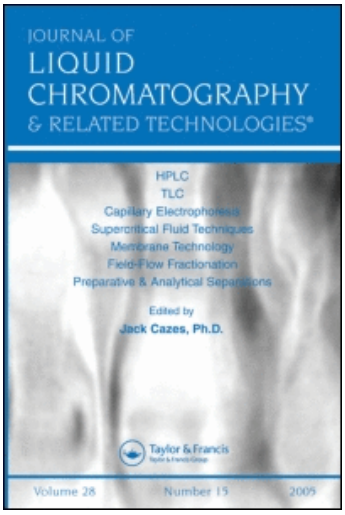

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Effect of Tertiary Alcohol Additives on Enantioselectivity of the Chiral-AGP Column

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Abstract: The chiral-AGP column is a protein based HPLC column widely used for analysis of chiral compounds in pharmaceutical and pharmacological applications. Organic solvents are frequently used as mobile phase additives to control analyte retention. In many cases, switching from one solvent additive to another can influence the enantioselectivity as well as retention. The group of solvents typically used as mobile phase additives includes methanol, ethanol, 1-propanol, 2-propanol, and acetonitrile. In this study, the column was used to resolve four different *N*-substituted amino acid derivatives. The mobile phase consisted of a pH 7 phosphate buffer with the addition of organic solvent to control retention. During method optimization, nine different organic solvent additives were compared, including two tertiary alcohols. For three of the four analytes, the tertiary alcohol additives provided significantly higher enantioselectivity than any of the commonly recommended solvent additives, affording enantioselectivities in the range of 1.4 to 3.8.

Keywords: Chiral-AGP, Enantioselectivity, Tertiary alcohols, Mobile phase composition

INTRODUCTION

The chiral-AGP column is a commercially available protein based HPLC column widely used in the pharmaceutical industry.[1] It can resolve a diverse array of chiral compounds and is compatible with aqueous eluents.[2,3] To adjust

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analyte retention, organic solvent additives are commonly used with the chiral-AGP column.[4–7] In many cases, organic solvents will also affect the enantioselectivity, and significant improvement in the enantioseparation may occur when switching from one organic solvent to another.[6,8–16] The first choice of solvent recommended by the column supplier is 2-propanol, which frequently gives favorable results.[1,3,7,8,17] Other recommended solvents are methanol, ethanol, 1-propanol, and acetonitrile. Additional solvent additives have also been investigated, including tetrahydrofuran, propionitrile, dimethylsulfoxide, and the alcohols 1-butanol, 2-butanol, 1-pentanol, and 1-octanol,[9,16,18–21] as well as the diols ethylene glycol, propylene glycol, and 1,2-butanediol.[1,9]

The fact that tertiary alcohols have not yet been investigated with the chiral-AGP column is surprising, considering the frequently superior enantioselectivity observed with the secondary alcohol 2-propanol. If the branched structure of 2-propanol is involved in enantioselectivity effects, it follows that tertiary alcohols may provide even greater influence on enantioselectivity. Studies involving the tertiary alcohol *t*-butanol have been reported for normal phased chiral separations, although the number of reports is very limited. In one study utilizing a Pirkle type stationary phase, it was found that *t*-butanol afforded higher enantioselectivity than 2-propanol.[22] In another study involving a polysaccharide type stationary phase, the investigators reported that branched alcohols *t*-butanol and 2-propanol caused more significant changes to the stationary phase compared to primary alcohols, based on solid state NMR measurements.[23] With regard to reversed phase chiral separations, we are aware of only one report involving the use of *t*-butanol, in which an ovomucoid type column was studied.[24]

We recently utilized the chiral-AGP column for optical purity analysis of compounds **I**–**IV** (Figure 1). This column was selected because **II** was known to be resolved using the chiral-AGP column,[25] and the ability to resolve other *N*-substituted amino acid derivatives was previously demonstrated.[8,26] During method optimization, the effect of organic solvent additives was examined. In addition to the five recommended solvents (methanol, ethanol, 1-propanol, 2-propanol, and acetonitrile), the solvents 1-butanol, 2-butanol (*racemic*), *t*-butanol, and *t*-amyl alcohol were also included for a total of nine solvents. While 1-butanol and 2-butanol have been previously investigated,[20,21] to the best of our knowledge tertiary alcohols have not been previously tested with the chiral-AGP column. The effects of the solvent additives on enantioselectivity for compounds **I**–**IV** are presented in this report.

