factors was also studied.

2. Experimental

2.1. Instrumentation

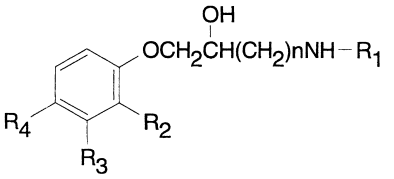
The chromatographic system consisted of a Binary LC pump 250 (Perkin-Elmer, Norwalk, CT, USA), an AS-3000 autosampler (Spectra-Physics Analytical, San Jose, CA, USA) and an LC detector Chrompack UV–Vis (Chrompack, The Netherlands). The Hypercarb column (100×4.6 mm, 5 μm), consisting of porous graphitized carbon (PGC) support was purchased from Hypersil (UK). The temperature of the column and solvent reservoir was maintained by a waterbath (Grant 6; Cambridge, UK). The mobile phase flow-rate was kept constant at 2.0 ml min^{-1}. The analyte solutions, injected twice, and the mobile phases were all freshly prepared. The solutes were detected at 272 nm unless otherwise stated. The injection volume was 20 μl and the sample concentration was around 0.1 m*M* for all the solutes.

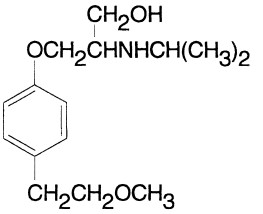
2.2. Chemicals

Methanol (of LiChrosolv quality) and sodium hydroxide were obtained from Merck (Darmstadt, Germany). All solutes, structures in Fig. 1, were synthesized at AstraZeneca R&D (Mölndal, Sweden). The two chiral counter ions, i.e., *N*-benzyloxycarbonyl-L-aspartyl–L-proline (*Z*-L-Asp–L-Pro) and *N*-benzyloxycarbonyl-L-aspartyl–D-proline (*Z*-L-Asp–D-Pro), structures in Fig. 1, were prepared at the Department of Medicinal Chemistry at AstraZeneca R&D.

2.3. Synthesis of *N*-benzyloxycarbonyl-L-aspartyl–L-proline and *N*-benzyloxycarbonyl-L-aspartyl–D-proline

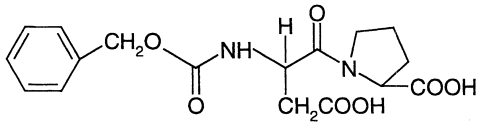
Z-L-Asp–L-Pro was prepared by dissolving (*N*-benzyloxycarbonyl-O_4-*tert.*-butyl)-L-aspartyl-L-proline (12 mmol) in 50 ml of trifluoroacetic acid and 50 ml of methylene chloride. The mixture was allowed to stand at room temperature for 4 h. It was evaporated at reduced pressure and the residue was freeze–dried from water–acetonitrile several times to get rid of traces of trifluoroacetic acid. Proton





Solute 12

Solute. No	N	R_1	R_2	R_3	R_4	k_1	α
1	1	$CH(CH_3)_2$	H	H	H	5.81	1.33
2	1	$CH(CH_3)_2$	$CH_2CH_2OCH_3$	H	H	16.6	1.24
3	1	$CH(CH_3)_2$	H	$CH_2CH_2OCH_3$	H	12.6	1.34
4	1	$CH(CH_3)_2$	H	H	$CH_2CH_2OCH_3$	14.5	1.36
5	2	$CH(CH_3)_2$	H	H	$CH_2CH_2OCH_3$	16.4	1.0
6	3	$CH(CH_3)_2$	H	H	$CH_2CH_2OCH_3$	26.8	1.0
7	1	$CH_2CH_2CH_3$	H	H	$CH_2CH_2OCH_3$	20.1	1.41
8	1	$C(CH_3)_3$	H	H	$CH_2CH_2OCH_3$	10.6	1.51
9	1	$CH(CH_3)_2$	H	H	OCH_3	17.4	1.36
10	1	$CH(CH_3)_2$	H	H	CH_2CONH_2	9.68	1.30
11	1	$CH(CH_3)_2$	CH_2CHCH_2	H	H	15.9	1.32
12						11.6	1.0



