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Comparative study of coated and immobilized polysaccharide-based chiral stationary phases and their applicability in the resolution of enantiomers☆

L. Thunberg, J. Hashemi, S. Andersson*

Astra Zeneca R&D Mölndal, Medicinal Chemistry, SE-431 83 Mölndal, Sweden

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ABSTRACT

The enantioselective and chromatographic properties of Chiralpak AD and Chiralpak IA as well as those of Chiralcel OD and Chiralpak IB have been evaluated using a set of 48 compounds that differ in their physical and chemical properties. The impact of the different immobilisation methodologies of the chiral polysaccharide, i.e., coated or immobilized on retention and enantioselectivity was studied. The study on immobilized chiral stationary phases (CSPs) was expanded to also include mobile phases containing mixtures of alkanes and more non-conventional solvents such as ethyl acetate, ethers, acetone and dichloromethane. In this paper we report some of the general trends observed for the 48 racemic compounds with respect to retention, α and R_s. Further, the impact of the immobilisation methodology and the choice of the mobile phase on the elution order of the enantiomers is also discussed.

1. Introduction

Studies of the differences in the pharmacological activities of stereoisomers of a chiral drug are necessary in the drug development process and also required by the regulatory bodies. Enantiomers can differ in their potency and selectivity, and they might show different pharmacokinetics. Sometimes toxity can be linked to one of the enantiomers [1–3]. Thus, the biological activity of the individual enantiomers needs to be established before choosing a candidate drug for further development. To this end, both enantiomers need to be isolated in sufficient quantity and purity.

During the past decade, liquid chromatography on chiral stationary phases (CSPs) has advanced considerably and become one of the most powerful methodologies to obtain pure enantiomers [4–8]. Development of more efficient CSPs and technology has been crucial for this progress. The CSPs most commonly used are based on silica coated with chiral polysaccharide derivatives. These CSPs are able to resolve a large variety of structurally different compounds and generally depict high loading capacities making them suitable for application in preparative scale resolution of enantiomers [9–12]. Since the polysaccharides are coated on silica, these CSPs can only be used with a limited range of solvents as mobile phases, generally alcohols, acetonitrile, alkanes and mixtures there of. Other solvents, like ethers, esters, ketones and chlorinated alkanes, dissolve the polysaccharides and wash the selector off the silica. In order to overcome this limited chemical stability, immobilisation of the polysaccharide onto silica has given CSPs allowing the use of a much broader range of solvents [13–22]. The freedom of choice of solvents can lead to different enantioselectivity and elution order of the individual isomers. Also, the expanded solvent range increases the ability to balance solubility of the compound, retention time and the resolution and thus optimisation of productivity, a key measure especially when large amounts of enantiomers need to be resolved.

In this work the chromatographic properties of two pairs of coated and immobilized phases have been compared. Chiralcel OD-H and Chiralpak IB contain the same chiral selector, 3,5-dimethyl carbamoyl derivative of cellulose, however, the former contains the coated derivatized polysaccharide while in the later the polymer is immobilized on the silica matrix. Similarly, Chiralpak AD-H and Chiralpak IA contain the 3,5-dimethyl carbamoyl derivative of amylose as the chiral selector but differ in the immobilisation methodology. The influence of the immobilisation methodology of the chiral selector on retention and enantioselectivity of 48 different racemic compounds was evaluated using alcohols, acetonitrile and mixtures of alkanes and alcohols as mobile phases. The study on immobilized CSPs was expanded to also include mobile phases containing mixtures of alkanes and more non-conventional solvents

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* Corresponding author. Tel.: +46 31 776 16 25; fax: +46 31 776 38 68.
E-mail address: Shalini.Andersson@astrazeneca.com (S. Andersson).

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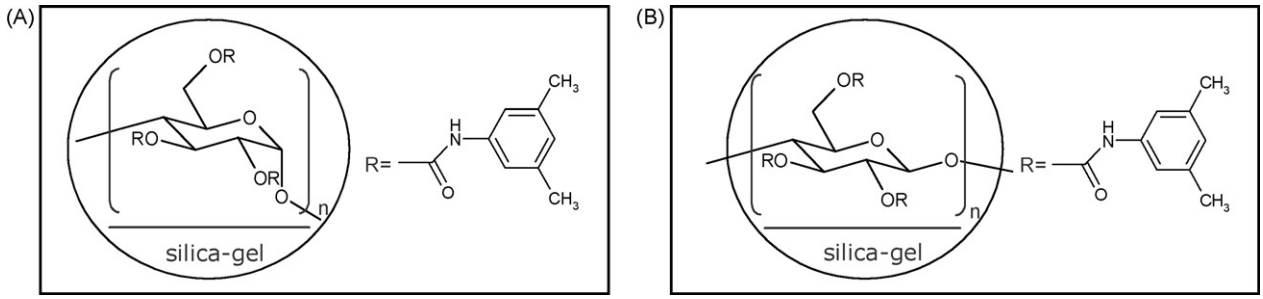

Fig. 1. The chiral selectors based on tris(3,5-dimethylphenylcarbamate) of (A) amylose and (B) cellulouse.

such as, ethyl acetate, tetrahydrofurane (THF), acetone, methyl *tert*-butyl ether (MTBE) and dichloromethane (DCM).

2. Experimental

2.1. Instruments

Analytical chromatography was performed on a system with a Waters 2695 Separations Module and a Waters 996 Photodiode Array Detector (Waters, Milford, MA, USA). The system was also equipped with an Advanced Laser Polarimeter (PDR-Chiral Inc., Lake Park, FL, USA) and gave the sign of the optical rotation, measured at 670 nm. For chromatographic data collection Empower Pro software build 1154 (Waters, Milford, MA, USA) was used.

