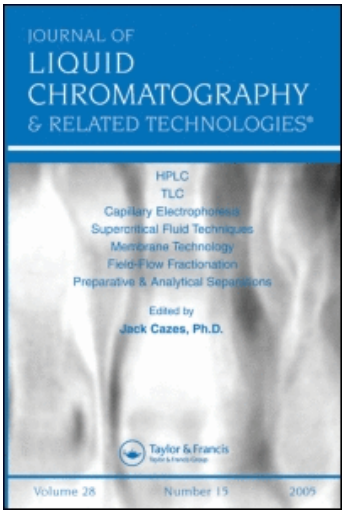

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Mechanistic Studies of the Separation of an HIV Protease Inhibitor from Its Piperazine Diastereomer by Reversed Phase High Performance Liquid Chromatography

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ABSTRACT

The HPLC separation and retention behavior of the piperazine diastereomers of an Human Immunodeficiency Virus (HIV) protease inhibitor on a commercially available C_{18} chromatographic phase is discussed. The pH and mobile phase composition have the greatest effect on the selectivity of this system. Evidence is presented that the selective interaction involves hydrogen bonding. As the mobile phase pH was varied, a reversal in the elution order corresponding to the change in the protonation state of the molecule is observed. It is proposed, that the selectivity is governed by coordination of the active piperazine amine as a proton donor or proton acceptor with the organic modifier, while interacting with the stationary phase. It is shown, that longer chain alcohols

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produce poorer selectivities than shorter chain alcohols. It is proposed, that this is because it is more difficult for the longer hydrophobic chains to form stable complexes with the solute through hydrogen bonding. The hydrogen bonding moiety of the solute may, thus, coordinate with water and not selectively interact with the stationary phase. The effects of stationary phase chemistry, ionic strength, and modifier strength are also described.

Key Words: Diastereomer selectivity; HIV protease inhibitors; Hydrogen bonding; pH effects; Piperazine; Reverse phase HPLC; Solvation effects.

INTRODUCTION

Protease inhibition is one therapeutic target in the control of the Human Immunodeficiency Virus (HIV). Second generation protease inhibitor drugs, able to inhibit variant HIV strains immune to marketed first generation compounds, such as ritonavir and indinavir, have been developed. Compound A (Fig. 1) is one such drug candidate.

Compound A has five chiral centers. Diastereomeric impurities affect the drug potency and may affect drug toxicity as well. Protease inhibitors are generally required in high dosages, so even low level impurities can still represent relatively high absolute amounts of the impurity upon ingestion.

The synthesis of Compound A has been described.[1,2] In practice, it is made via convergent synthesis; three intermediates are first prepared and subsequently coupled to make the active drug. Diastereomeric purity of the drug substance is controlled by establishing assays for the chiral and diastereomeric purity of each of the intermediates. The non-desired minor enantiomer of the piperazine intermediate, *R*-piperazine, couples to a second

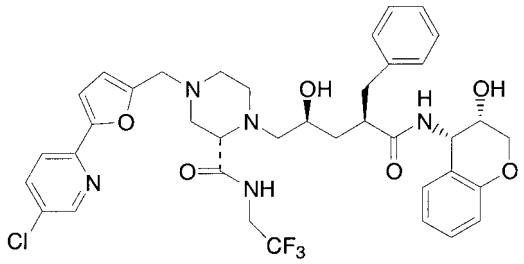

Figure 1. Piperazine diastereomers of Compound A. *S*-piperazine (active): trifluoroethylamide into the page; *R*-piperazine (impurity): trifluoroethylamide out of the page.

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intermediate with kinetics similar to the desired *S*-piperazine intermediate. Due to its structural similarity to *S*-piperazine, it is difficult to reject the *R*-piperazine analog of Compound A after the coupling. Control and accurate quantitation of the diastereomer is critical in determining drug potency and assessment of drug safety.

In this work, the selectivity and retention behavior of the *R*- and *S*-piperazine diastereomers of compound A is presented under reverse phase chromatographic conditions.

EXPERIMENTAL

HPLC experiments were carried out using a Thermo Separations Products (TSP-Sunnyvale, CA) P-4000 pump, a TSP AS-4000 autosampler, and a TSP UV-1000 absorbance detector. Absorbance was monitored at 210 nm for all experiments. All data were collected and processed using the Perkin Elmer (Cupertino, CA) Nelson TurboChrom chromatographic software. The separation temperatures were maintained with a CER temperature controller (Mahwah, NJ).