Z-asparatyl-proline

Fig. 1. Solute structures and effect enantioselective retention.

nuclear magnetic resonance (NMR) (C^2HCl_3) δ 8.4 (broad), 7.3 (5 H, m), 6.0 (1 H, d), 5.1 (2 H, dd), 5.0 (1 H, m), 4.5 (1 H, m), 3.9 (2 H, m), 3.0 (1 H, m), 2.8 (1 H, m), 2.4–1.9 (4 H, m).

(*N*-Benzyloxycarbonyl-O_4-*tert.*-butyl)-L-aspartyl-L-proline was prepared from *N*-benzyloxycarbonyl-L-aspartic acid 5-*tert.*-butyl-1-hydroxysuccinimide diester (12 mmol), L-proline (46 mmol), and sodium hydrogencarbonate (46 mmol) in 125 ml of tetrahydrofuran and 125 ml of water. The mixture was stirred vigorously overnight. Tetrahydrofuran was evaporated at reduced pressure and the aqueous phase was washed with methylene chloride. The aqueous phase was subsequently acidified to pH 2 with hydrochloric acid and extracted three times with ethyl acetate. The combined organic phase was washed with water, dried over anhydrous sodium sulfate and evaporated at reduced pressure. The

obtained product could be used without further purification.

Proton NMR (C^2HCl_3) δ 9.5 (1 H, broad), 7.3 (5 H, m), 6.0 (1 H, d), 5.1 (2 H, dd), 4.9 (1 H, m), 4.5 (1 H, m), 3.8 (2 H, m), 2.7 (1 H, m), 2.6 (1 H, m), 2.2–1.8 (4 H, m), 1.4 (9 H, s).

Z-L-Asp–D-Pro was prepared from 3.6 mmol of (*N*-benzyloxycarbonyl-O_4-*tert*.-butyl)-L-aspartyl-D-proline in the same way as *Z*-L-Asp–L-Pro. Proton NMR (MeO^2H) δ 7.9 (broad), 7.4 (5 H, m), 5.1 (2 H, s) 4.9 (1 H, m), 4.4 (1 H, m), 3.7–3.6 (2 H, m), 2.9–2.7 (2 H, m), 2.3–1.8 (4 H, m).

(*N*-Benzyloxycarbonyl-O_4-*tert*.-butyl)-L-aspartyl-D-proline was prepared in the same way as (*N*-benzyloxycarbonyl-O_4-*tert*.-butyl)-L-aspartyl-D-proline from *N*-benzyloxycarbonyl-L-aspartic acid 5-*tert*.-butyl-1-hydroxysuccinimide diester and D-Pro. Proton NMR (C^2HCl_3) δ 7.4 (5 H, m), 5.7 (1 H, d), 5.1 (2 H, dd), 4.9 (1 H, m), 4.5 (1 H, m), 3.9–3.7 (2 H, m), 2.8 (1 H, m), 2.6 (1 H, m), 2.4–2.0 (4 H, m), 1.4 (9 H, s).

2.4. Liquid chromatography methods

The retention factor, k was defined as $k=t_R/t_0-1$ where t_0 was the transport time from injection to the detector cell by a non retained component. t_0 was calculated from the first disturbance of the baseline obtained after injection (0.35 min). The separation factor, α, was calculated by the k for the later eluting enantiomer over k for the faster eluting enantiomer.

When using *Z*-L-Asp–L-Pro or *Z*-D-Asp–L-Pro an equilibration procedure of about 30 column volumes of mobile phase passing through the column is required to give stable chromatographic systems. A procedure that we used to restore old columns is to wash with 250 column volumes of 20 m*M* sodium hydroxide in methanol at 40°C.