2.2. Chemicals

The solvents used for chromatography were of HPLC grade and were purchased from Sigma–Aldrich (Seelze, Germany). The ethanol was purchased from Kemetyl (Haninge, Sweden). Formic acid (p.a. grade) was obtained from Riedel-de Haën (Seelze, Germany) and triethylamine (p.a. grade) was from Fluka (Buchs, Switzerland). Most of the analytes were synthesised at AstraZeneca R&D (Mölndal, Sweden). Quinidine, 3,4-dihydroxymandelic acid, ethyl mandelate, 2-phenylpropionic acid and 4′,5,7-trihydroxyflavanone were purchased from Sigma–Aldrich (Seelze, Germany). Mandelic acid and methyl phenyl sulfoxide were obtained from Fluka (Buchs, Switzerland) and Fmoc-Ala-OH, Fmoc-Phe-OH and Z-Ala-OH were obtained from Bachem (Bubendorf, Switzerland).

2.3. Chromatography

The columns Chiralpak AD-H, Chiralpak IA, Chiralcel OD-H and Chiralpak IB were obtained from Chiral Technologies Europe (Illkirch Cedex, France). The CSPs Chiralpak AD-H and Chiralcel OD-H are coated on silica and are based on tris (3,5-dimethylphenylcarbamate) of amylose and cellulose, Fig. 1. Chiralpak IA and Chiralpak IB contain the corresponding chiral selectors as in Chiralpak AD-H and Chiralcel OD-H, respectively, but the polysaccharides are immobilized onto silica. The dimension of the columns was 250 mm × 4.6 mm i.d. and the particle size was 5 μm. Unless otherwise stated, the flow rate was 1 ml/min. Chromatography was carried out at room temperature and a 10 μl solution (1 mg/ml in 2-propanol) was injected. The mobile phases were based on solvents applicable on the coated and immobilized polysaccharide CSPs, respectively, Table 1. For in depth studies (Sections 3.2 and 3.3) the amount of the polar component in the mobile phase was chosen to give an approximate retention time of 10 min for the first eluted enantiomer. The sign of the optical rotation of the resolved enantiomers was measured online. When necessary, the elution order was confirmed by spiking with one of the pure enantiomers.

The chemical and mechanical stability of the columns was followed by chromatography of *trans*-stilbene oxide (TSO) during period of 140 days. Differences in retention, selectivity and resolution were determined at a 20-day interval by injecting 10 μl of a 1 mg/ml solution of TSO. After an initial loss in resolution on the Chiralcel OD-H column, all three parameters were found to be relatively constant during the 140-day period. Similar degradation was also found on the Chiralpak AD-H column, but first after 100 days. The columns with immobilized CSPs showed only small variations in the stability through out the 140-day period.

2.4. Analyte selection and analytical chromatographic strategy

Out of about 200 racemic pharmaceutical compounds available, 48 were selected to represent a large variety of different physical and chemical properties. PCA plots calculated with the software SIMCA-P+ (Umetrics, Umeå, Sweden) wherein 40 different physical and chemical properties were tabulated were utilized for the selection of the analytes. The *x*-axis summarises differences in size, i.e., the molecular weight and volume of the analytes chosen while the *y*-axis shows the difference in polarity mainly based on hydrogen bonding ability and polar surface area. The 48 compounds were classified as being acidic (18), basic (15) and neutral (15), Fig. 2. Evaluation of the retentive and enantioselective properties of the four CSPs was initially performed with three different mobile phases, 20% 2-propanol in heptane, 15% ethanol in heptane and 100% ethanol.

The results were exported into the software Spotfire DecisionSite 8.1.1. (Spotfire AB, Gothenburg, Sweden) in order to visualize differences in retention factor, selectivity factor, resolution and number of theoretical plates. Of the compounds giving most interesting results, regarding both retention and selectivity, 10 were selected (three acidic, three basic and four neutral compounds), Fig. 3. These compounds were screened on all the four CSPs with a variety of solvents as mobile phases. The analytes were initially eluted by a gradient whereby the most polar solvent was increased linearly from 5% to 95% during a 60 min run. The results obtained

Table 1
Examples of solvents applicable as mobile phases on the CSPs with coated and immobilized derivatized polysaccharides, respectively

Coated immobilized	Immobilized	
Polar solvents applicable		
Methanol	Ethanol	Dioxane
Ethanol	Ethanol	Ethyl acetate
Propanol (1-, 2-)	Propanol (1-, 2-)	Acetone
Acetonitrile	Acetonitrile	CH_2Cl_2
	THF	$CHCl_3$
	MTBE	

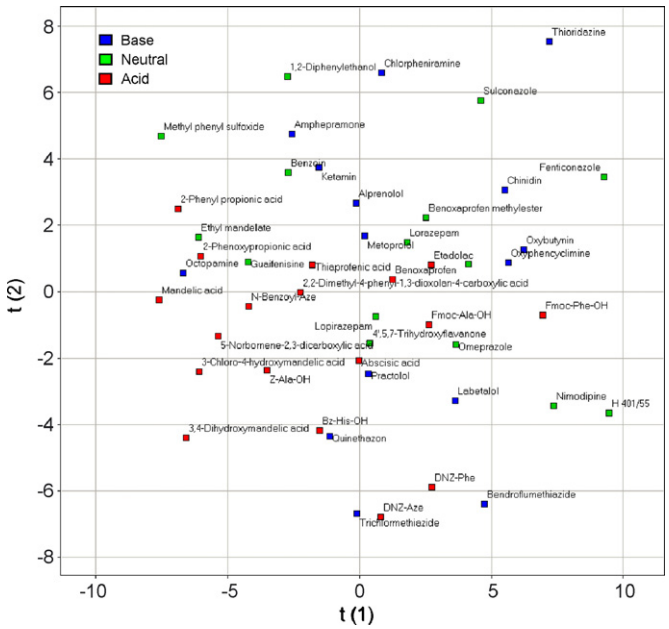

Fig. 2. The 48 compounds selected in the principal component analysis of 200 compounds. Increasing size (molecular weight and volume) from left to right on $t(1)$-axis and decreasing polarity (H-bond donor/acceptor and polar surface area) upwards on $t(2)$-axis.

from the gradient elutions were then transferred to isocratic runs with the aim to obtain similar retention times of the first eluted enantiomer for all compounds studied. In all cases, the retention time was optimised to between 8.5 and 11.5 min thus allowing the enantiomers to have comparable and ample time to interact with the chiral selector.