EXPERIMENTAL

Chemicals

Monobasic sodium phosphate monohydrate (ACS grade), methanol, and acetonitrile (both HPLC grade) were from Fisher Scientific (Fair Lawn, NJ,

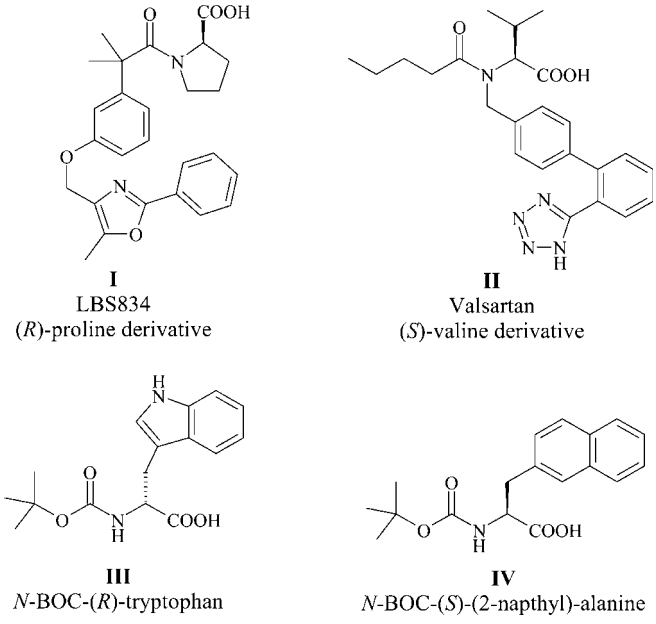

Figure 1. Amide and carbamate amino acid derivatives **I**–**IV** under investigation as analytes for the Chiral-AGP column.

USA). Ethanol (200 proof) was from Pharmco (Brookfield, CT, USA). *t*-butanol (ACS grade) and all other solvents (>99% purity) were from Aldrich (Milwaukee, WI, USA). Dibasic sodium phosphate (AR grade) was from Mallinckrodt (Phillipsburg, NJ, USA). The amino acid derivatives **I** and **II** are currently under development and were synthesized in-house at Novartis Pharmaceuticals Corporation. For *N-t*-BOC-tryptophan (**III**), the *R*-enantiomer was obtained from Bachem (King of Prussia, PA, USA) and the *S*-enantiomer from Aldrich. For *N-t*-BOC-(2-naphthyl)-alanine (**IV**), the *R*-enantiomer was from Aldrich and the *S*-enantiomer from Fluka (Milwaukee, WI, USA).

Instrumentation and Chromatographic Setup

The chromatographic system consisted of an Alliance HPLC separation module and a model 996 photodiode array UV detector (Waters Corp., Milford, MA, USA). Electronic data acquisition and instrument control was accomplished using Empower software (Waters). HPLC column was chiral-AGP (Regis Technologies, Inc., Morton Grove, IL, USA) of dimensions 15 mm (L) x 4.0 mm (I.D.) with 5 μm particles. The column was maintained at 25°C and the flow rate kept at 0.5 mL/min for all

experiments. Injection volume was 2 μL. Chromatographic calculations were performed using the Empower software. For calculating retention (k), the column hold-up time of 2.90 minutes was used based on injection of water. Resolution (R_S) and efficiency (N) were calculated by USP methods.

Mobile Phase Preparation and General Procedure

Mobile phase buffer was prepared by dissolving 1.70 g of monosodium dihydrogen phosphate monohydrate and 1.70 g disodium hydrogen phosphate (anhydrous) into one liter of water. The pH of this buffer was maintained within the limits of 6.95 and 7.00 when measured with a pH meter, and no pH adjustment was made. For mobile phases containing *t*-butanol, the loosely capped bottle of *t*-butanol was first placed in a 40°C water bath until the contents melted (this procedure was performed in a fume hood). The desired volume of liquid *t*-butanol was then poured into a graduated cylinder, and the measured aliquot was poured into the final mixing vessel. Any solidified material remaining in the graduated cylinder was transferred to the final container by several rinses with buffer, which had been premeasured in another graduated cylinder.