3. Results and discussion

3.1. Effect on enantioselectivity by charge of the anionic chiral counter ion

The effect of charge of the chiral counter ion was studied using a mobile phase with a 5 m*M* concentration of *Z*-L-Asp–L-Pro, Fig. 2. Addition of different amounts of sodium hydroxide to the respective mobile phase was used to control the charge of the chiral counter ion. By the addition of 3 m*M* sodium hydroxide to the mobile phase the enantiomer of the tested solute, metoprolol, was retained, retention factor of about 5. However, by using the mono-anionic form of the CMPA no enantioselectivity was observed. By adding 6.2 m*M* sodium hydroxide the CMPA is partly present in its di-anionic form. The retention factor then increased in a selective way for the two enantiomers of metoprolol, resulting in an enantiomeric separation, Fig. 2. The (*S*)-form of metoprolol elutes before the (*R*)-form. A further increase of the CMPA concentration, up to 9 m*M*, resulted in higher retention and selectivity factors. A mobile phase with a ratio, between sodium hydroxide and CMPA concentrations, of 3 was also tested. The retention of the enantiomers of metoprolol then decreased and enantioselectivity was lost, Fig. 2. A reason for this effect is that the solute enantiomers are retained in their uncharged form and the ability to form diatereoisomeric complexes with the chiral counter ion is lost. A ratio between sodium hydroxide and the CMPA over 2 gives an apparent pH that is basic resulting in uncharged solutes. The results showed that to obtain enantioselectivity a di-anionic CMPA and a positively charged solute are required.

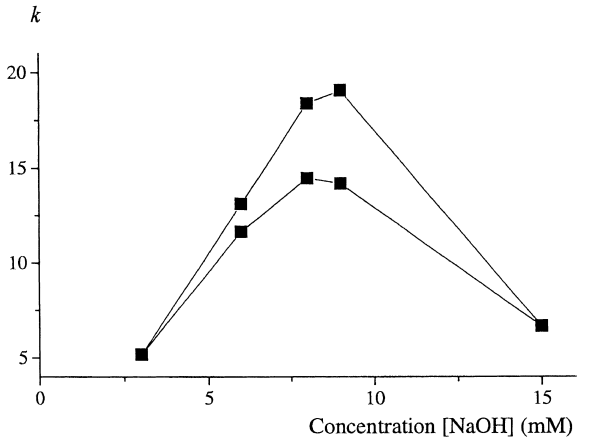


Fig. 2. Effect of charge of the anionic chiral counter ion on enantioselectivity. Solid phase: Hypercarb (5 μm, 100×4.6 mm). Mobile phase: 5 m*M* *Z*-L-Asp–L-Pro and *X* m*M* NaOH in methanol. Column temperature: 30°C. Flow-rate: 2.0 ml min^{-1}. Solute: (*R*,*S*)-metoprolol.

The obtained enantioselectivity might be caused by differences in adsorption of the diastereoisomeric complexes but also by the fact that a chiral pseudo-stationary phase is formed. By using *Z*-L-Asp–L-Pro instead of *Z*-Gly–L-Pro about five times more of the chiral counter ion is adsorbed, about 7 mg. This indicates that the mechanism giving enantioselectivity might be the formation of a chiral pseudo-stationary phase when using *Z*-L-Asp–L-Pro as CMPA. Due to the adsorption of *Z*-L-Asp–L-Pro a system peak is created when injecting solutions that deviate from the mobile phase composition. As the solutes were dissolved in pure methanol a negative system peak arising from the chiral counter ion was detected around 2–3 min at a flow-rate of 2 ml min^{-1}. The solutes were to a higher extent adsorbed onto the PGC surface and no interference with the system peak was observed.