3. Results and discussion

3.1. Screening of 48 compounds on four different CSPs

The 48 racemic compounds selected were screened for enantioselectivity on the four CSPs with three general mobile phases,

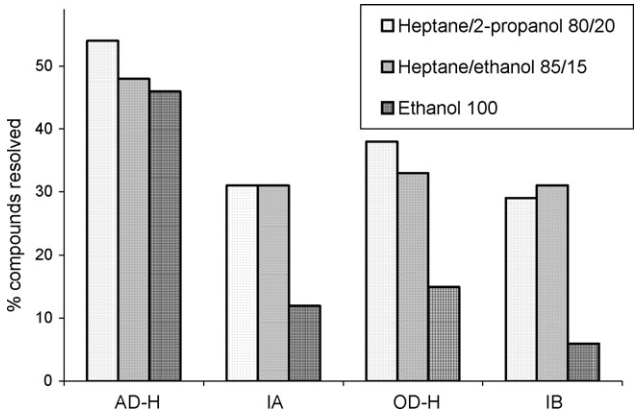

Fig. 4. The percentage compounds separated on the columns with three different mobile phases.

20% 2-propanol in heptane, 15% ethanol in heptane and 100% ethanol. Under these conditions, Chiralpak AD-H separated ca 50% of all the compounds studied ($\alpha > 1.0$), Fig. 4. Chiralpak IA, based on the same chiral selector albeit immobilized to the silica surface, separated only about 30% of the compounds with mobile phases based on mixtures of heptane and alcohols. Similar results were obtained with Chiralcel OD-H and Chiralpak IB, which contain the same chiral selector that is coated or immobilized on silica, respectively. The most significant difference was observed using pure ethanol as mobile phase. For all CSPs except Chiralpak AD-H, drastic loss in enantioselectivity was observed, indicating that hydrogen bonding plays an important role in the chiral recognition of the individual isomers.

The retention of the first eluted enantiomer and selectivity factors obtained for the 48 compounds on Chiralpak AD-H and Chiralpak IA when using 20% 2-propanol in heptane as mobile phase are shown in Fig. 5. Under these conditions most compounds were retained more strongly on the immobilized phase than on the coated; especially in the case of the acidic compounds such as the FMOC-derivatives of amino acids. However, most compounds were generally resolved with higher selectivity on Chiralpak AD-H than on Chiralpak IA. This suggests that there is a greater degree

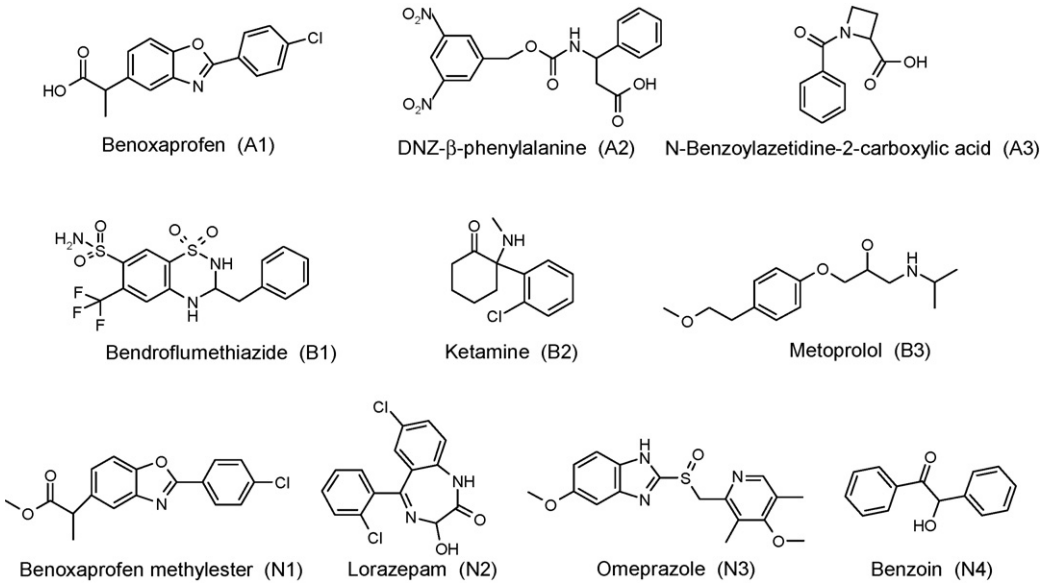

Fig. 3. The racemic compounds selected for analysis.