For each compound **I**–**IV** in Figure 1, a test mixture of both enantiomers was prepared in a 7:3 enantiomeric ratio so that relative peak areas could be used to confirm the identify of the individual enantiomers. Diluent was 1:1 (v/v) acetonitrile/buffer. The amounts injected and the detection wavelengths are shown in Table 1. Since the retention and resolution obtained from protein based columns may vary considerably with sample loading,[27–29] the same solutions were injected for each mobile phase condition to ensure a valid chromatographic comparison between solvent additives. Experiments were run with several mobile phases having different solvent content, allowing enantioselectivity (α) to be calculated across a range of k values.

Table 1. Amount of each compound injected and detection wavelengths

Structure & configuration of major enantiomer	Detection wavelength (nm)	Amount injected (μg)		
		Major enantiomer	Minor enantiomer	Total
(*R*)-I	273	0.91	0.38	1.29
(*S*)-II	254	1.74	0.78	2.52
(*R*)-III	225	0.42	0.19	0.61
(*S*)-IV	225	0.37	0.16	0.53

RESULTS AND DISCUSSION

Effect of Solvents on Enantioselectivity

The effect of solvent additives on the enantioseparation of **I** is shown in Figure 2. Due to the high retention of **I**, it was essential to add organic solvent to the mobile phase to obtain reasonable retention times. Significant variation in α occurred from one solvent to another. However, for a given solvent, very little variation occurred as the amount of solvent was changed. Thus, the α values in Figure 2 appear approximately as a horizontal line for each solvent. The tertiary alcohols showed the highest α values, followed by secondary alcohols 2-butanol and 2-propanol, which showed higher enantioselectivity compared to the remaining solvents. For alcohols having a given number of carbon atoms, the trend was increasing α for higher order alcohols (2-propanol > 1-propanol, and *t*-butanol > 2-butanol > 1-butanol). These data suggest that the enantioselectivity enhancement is related to the branched structure of the alcohol, and not simply to the number of carbon atoms.

Figure 3 shows the α values for **II**. This compound was more weakly retained than **I** and, therefore, it was possible to chromatograph **II** in buffer alone, as well as mobile phases containing organic solvent. As previously reported,[25] excellent enantioseparation of **II** was obtained with 2-propanol. However, Figure 3 shows the highest α values were again observed with the tertiary alcohols, as was the case for **I**. With regard to alcohol type (1°, 2°, or 3°), the trend for **II** was also the same as for **I** (3° > 2° > 1°). Some improvement in α was observed for 1-butanol and 2-butanol compared to

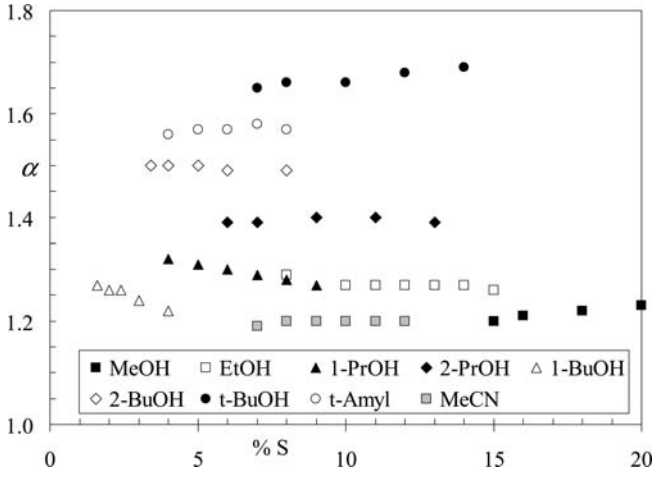

Figure 2. Enantioselectivity (α) *vs.* per cent added solvent for compound **I**.

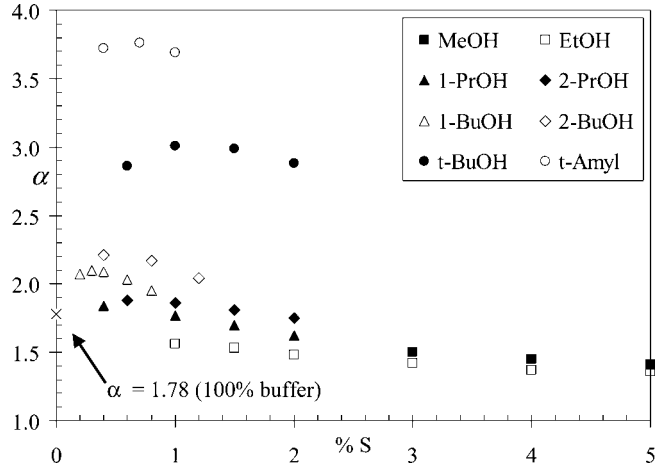

Figure 3. Enantioselectivity (α) vs. per cent added solvent for compound **II**.