To prepare the mobile phases, an aqueous solution was first prepared using potassium dihydrogen phosphate (Acros Chemicals, Springfield, NJ) and adding appropriate volumes of high purity phosphoric acid (Aldrich 85% concentrated, Milwaukee, WI) until the desired pH was reached. A Fisher Scientific (Springfield, NJ) Accumet Research AR 15 pH meter, equipped with a Corning (Corning, NY) Rugged Bulb pH electrode was used. Optima™ grade methanol was purchased from Fisher (Springfield, NJ), and Omnisolve™ grade acetonitrile was purchased from EM Science (Darmstadt, Germany). The water was deionized using a Hydro™ Ultrapure water deionizer (Garfield, NJ). The buffer was mixed on-line to the prescribed volume % with the chosen organic modifier(s). *R*- and *S*-piperazine diastereomers were synthesized by Merck Process Research (Rahway, NJ).

Although several kinds of C_{18} phases were investigated, it was found that neither the degree of carbon loading nor the characteristics of the silanol endcapping had an effect on the retention or selectivity in this system. The following chromatographic columns were used to investigate the effect of endcapping chemistry on the selectivity: YMC Pro C_{18}, YMC-Pack ODS-AL, YMC-Pack C_8, YMC-Pack ODS-AQ, all from Waters Corporation (Milford, MA), and Inertsil ODS-2 (a highly end-capped C_{18} phase from Metachem in Torrance, California). Each column was 4.6×250 mm, packed with 5 µm, 120 Å silica particles. For investigations of pH, alcohol and modifier effects, and temperature studies, the YMC Pro C_{18} stationary phase was used.

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The column was equilibrated with mobile phase for 30 min at a flow rate of 1.5 mL/min once the mobile phase composition was changed. The column dead time, t_0, was estimated by recording the first baseline perturbation. Duplicate injections (10 μL) were performed at each mobile phase concentration. The retention factor for each diastereomer was calculated as:

$$k' = \frac{(t_r - t_0)}{t_0}$$

where t_r is the retention time of the analyte and the t_0 is the column void time. The void time was estimated by observing baseline perturbation. The selectivity factor was calculated as the ratio:

$$\alpha = \frac{k'_2}{k'_1}$$

where k'_1 is the retention factor of the *S*-piperazine diastereomer and k'_2 is the retention factor of the *R*-piperazine.

RESULTS AND DISCUSSION

Effect of Alcohols

A variety of different retention models have been proposed to explain and predict retention and selectivity in reversed-phase liquid chromatography (RPLC).[3–10] The most widely applied model in the optimization of separations is the linear solvent strength model developed by Snyder and co-workers.[11–13] This model predicts a logarithmic dependence of retention to the concentration of an organic solvent or a stronger solvent. According to this theory, hydrophobic or solvophobic interactions result from repulsive forces between a polar solvent and the nonpolar solute and stationary phase. The driving force in the binding of the solute to the stationary phase is the decrease in the area of the nonpolar segment of the solute exposed to the solvent, which is also thought crucial to selectivity.

It has been reported that alcohols can play a significant role in enantiomeric separations.[14,15] In this study, methanol, ethanol, and propanol were used to study the effect of alcohols on separation of two diastereomers that vary by a single chiral center in a five chiral center solute. The effect of the alcohols on retention factor and diastereomeric selectivity in a ternary mobile phase system are shown in Figs. 2 and 3. The molar ratios of alcohols to buffer were varied at a constant concentration of acetonitrile (7.7 M). The logarithmic

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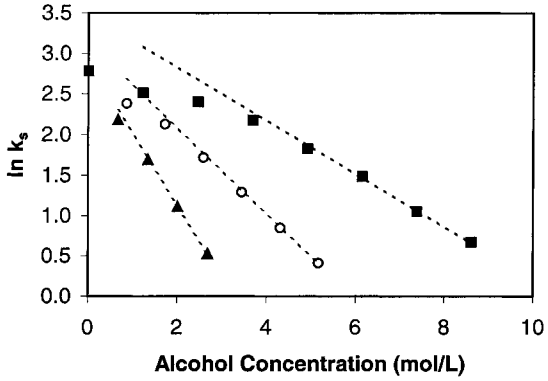

Figure 2. Effect of alcohol on retention factor. Conditions: 250 × 4.6 mm YMC Pro C_{18} column packed with 5 μm porous (120 Å, C_{18}) particles, 25°C; flow rate: 1.5 mL/min; 2 mM K_2HPO_4 (pH 6.4) as the buffer; the molarity of alcohol was varied at a constant acetonitrile molarity (7.7 M); (■) methanol; (○) ethanol; (▲) *n*-propanol.

retention factor decreases linearly with an increase in alcohol concentration (Fig. 2) when the alcohol concentration is higher than a certain value (e.g., >4.9 M methanol; >1.7 M ethanol; >0.67 M isopropanol). This observation is in agreement with the prediction of the linear solvent strength model. Based on this model, the hydrophobic interaction decreases as the ratio of alcohol to water increases, since the alcohol is a stronger solvent than water.