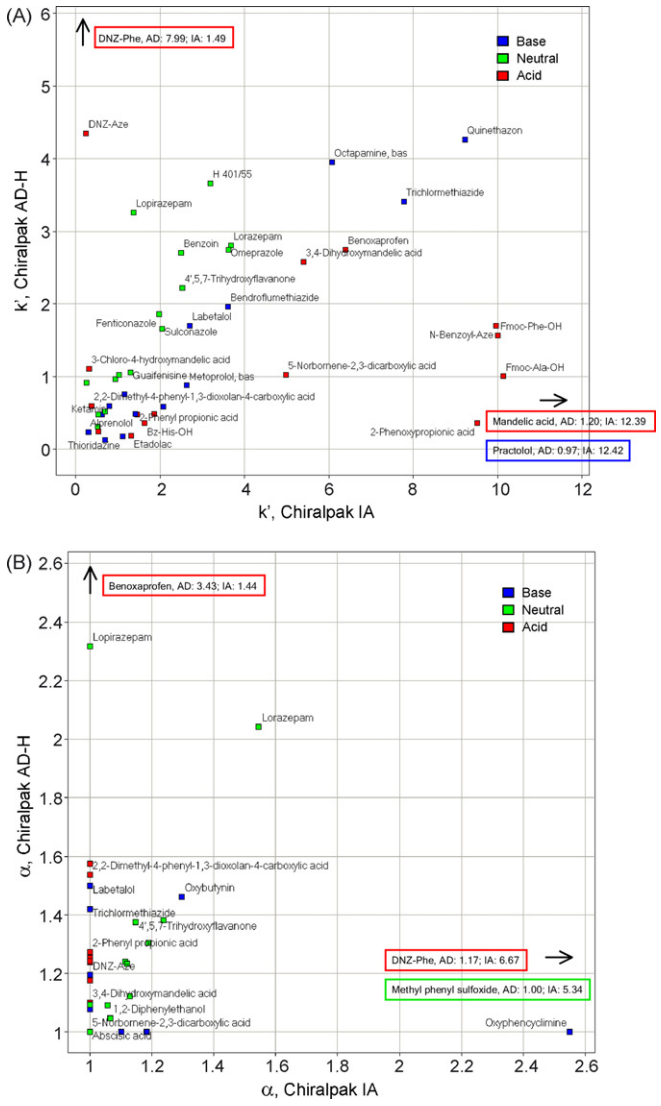

Fig. 5. Illustration of the differences in (A) retention factors (k'_1) and (B) selectivity factors on Chiralpak AD-H and Chiralpak IA with 20% 2-propanol in heptane as mobile phase.

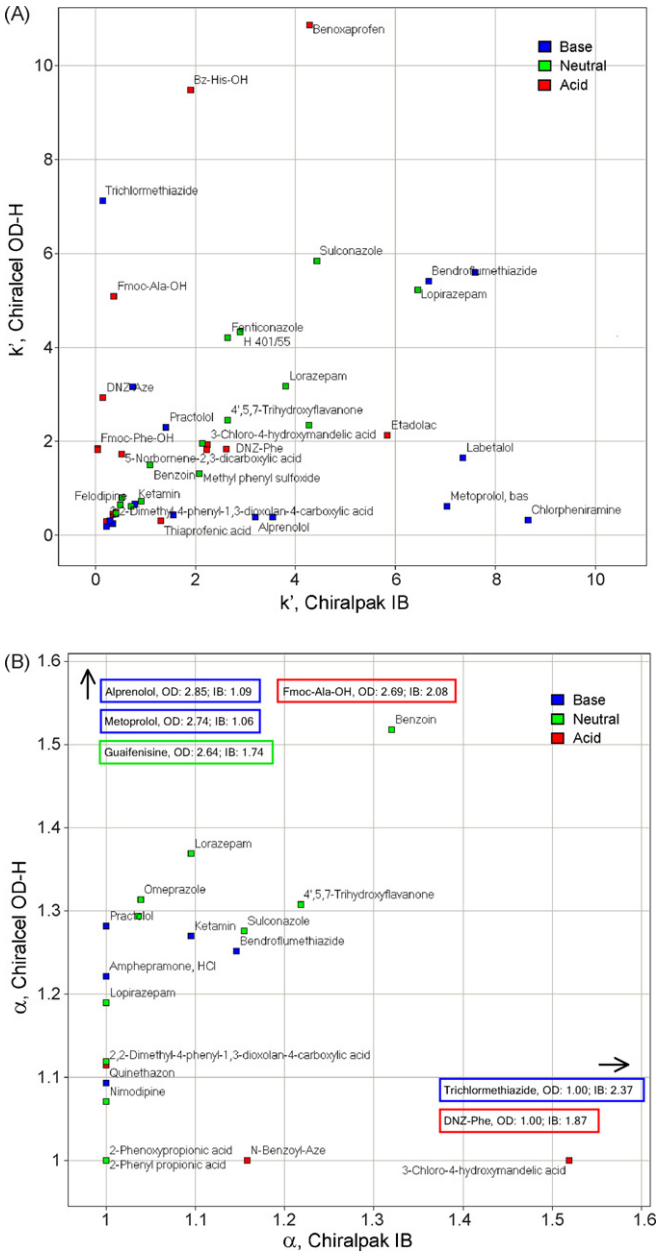

Fig. 6. Illustration of the differences in (A) retention factors and (k'_1) (B) selectivity factors on Chiralcel OD-H and Chiralpak IB with 20% 2-propanol in heptane as mobile phase.

of achiral interactions involved on the immobilized CSP leading to stronger retention of the enantiomers but lower enantioselectivity.

Similar studies on the Chiralcel OD-H and Chiralpak IB columns showed higher k'-values of the acidic compounds on the coated phase Chiralcel OD-H, while the basic and neutral compounds were more strongly retained on the immobilized phase Chiralpak IB, Fig. 6. In general, Chiralcel OD-H showed higher enantioselectivity than Chiralpak IB for most compounds.

3.2. Effects on retention and selectivity of 10 selected racemates using a broader range of mobile phases

To better understand the impact of the immobilisation methodology on the retentive and enantioselective properties of the studied CSPs, the chromatographic behavior of 10 of the 48 compounds was studied in more depth. The racemates, three acidic, three basic and four neutral shown in Fig. 3, were chosen mainly due to differences in chromatographic behavior as well as in the physico-chemical properties (see Fig. 2). An extended range of the mobile phases applicable on all CSPs, i.e., 20% 2-propanol in heptane, 15% ethanol in heptane, 100% ethanol, 100% methanol and 100% acetonitrile were used.