buffer alone, but addition of 1-propanol and 2-propanol showed very little improvement, and methanol and ethanol caused α to decrease. For **II**, acetonitrile caused a reversal of elution order and resulted in very broad peaks unsuitable for quantitative analysis. For this reason the α values for acetonitrile were not included in Figure 3. Although uncommon, other examples of elution order reversal have been observed with the chiral-AGP column when changing from 2-propanol to acetonitrile.[16,27,30]

Figure 4 shows the α values obtained for **III**. This compound showed the least retention of the four compounds. Therefore, only small amounts of organic solvents were added to the mobile phase. Nevertheless, some of the solvents had a very significant effect on α despite being present at low levels. In particular, *t*-butanol and *t*-amyl alcohol showed a significant increase in α, even as k was decreasing. Methanol, ethanol, 2-propanol, and 2-butanol appeared to affect only k and had little or no influence on α compared to 100% buffer mobile phase. The longer chain linear alcohols 1-propanol and 1-butanol caused α to decrease significantly.

Figure 5 shows the α values for **IV**, which showed similar retention to **II**. For compound **IV**, significant losses in α occurred for all solvent additives with the exception of small amounts of ethanol (1 to 2%), which caused only a marginal improvement compared to buffer alone. This example shows that the enhancement of α by the tertiary alcohols is analyte dependent and not a general phenomenon. The significantly different enantioselectivity behavior between **III** and **IV** is interesting when considering the similarity in molecular structure of the two compounds (Figure 1). This illustrates how, for the chiral-AGP column, determining the best solvent additive for a particular chiral separation remains largely a trial and error approach.

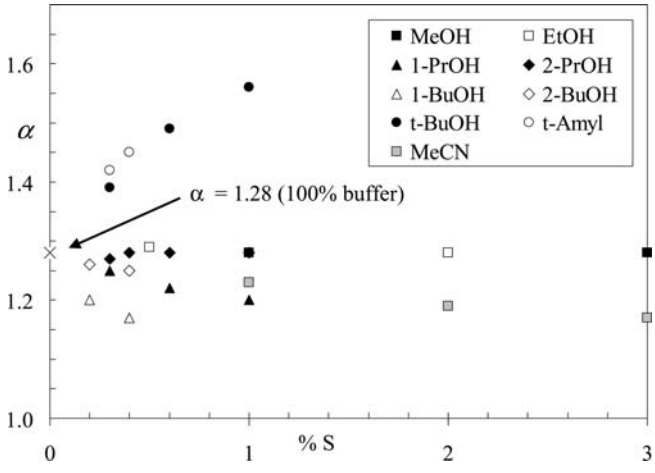

Figure 4. Enantioselectivity (α) vs. per cent added solvent for compound **III**.

Other Factors Influencing Chromatographic Resolution

Improved enantioselectivity is of little value if excessive peak broadening occurs at the same time. For this reason, chromatographic efficiency and resolution were also evaluated. The most significant variations in efficiency occurred for the more strongly retained enantiomers of **I** and **II**. Figure 6 shows *N* values for the second eluting peak of **I**. The relatively low efficiency is not unusual for this type of column.[4,27,29,31] It is evident from Figure 6 that

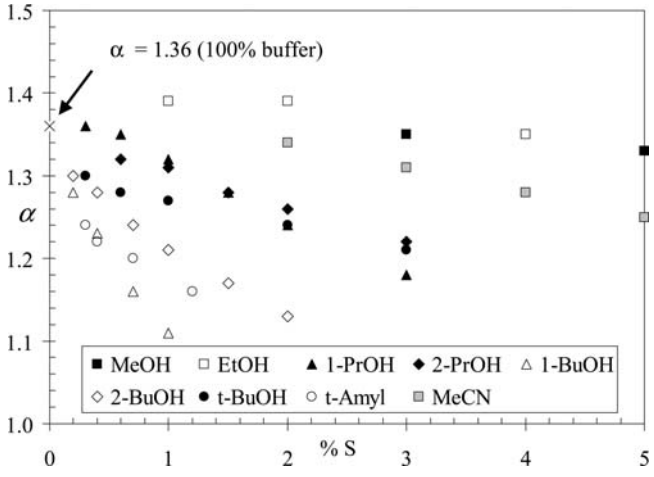

Figure 5. Enantioselectivity (α) vs. per cent added solvent for compound **IV**.