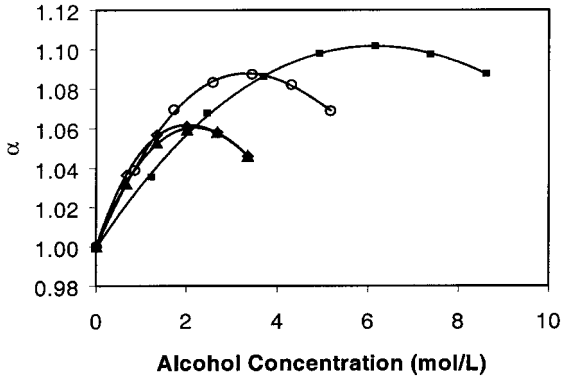

Figure 3. Effect of alcohol on selectivity factor. Conditions are the same as in Fig. 2. (■) methanol; (○) ethanol; (▲) *n*-propanol; (□) *i*-propanol.

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However, when the ratios are lower than those values, retention factor values are lower than theoretically expected. These results may be explained by the existence of preferential solvation in the mobile phase medium.[16] The strongest solvating can occur for some substances at a certain composition of binary mixtures.[17] For example, the preferential solvation of acetate ions reaches a maximum at an acetonitrile to water ratio of ~4 : 1.[18] Similarly, the preferential solvation for piperazine diastereomer molecules may exist at a certain ratio of alcohol to water in a ternary mobile phase system. This may lead to a decrease in repulsive force that drives the solute molecules to be retained in the stationary phase. In other words, it is more difficult for the highly solvated solute molecules to enter the stationary phase, which can result in the lower-than-predicted values of retention factor. Cheong and Carr observed that the changes in retention factors upon changing the mobile-phase composition were highly correlated with the solute's mobile-phase activity coefficients.[19] A highly solvated solute can possess a low activity coefficient.

In a pH 6.4 buffered aqueous-alcohol system, the selectivity factor, α increases with increasing ratio of alcohol to buffer, reaches a maximum, and then decreases (Fig. 3). The selectivity decreases from methanol to ethanol to isopropanol. The effect is less pronounced with longer chain alcohols, possibly because hydrogen bonding is more difficult as the alcohols become bulkier. There is no significant difference in selectivity between *i*-propanol and *n*-propanol.

One possible explanation for this behavior is to consider a model that treats compound A and the alcohol, as a coordinated complex interacting with the stationary phase as an entity. Strong hydrogen bonding modifiers like methanol form tight associations with the solute, and as an entity is able to interact with the stationary phase, engaging in hydrophobic interactions that lead to selectivity. Because the association of the other alcohols with the solute through hydrogen bonding is weaker than that of methanol, those complexes have less interaction with the stationary phase and, thus, the selectivity is poorer. In the presence of these other alcohols, the solute may instead form a hydrogen bond complex with water molecules, effectively disengaging itself from selective interactions.

These results may also be interpreted by considering preferential solvation and hydrophobic interactions. At low ratios of alcohol to water, the contribution of preferential solvation to selectivity may be dominant and it may increase as the alcohol concentration increases. At high ratios of alcohol to water, the contribution of solvent strength or polarity to selectivity may be dominant; in this case, the selectivity usually decreases with an increase in solvent strength. When the solvent strength increases from methanol to ethanol to isopropanol the selectivity decreases in the same order. A maxima

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in selectivity is observed when the ratio of alcohol to water, necessary for optimal preferential solvation, is not overcome by hydrophobic elution. Therefore, 3.7–7.4 M methanol (15–30% v/v) can be used, in practice, to obtain high selectivities in this ternary system.

Effect of pH

Compound A contains two basic substituents, a piperazine subunit and a pyridine ring. Because of these structural elements, the mobile phase pH can have a marked effect on both selectivity and retention. In each of the solvent systems investigated (aqueous methanol, aqueous acetonitrile, and aqueous acetonitrile/methanol), there is an observed shift in the elution order of the piperazine diastereomers as the pH is changed (Fig. 4). These data also provide some insight as to the possible location of the stereoselective interaction between methanol and the solute. To rationalize the transient nature of this selectivity, the titrimetric behavior of this compound should first be considered.