A similar trend as already observed when using 2-propanol in heptane as mobile phase was seen for the limited test set of 10 compounds, i.e., the retention factors were generally higher on Chiralpak IA than on Chiralpak AD-H, Figs. 5 and 7A. However, when using the other mobile phases most compounds were more strongly retained on the coated phase, Chiralpak AD-H; especially in the case of the neutral compounds. Also, the selectivity factors were generally higher on Chiralpak AD-H than on Chiralpak IA when using the mobile phases applicable on both phases, Figs. 5 and 7B. Somewhat different behavior was observed with pure methanol or acetonitrile as the mobile phase, whereby Chiralpak IA showed higher enantioselectivity than Chiralpak AD-H for some of the compounds. Compared to its methyl ester (N1), the free carboxylic acid

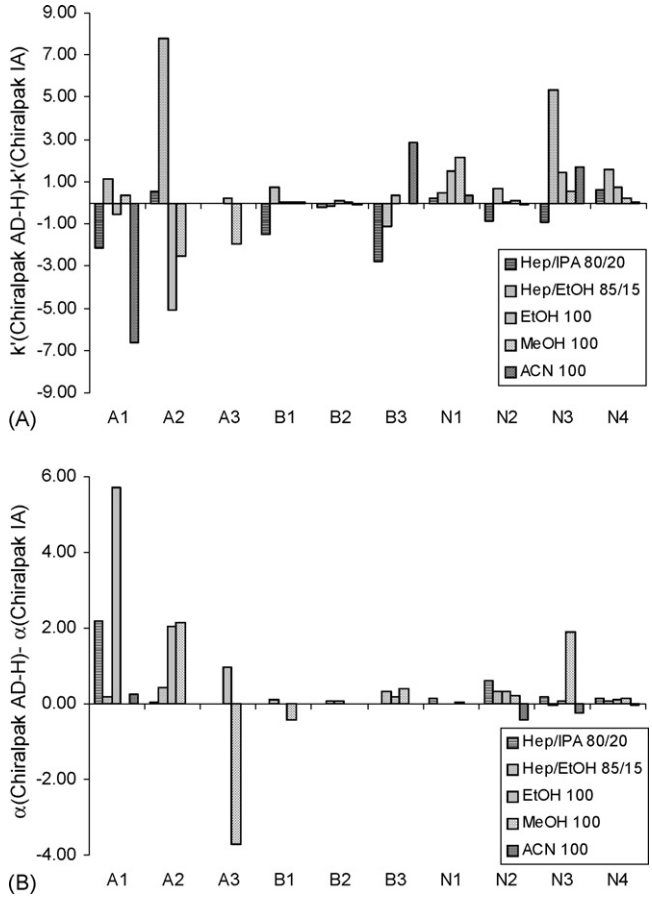

Fig. 7. The influence of the mobile phases applicable on all columns on (A) retention (k'_1) and (B) enantioselectivity on Chiralpak AD-H and Chiralpak IA. α(Chiralpak AD-H) − α(Chiralpak IA) = 0 indicates no separation on both CSPs.

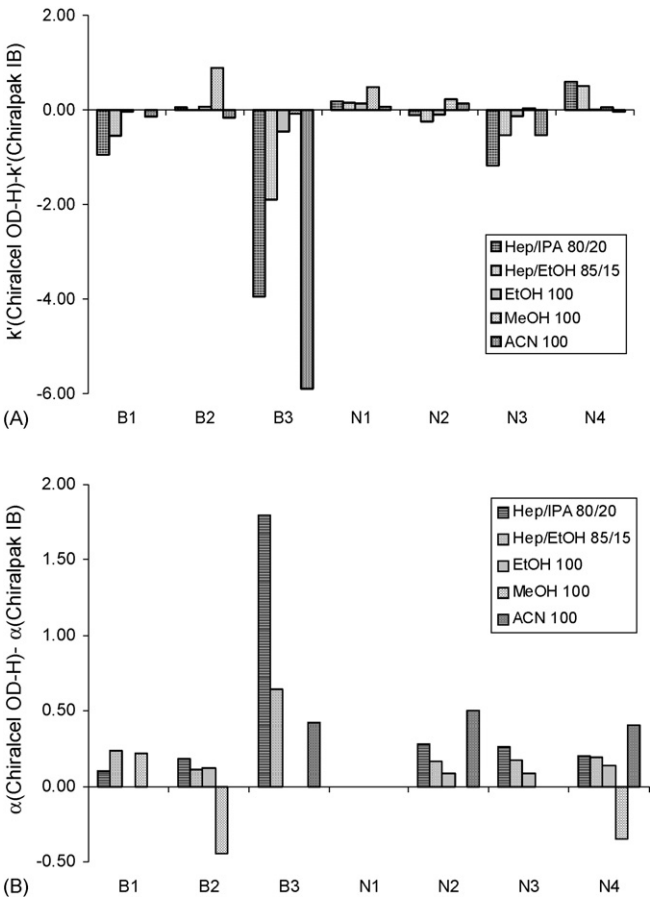

Fig. 8. The influence of the mobile phases applicable on all columns on (A) retention (k'_1) and (B) enantioselectivity on Chiralcel OD-H and Chiralpak IB. α(Chiralcel OD-H) − α(Chiralpak IB) = 0 indicates no separation on both CSPs.

benoxaprofen (A1) shows a drastic increase in retention on Chiralpak IA with the aprotic solvent acetonitrile but very little difference in the α-value. This observation is in line with what we have seen in the more general study with the 48 compounds (Section 3.1). No clear trend on retention was observed in polar organic mode. Retention of the analytes studied depended on both the structure and the type of solvent chosen, i.e., methanol, ethanol and acetonitrile (Fig. 7A).

With the exception of Metoprolol (B3), the retention factors were similar on Chiralcel OD-H and Chiralpak IB for all compounds studied, Figs. 6 and 8A. Due to exceptionally long retention times on both columns, the acidic compounds were excluded in Fig. 8. In general, the selectivity factors were higher on Chiralcel OD-H than on Chiralpak IB, Figs. 6 and 8B. The immobilized phase showed higher enantioselectivity for only two of the compounds, namely, Benzoin (N4) and Ketamine (B2) with methanol as the mobile phase. In contrast, the enantiomers of Metoprolol (B3) showed a remarkable separation on the coated CSP although the isomers were retained much more strongly on Chiralpak IB. For most compounds, mobile phases based on 2-propanol or ethanol in heptane were favorable to obtain resolution on the coated phases.

3.3. Impact of extended range of solvents on selectivity and resolution

The greatest advantage of immobilized CSPs is the unlimited range of organic solvent combinations that can be utilized for the chromatographic separation of racemic compounds. One is no longer restricted to mobile phases containing alcohols, acetonitrile and alkanes. The mobile phases can also be based on mixtures of alkane and one of the more non-conventional solvents such as ethyl acetate, THF, acetone, methyl *tert*-butyl ether or dichloromethane. One of the advantages of using these solvents as mobile phases, especially in preparative chromatography, is the possibility to attain much higher solubility of the racemate in the mobile phase, reducing the risk of injection effects.