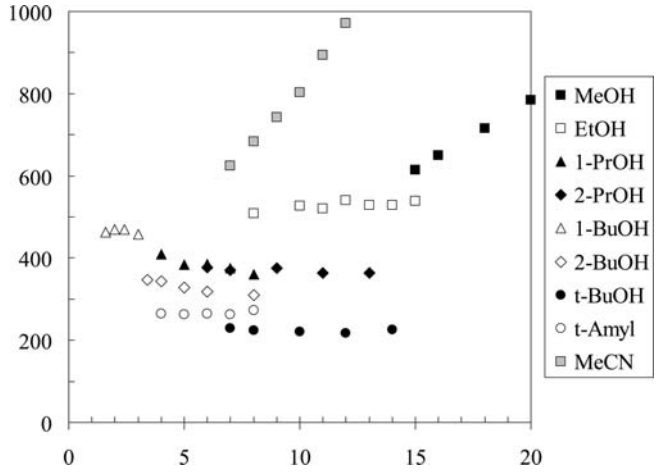

Figure 6. Chromatographic efficiency (N) vs. per cent added solvent. The N values pertain to the second eluting enantiomer for compound **I**.

the solvent additives caused significant variation in N for the second eluting peak. Such solvent related changes have been observed for other compounds with the chiral-AGP column.[20,32] Interestingly, the solvent additives which showed the highest α values (both tertiary alcohols and 2-butanol) showed the lowest N values. However, N did not impact R_S nearly as much as α. This is illustrated in Figure 7, which shows R_S values for **I**. Despite the lower N values obtained with the tertiary alcohols and

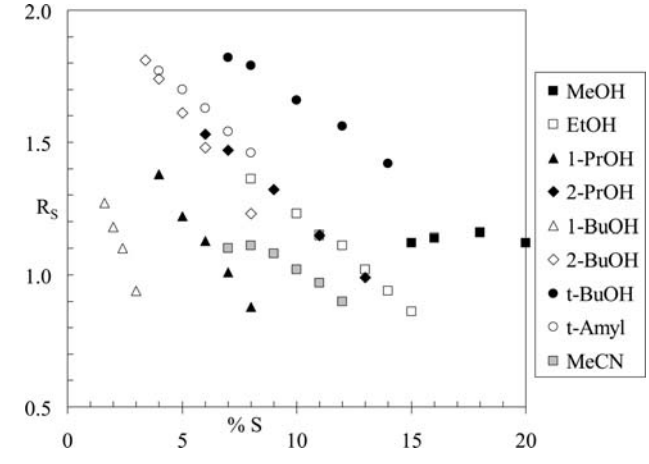

Figure 7. Chromatographic resolution (R_S) vs. per cent added solvent for compound **I**.

2-butanol, these same solvents afforded higher R_S values than any of the other solvents. This indicates that α is the dominant factor in the chromatographic resolution of **I**, and consequently R_S values show very similar trends as was observed for α with respect to solvent type (Figures 2 and 7). Similar behavior was observed for **II**. As observed for **I**, variations in *N* were much less influential on R_S than variations in α, and the tertiary alcohols afforded the highest R_S for **II** despite having the lowest *N* values for the second eluting enantiomer. Figure 8 shows chromatograms obtained for **II** using 2- propanol and *t*-butanol as mobile phase additives, illustrating the superior separation obtained with *t*-butanol compared to the most frequently used solvent additive 2-propanol.

For **III**, the *N* values for both enantiomers increased upon the addition of organic solvent and the effect was similar for all of the solvents. Consequently, for **III** the same trends in both R_S and α were observed. The tertiary alcohols again afforded higher resolution than any of the other solvent additives as was the case for **II**. Figure 9 shows chromatograms obtained for **III**, illustrating the superior separation using *t*-butanol compared with 2-propanol. For compound **IV**, the values of R_S also showed the same trends as α. In this case, ethanol was the only solvent showing any significant improvement in resolution for compound **IV**.