The diastereoisomer, *Z*-L-Asp–D-Pro was also tested as CMPA. The enantiomers of metoprolol, atenolol and alprenolol were all separated with the same retention order as above using 5 m*M* of CMPA and 9 m*M* of sodium hydroxide in methanol as the mobile phase. However, the obtained selectivity factors were lower, less than 1.1. Contrary to these results, a previous study showed that *Z*-L-Glu–L-Pro but not its diastereoisomer *Z*-L-Glu–D-Pro could be used to separate enantiomers of amines [18]. The differences in enantioselectivity using the two diastereoisomers might be caused by differences in complex-formation constants in the mobile phase and also by differences in adsorption constants of the formed diastereoisomeric complexes to the non-chiral PGC surface.

3.2. Effect of chiral counter ion concentration on enantioselectivity

The effect of CMPA concentration on the retention factors was studied. The ratio between CMPA and sodium hydroxide concentration was kept constant at 1.6. This means that 60% of total CMPA concentration is present in its di-anionic form and the rest is present as the mono-anionic form. The total CMPA concentration was increased from 2.5 to 16 m*M*, Fig. 3. The retention as well as the separation factors decreased slightly as the total CMPA concentration increased. This indicates that the di- and mono-anionic form compete, probably as an ion-pair with the sodium ion, with the diastereoisomeric complexes for the limited amount of adsorption sites. However, broad and asymmetrical solute peaks were noticed at lower total CMPA concentrations. An increase in CMPA concentration favors peak performance and therefore also the resolution between the two enantiomers of metoprolol.

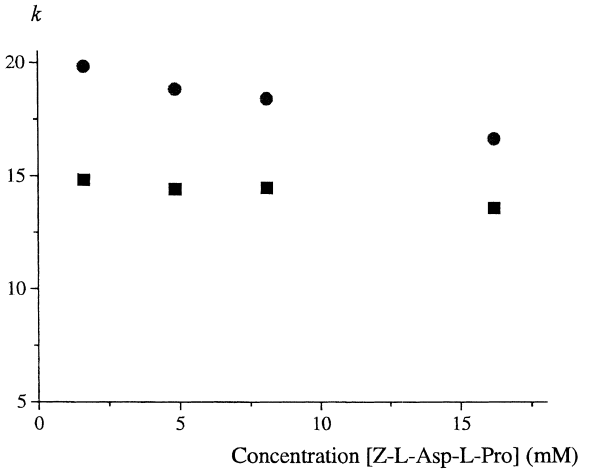


Fig. 3. Influence of counter ion concentration on the enantioselective retention. Solid phase: Hypercarb (5 μm, 100×4.6 mm). Mobile phase: *X* m*M* *Z*-L-Asp–L-Pro and 1.6 times *X* m*M* NaOH in methanol. Flow-rate: 2.0 ml min^{-1}. Solute: (*R*,*S*)-metoprolol.

3.3. Solute structure and enantioselectivity

The influence of solute structure on enantioselectivity using Hypercarb as solid phase is shown in Fig. 1. A mobile phase of 5 m*M* *Z*-L-Asp–L-Pro and 9 m*M* sodium hydroxide in methanol was used to enantioseparate the tested racemic amino alcohols. Nine out of 12 studied amino alcohols were baseline resolved, among them atenolol and metoprolol, for chromatogram see Fig. 4. The enantioselectivities were only slightly affected by type substituent attached at different positions in the aromatic ring, solute Nos. 1–4, 10 and 11. However, the retention factors varied and was lowest for the solute lacking substituent in the aromatic ring, solute No. 1. The distance between the chiral center and the amine function has a dramatic effect on enantioselectivity, solute Nos. 4–6. Enantioseparation was only obtained for the solute with one methylene group,