Three of the ten racemates studied before were used as model compounds to try to further understand the impact of the immobilisation methodology on the interactions involved between the isomers and the chiral selectors. Omeprazole and Ketamine were investigated on Chiralpak AD-H and Chiralpak IA and DNZ-β-phenylalanine on Chiralcel OD-H and Chiralpak IB. Mobile phase mixtures of heptane/2-propanol and heptane/ethanol were used for chromatography on both the coated and immobilized CSPs. The range was then extended to mixtures of heptane and one of the polar component ethyl acetate, THF, acetone, MTBE or DCM for studies on Chiralpak IA and Chiralpak IB.

Omeprazole was well resolved on Chiralpak AD-H with both 2-propanol (R_S = 4.2) or ethanol (R_S = 6.5) as the polar component in the mobile phase, Fig. 9A. However, a fivefold difference in resolution is observed on Chiralpak IA when 2-propanol is replaced with ethanol in the mobile phase. Also, use of ethanol instead of 2-propanol in the mobile phase gave an opposite elution order with the (−)-(*S*)-Omeprazole eluting first. This is in agreement with the results obtained in our earlier studies [23]. Use of non-conventional organic solvents did not improve the resolution, although mobile

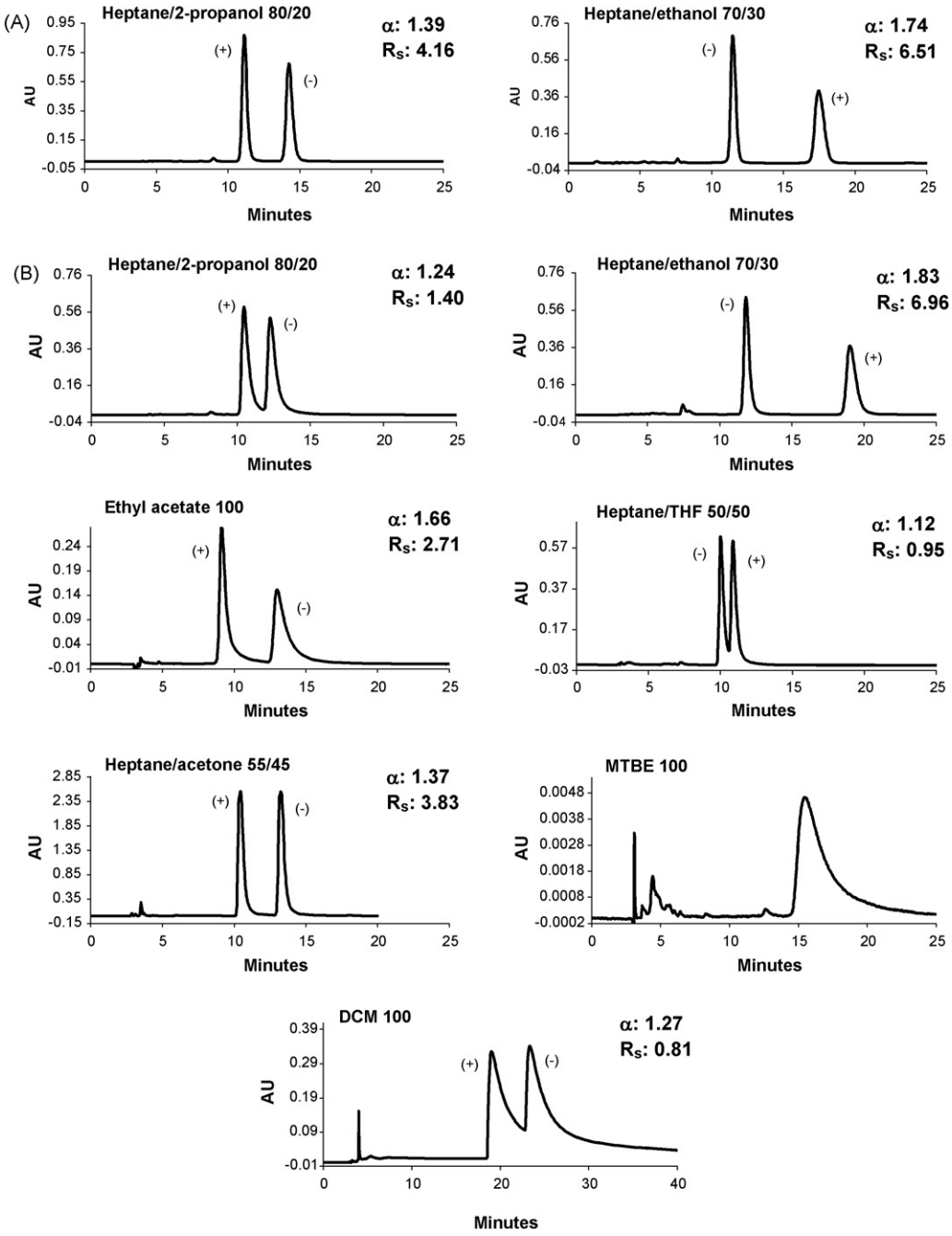

Fig. 9. Chromatograms of Omeprazole on (A) Chiralpak AD-H and (B) Chiralpak IA.

phases based on acetone (45% in heptane) and ethylacetate did give reasonable separation and peak shape of the enantiomers, Fig. 9B. Pronounced tailing of the peaks was observed with ethyl acetate, MTBE and DCM the mobile phase. Indeed, all selectivity is lost with MTBE as the mobile phase even though the racemate is well retained. Addition of ethanol (3%) in MTBE as the mobile phase showed very little effect on tailing and gave no separation of the enantiomers. However, increasing the proportion of ethanol to 5% in MTBE gave an $\alpha = 1.32$ and a resolution of 3.16 which is comparable to what was observed with acetone (45%) in heptane. Obviously, the use of protic solvents in the mobile phase help decrease the amount of achiral interactions between the Omeprazole enantiomers and the CSP thus enhancing enantioselectivity. Interestingly, THF as the mobile phase component gives the opposite elution compared to other aprotic solvents DCM, ethyl acetate and acetone.