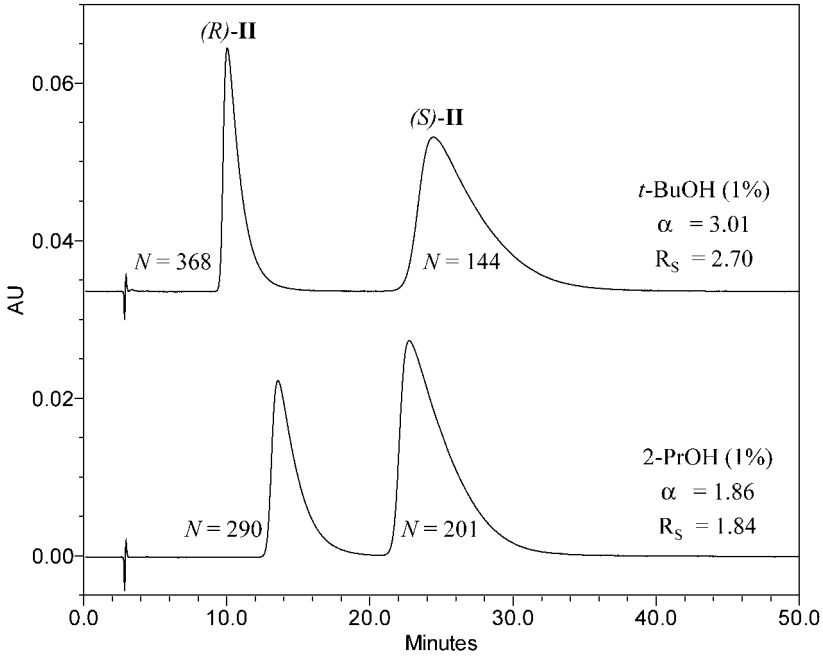

Figure 8. Chromatograms for compound **II** obtained using mobile phase additives of *t*-butanol (top) and 2- propanol (bottom).

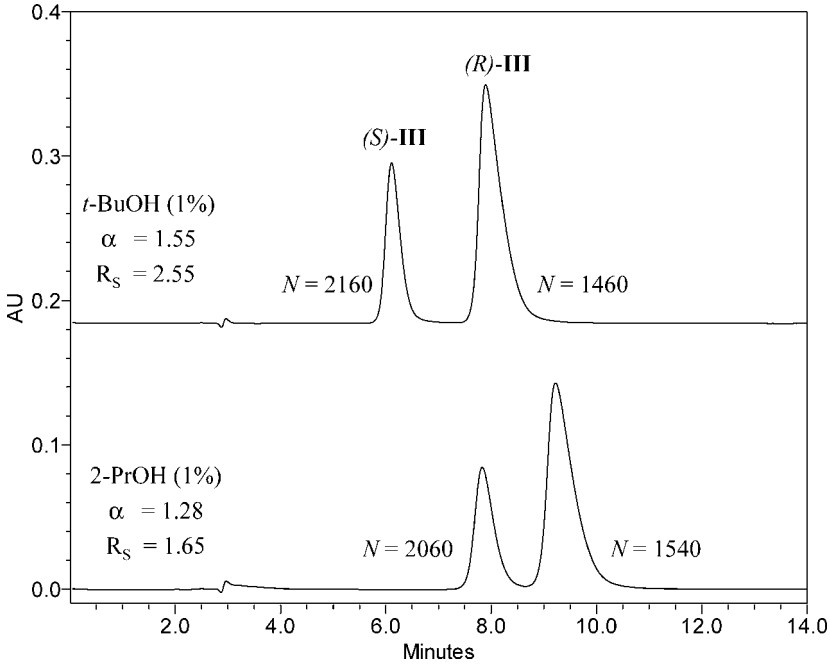

Figure 9. Chromatograms for compound **III** obtained using mobile phase additives of *t*-butanol (top) and 2-propanol (bottom).

Relative Eluting Power of Solvents

Examination of plots of log (*k*) vs. per cent solvent in the mobile phase (Figure 10) is a convenient way to compare the eluting power of the nine solvent additives. The relative eluting power of the primary and secondary type alcohols followed the general trend of 1-butanol > 2-butanol > 1-propanol > 2-propanol > ethanol > methanol. This order is consistent with other studies in which the effect of solvents on retention has been systematically investigated with the chiral-AGP column.[20,21,31] Acetonitrile was comparable in strength to ethanol for **III** & **IV**, but behaved as a slightly weaker solvent for **II** (between methanol and ethanol) and as a stronger solvent for **I** (comparable to 2-propanol).