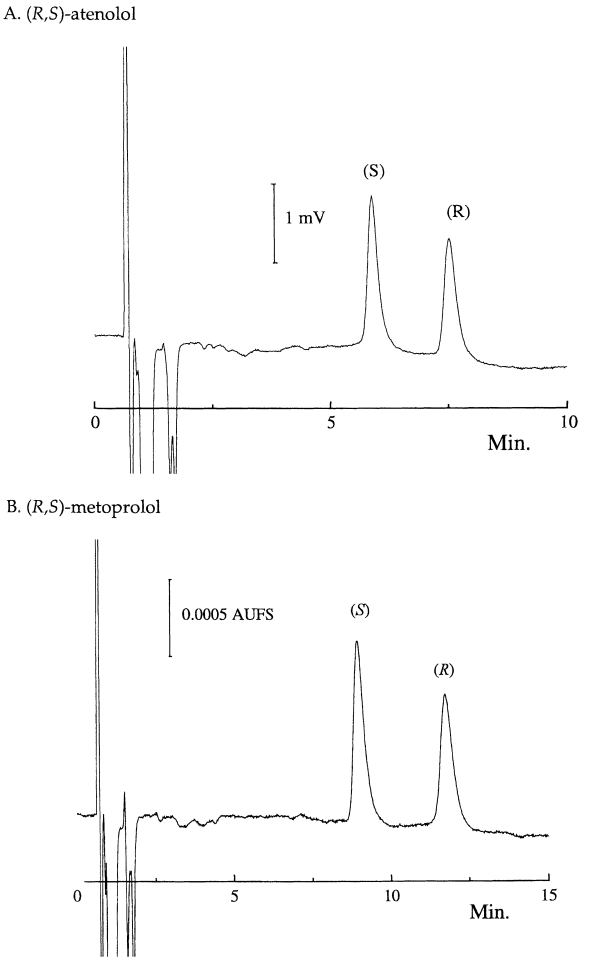


Fig. 4. Enantioseparation of atenolol and metoprolol. Solid phase: Hypercarb (5 μm, 100×4.6 mm). Mobile phase: 5.0 m*M* *Z*-L-Asp–L-Pro and 9.0 m*M* NaOH in methanol. Flow-rate: 2.0 ml min^{-1}. Column temperature: 40°C.

solute No. 4. Retention for these three solutes increases with increased distance and therefore with increased hydrophobicity. Retention also increases with decreasing bulkiness of the alkyl group attached to the nitrogen atom, solutes 4, 7 and 8. However, the enantioselectivity increased in the order isopropyl<*n*-propyl<*tert*.-butyl. This finding was in agreement with the results obtained using *Z*-L-glutamyl–L-proline as chiral mobile phase additive [18]. The chromatographic results obtained in this study showed that *Z*-L-Asp–L-Pro gave higher enantioselectivities for amino alcohols than the modified dipeptides *Z*-L-Glu–L-Pro and *Z*-Gly–L-Pro [18]. The reason for this is that the diastereoisomeric complexes formed between the enantiomeric solutes and the chiral counter ion discriminate to different extents. Future experiments using, e.g., NMR spectroscopy and molecule modeling could give deeper knowledge about ion-paring constants as well as information about the appearance of the formed diastereoisomeric complexes. By using an optimized chromatographic system all four stereoisomers of an analogue to metoprolol could be separated, Fig. 5.

3.4. Effect of column temperature on stereoselective retention

The influence of column temperature on the retention factors on Hypercarb was studied using a mobile phase of 5 m*M* *Z*-L-Asp–L-Pro and 9 m*M* sodium hydroxide in methanol. The results are presented as Van 't Hoff plots in Fig. 6. The common effects, i.e., reduced retention and also decreased enantioselectivity were observed at higher temperature for the tested amino alcohol, (*R*,*S*)-atenolol. The decrease in retention was less pronounced than for a similar system using *Z*-L-Glu–L-Pro as the chiral di-anionic counter ion on Hypercarb [18]. However,