The opposite was observed for the resolution of Ketamine enantiomers on Chiralpak AD-H and Chiralpak IA. Protic solvents such as 2-propanol or ethanol in heptane as mobile phases did not give baseline resolution of the Ketamine isomers, Fig. 10A and B. Use of aprotic solvents had a major impact on both enantioselectivity and resolution of the isomers of Ketamine and the highest resolution was obtained with 45% MTBE in heptane ($R_s = 6.7$). Thus, hydrogen bonding between the –NH group in Ketamin and the chiral selector is probably crucial for enantioselectivity. The elution order of the enantiomers is retained with all mobile phases studied except with 2-propanol as the polar component in the mobile phase whereby the (+)-enantiomer elutes first.

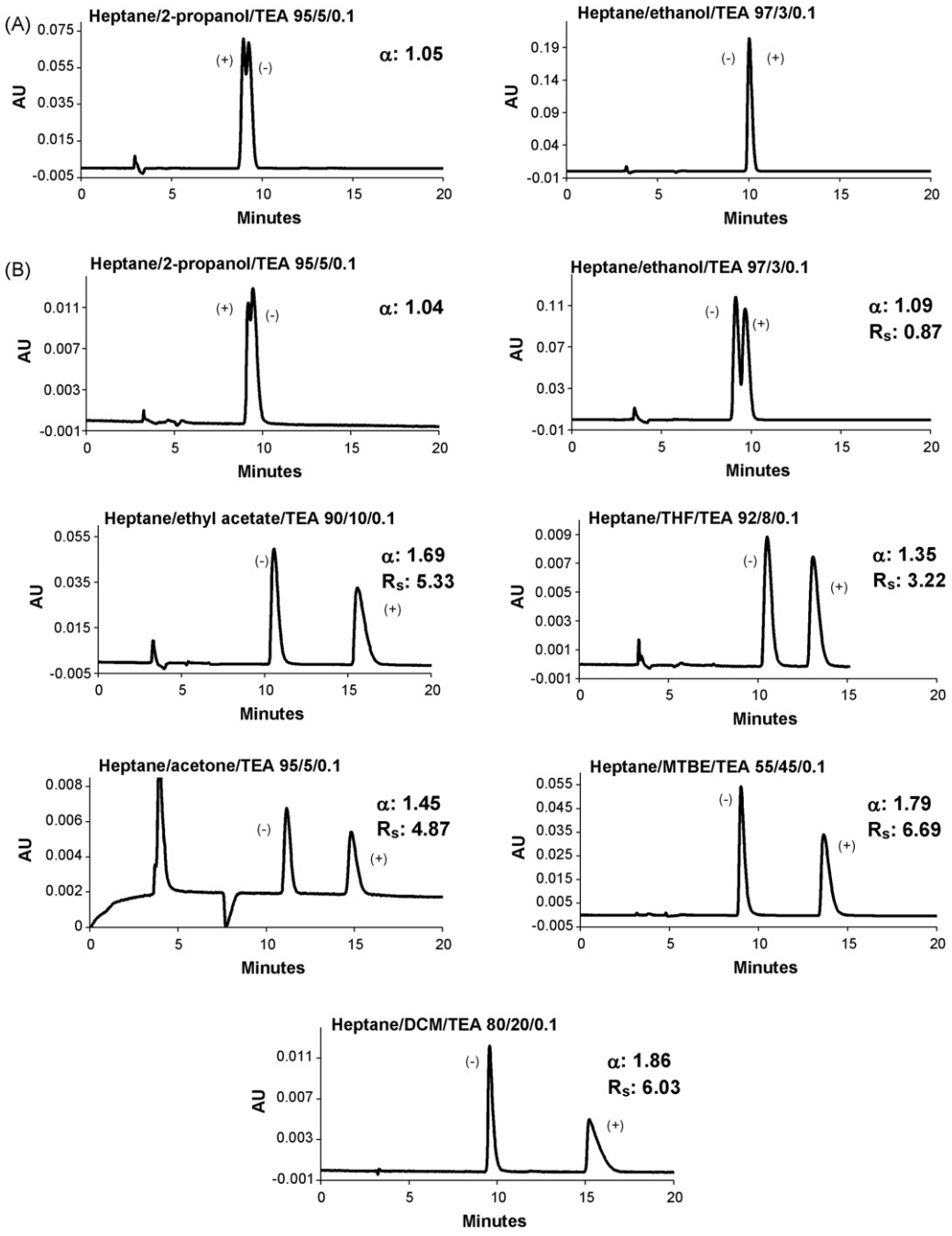

Fig. 10. Chromatograms of Ketamine on (A) Chiralpak AD-H and (B) Chiralpak IA.

In general, acidic compounds are not well resolved on the cellulosic phases Chiralcel OD-H and Chiralpak IB. However, we wished to study if the use of non-conventional solvents could help enhance enantioselective interactions for a model molecule, DNZ-β-phenylalanine with the chiral selector. A partial resolution of the enantiomers is observed on Chiralcel OD-H column using 2-propanol or ethanol in hexane as the mobile phase. However, DNZ-β-phenylalanine shows a much higher separation on the immobilized CSP Chiralpak IB especially with DCM, resulting in a resolution of 3.3, Fig. 11A and B.

Although the chiral selectors are the same, the coated and the corresponding immobilized CSPs generally show different enantioselectivity under same conditions. This indicates differences in the ability to access the chiral interactions sites in the selector due to conformational changes of the polysaccharide chains upon immobilisation. Since prediction of the best solvent for resolution of specific compounds is very difficult, the immobilized phases have to be screened with both mobile phases applicable on all columns as well as the more non-conventional solvents. The extended range of solvents applicable on the immobilized polysaccharide CSPs can increase the possibility to attain new or enhanced enantioselectivity and resolution.