In terms of eluting strength, the solvent which most closely matched *t*-butanol was 2-propanol. The stronger solvent *t*-amyl alcohol showed similar eluting strength to 2-butanol. However, both of the tertiary alcohols showed variations in strength compared to the other alcohols depending on the analyte. This is illustrated in Figure 11, which shows the relative amount of solvent (S_{Rel}) required to achieve the same *k* value (calculated from log (*k*) vs. %S data) for alcohols containing three or more carbon atoms. 2-propanol was arbitrarily selected as the

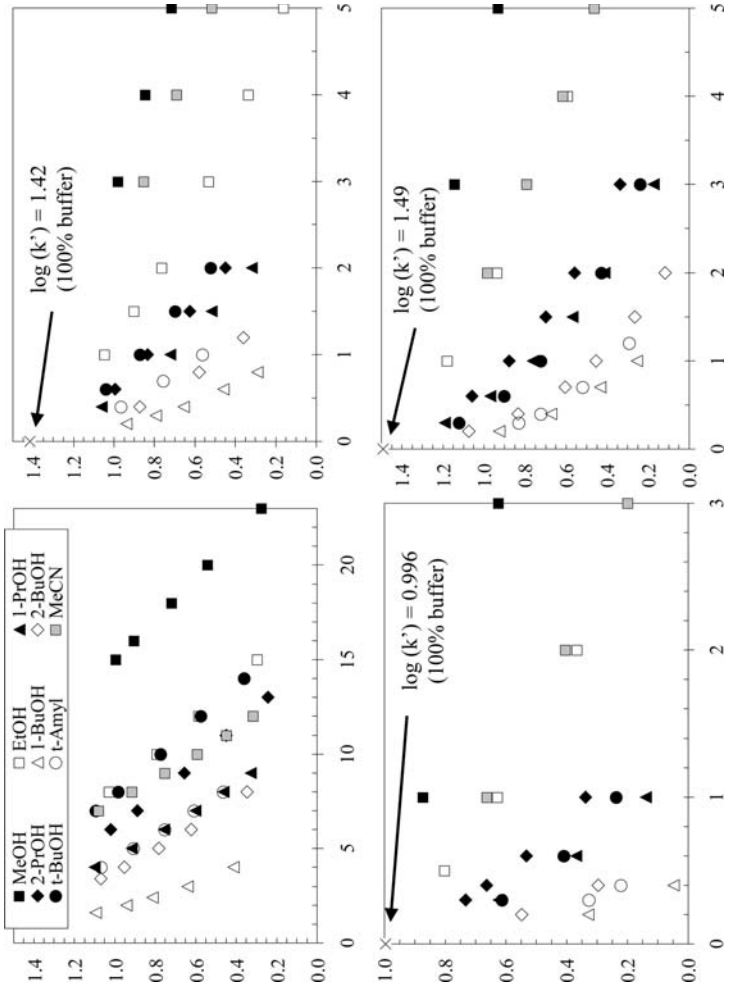

Figure 10. Plots of log (k) vs. per cent solvent added (%S) to mobile phase for compounds **I** (top left), **II** (top right), **III** (bottom left), & **IV** (bottom right) using the chiral-AGP column under pH 7 conditions. The log (k) values pertain to the more strongly retained enantiomer in all cases.

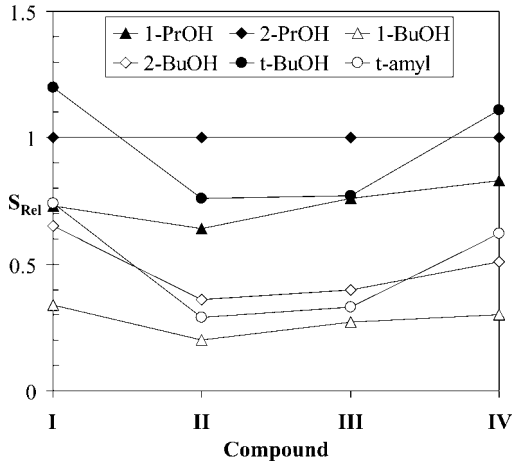

Figure 11. Relative amounts of solvent required (S_{Rel}) to obtain same retention obtained with 2-propanol. S_{Rel} values shown for each of the compounds **I–IV** (left to right). S_{Rel} values calculated from log(k) vs. *%S* data as shown in Figure 2.