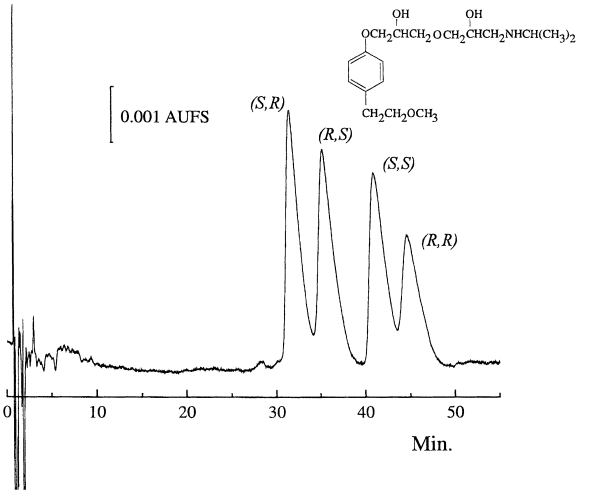


Fig. 5. Simultaneous separation of the enantiomers and the diastereoisomers of an amino alcohol. Solid phase: Hypercarb (5 μm, 100×4.6 mm). Mobile phase: 5.0 m*M* *Z*-L-Asp–L-Pro and 9.0 m*M* NaOH in methanol. Flow-rate: 2.0 ml min^{-1}. Column temperature: 40°C.

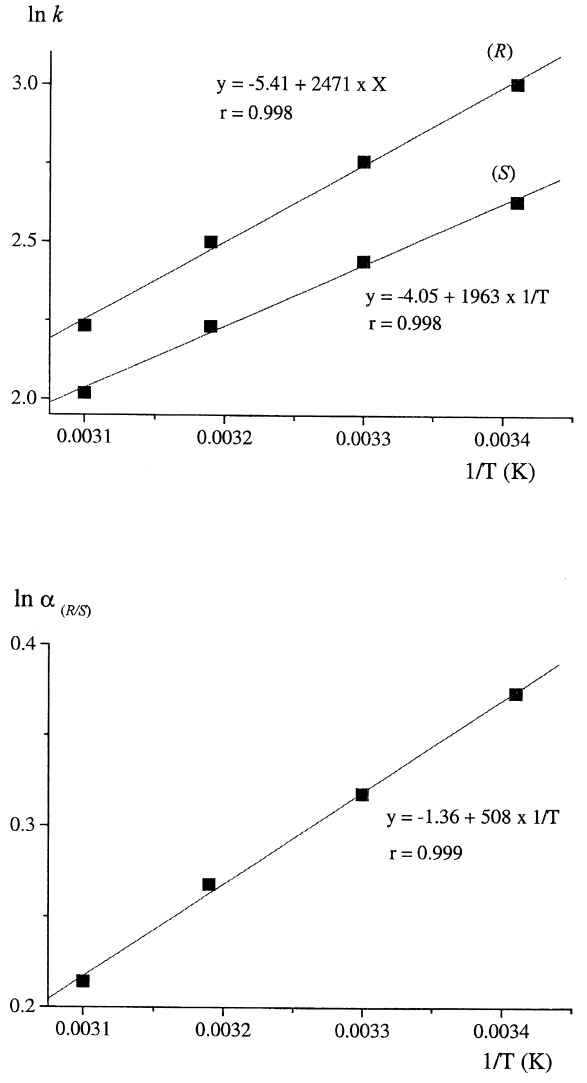


Fig. 6. Influence of column temperature on enantioselective retention. Solid phase: Hypercarb (5 μm, 100×4.6 mm). Mobile phase: 5.0 m*M* *Z*-L-Asp–L-Pro and 9.0 m*M* NaOH in methanol. Flow-rate: 2.0 ml min^{-1}. Solute: (*R*,*S*)-atenolol.

the decrease in retention was steeper than for a similar chromatographic system where a mono-anionic chiral counter ion was used [18]. The effect of column temperature on enantioselectivity was more pronounced for *Z*-L-Asp–L-Pro in comparison with the two chiral counter ions tested in a previous presentation [18].

4. Conclusion

A di-anionic chiral counter ion, *Z*-L-Asp–L-Pro was used to baseline resolve enantiomers of several amino alcohols. The study showed the necessity to titrate the counter ion with sodium hydroxide to its di-anionic form in order to optimize the resolution. Column temperature was shown to be a powerful tool to reduce sample analysis time without jeopardizing baseline resolution.

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