In extensive screens of racemates it has also been observed that some solvents can give reverse elution order of the enantiomers, for example THF in resolution of Omeprazole, Fig. 9. Changes in elution order can be very advantageous in preparative

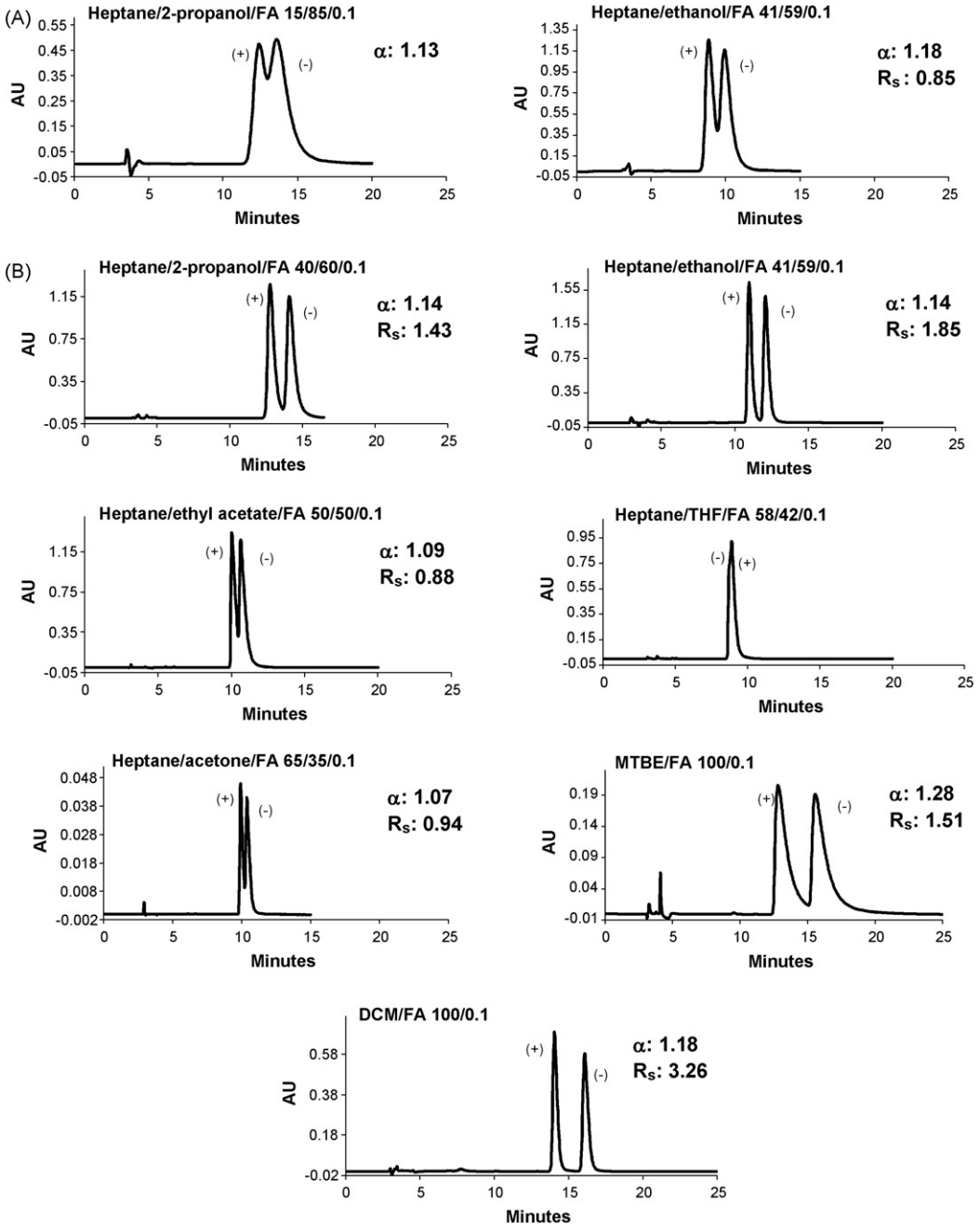

Fig. 11. Chromatograms of DNZ-β-phenylalanine on (A) Chiralcel OD-H and (B) Chiralpak IB.

chromatography when optimising the throughput of the required enantiomer.

4. Conclusions

Most of the 48 test compounds were retained more strongly on the immobilized CSP, Chiralpak IA, compared to the corresponding coated CSP Chiralpak AD-H with the generic mobile phase, 20% 2-propanol in heptane. A similar trend was observed for neutral and basic compounds on Chiralcel OD-H. Acidic compounds on the other hand show an opposite behavior on Chiralcel OD-H that has stronger retention for most compounds.

A majority of the racemic compounds were better resolved on Chiralpak AD-H than on Chiralpak IA. This suggests that there is a greater degree of achiral interactions involved on the immobilized CSP leading to stronger retention of the enantiomers but lower or similar enantioselectivity. This is clearly illustrated by the separation of Benoxaprofen and its methyl ester, whereby the free acid shows a drastic increase in retention on Chiralpak IA with the aprotic solvent acetonitrile but very little difference in the α-value.

Similarly, Chiralcel OD-H showed higher enantioselectivity than Chiralpak IB for most compounds when using mobile phases applicable to both CSPs.

The possibility to use an extended range of solvents in the mobile phase can be a great advantage and lead to enhancement of interactions between the chiral selector and the enantiomers. Use of aprotic solvents such as ethers can lead to much higher enantioselectivities as in the case of Ketamine on Chiralpak IA, whereby, a

sevenfold increase in the α-value is observed. In many cases, the elution order of the enantiomers is also changed and one needs therefore to keep close track of the elution order.

In our laboratories, the main advantage of the immobilized CSPs is not only the possibility to enhance enantioselectivity but also the ability to select the appropriate mobile phase that allows high solubility of the racemate to be resolved. This has a huge impact on productivity (Kg racemate/Kg CSP/day) of many preparative scale resolutions of enantiomers that we undertake during the drug development process and is a subject for another paper.

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