reference, and therefore all S_{Rel} values are unity for this solvent. Note that the same relative ranking of solvents is obtained among the primary and secondary alcohols for all of compounds **I–IV**, but the position of the tertiary alcohols shifts within this series depending on the compound, as indicated by the crossing of data lines in Figure 11. For **I**, the tertiary alcohols acted as weaker solvents, requiring relative larger amounts in the mobile phase to obtain comparable retention. Thus, for **I** the eluting strength increased according to the ranking *t*-butanol > 2-propanol > *t*-amyl ~ 1-propanol > 2-butanol > 1-butanol. Compound **IV** showed a similar trend as **I**. For **II**, the strength of tertiary alcohols showed a relative increase, leading to a change in order when ranked from weakest to strongest: 2-propanol > *t*-butanol > 1-propanol > 2-butanol > *t*-amyl > 1-butanol. A similar trend was seen for compound **III**. Perhaps the most interesting aspect of Figure 11 is the similarity in behavior of the two tertiary alcohols. Both showed similar shifts in S_{Rel} when moving from one compound to another, just as similar trends were observed for α values as described in the previous sections. This again suggests that it is the branched structure of the tertiary alcohols, which is responsible for the different selectivity and retention behavior.

The similar eluting strength of 2-propanol and *t*-butanol observed in the current study was previously reported for chiral separations using an ovomucoid type chiral column.[24] These investigators pointed out that the observed relative strength of *t*-butanol was lower than would be predicted by comparing log P values of the solvents. They proposed that steric

effects of the solvent molecule within the region of the chiral recognition site played an important role in retention, thereby explaining the lack of a simple correlation between solvent hydrophobicity and retention. The results of the current study suggest that similar phenomena may also occur with the chiral-AGP stationary phase when tertiary alcohol additives are used.

From a practical standpoint, knowledge of relative solvent strengths is valuable for method development purposes when screening several solvent additives with the chiral-AGP column. If the optimal percentage of 2-propanol in the mobile phase has already been determined for a particular compound, the data suggest that the same percentage is a reasonable starting point for *t*-butanol experiments. Similarly, an appropriate percentage for *t*-amyl alcohol pilot experiments can be estimated from 1-propanol or 2-butanol retention data, if available. This can potentially reduce the number of experiments required during method optimization.

CONCLUSION

The enantioseparation of four different *N*-substituted amino acid derivatives using the chiral-AGP column was studied. All four compounds were resolved using a mobile phase of pH 7 phosphate buffer with organic solvents added to adjust retention. The effect of nine different solvents was compared. The most interesting aspect of this work was enantioselectivity improvements obtained from tertiary alcohol additives in the mobile phase. For compounds **I**–**III**, α values were considerably higher when using tertiary alcohols compared to more commonly employed solvents such as methanol, ethanol, 1-propanol, 2-propanol, and acetonitrile. Both of the tertiary alcohols utilized in this study (*t*-butanol and *t*-amyl alcohol) showed similar retention and selectivity effects, suggesting that some property related to the structure of tertiary alcohols is responsible for these effects. In the case of **IV**, the lack of enantioselectivity enhancement by tertiary alcohols indicates that the effect is compound dependent, and does not occur in all cases. Also, it is important to note that all of the chiral compounds in this study were structurally related amino acid derivatives. Therefore, it is not known if the enhancement effect of tertiary alcohols will occur when separating other types of compounds with the chiral-AGP column.

The lack of any previous studies involving tertiary alcohol additives may be due to concerns about limited miscibility in water (*t*-amyl alcohol) or the inconvenience of working with a low melting solid material (*t*-butanol). However, these factors did not cause any problems during the course of this work. Preparation of *t*-butanol mobile phases was easily accomplished by simply premelting the solid, and the amount of *t*-amyl alcohol required in the mobile phase (8% v/v or less) was

below any miscibility limitations when mixed with a typical sodium phosphate mobile phase buffer. Therefore, we believe that the use of tertiary alcohol additives with the chiral-AGP column is worthy of further investigation, and additives such as *t*-butanol and *t*-amyl alcohol may provide a useful addition to the group of solvents typically used as mobile phase additives for chiral-AGP separations.

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