Although compound A has two tertiary piperazine amines, partially aqueous acid titrations of the compound in each of the mobile phase compositions showed only one equivalence point with an apparent pK_a of ~4.5, which can be attributed to one of these amines. The basicity of the compound is considerably less than what might be predicted from the titrimetric behavior of molecular piperazine (pK_as of 9.5 and 4.2), because each amine has an added degree of substitution. The ionization state of compound A depends upon the pH of the mobile phase; its ability to hydrogen bond depends upon the ionization state.

The pH–selectivity curves in Fig. 4 can be attributed to the transient nature of the ionization state of the solute, and the resulting erosion and reformation of hydrogen bonds. At pHs above ~5, the piperazine subunit is uncharged and able to form a selective hydrogen bond with a hydrogen donor like methanol; the selectivity remains essentially constant. As the pH is decreased below ~5, one of the piperazine amines becomes protonated, and the association between methanol (as the hydrogen donor) and piperazine (as the hydrogen acceptor) becomes weaker. As this ionization occurs, a new selective hydrogen bond association forms. The solute becomes the hydrogen donor, and methanol and acetonitrile become the acceptors. The selectivity advances in the opposite direction as the solute becomes fully protonated at aqueous pH ~ 3.0.

In the pH range between 3.0 and 2.0, a selectivity decrease is observed because the solute becomes highly polar and less hydrophobic; this is because the retention is practically lost. In this pH range, hydrogen bonding may still

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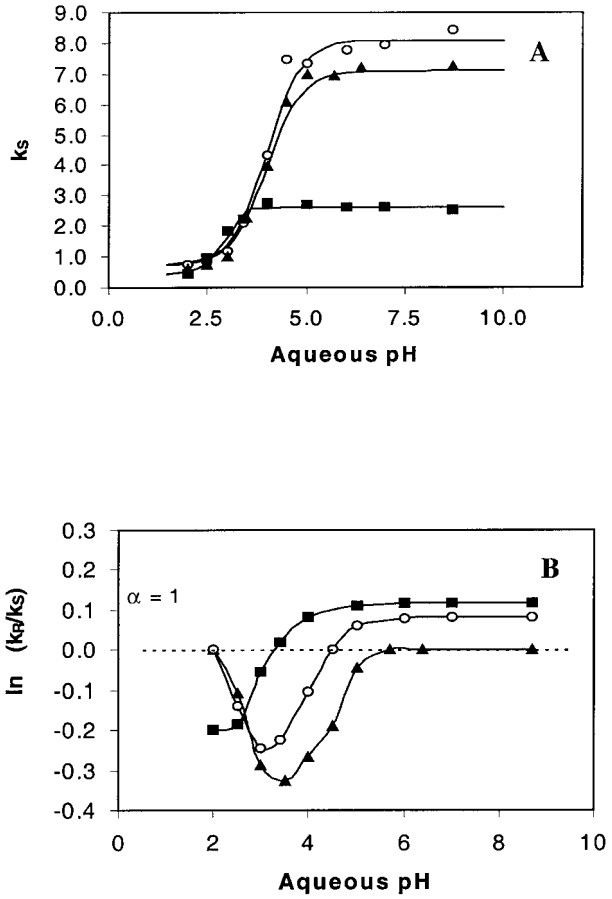

Figure 4. Effect of pH. (A) Effect of pH on retention (■) 19.1 M methanol in 2 mM K_2PO_4 buffer; (○) 3.7 M methanol/7.7 M acetonitrile in 2 mM K_2PO_4 buffer; (▲) 8.6 M acetonitrile in 2 mM K_2PO_4 buffer. (B) Effect of pH on ln (selectivity factor) 19.1 M methanol in 2 mM K_2PO_4 buffer; (○) 3.7 M methanol/7.7 M acetonitrile in 2 mM K_2PO_4 buffer; (▲) 8.6 M acetonitrile in 2 mM K_2PO_4 buffer. Other conditions are the same as in Fig. 2.

take place between the solute (as the hydrogen donor) and water (as a hydrogen acceptor), but this donor–acceptor complex has little affinity for the hydrophobic stationary phase.

The selectivity vs. pH relationship with the buffered acetonitrile mirrors that of buffered acetonitrile/methanol, albeit the curve is shifted to a higher

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pH. Above pH 6.0, no selectivity is observed. Acetonitrile does not form selective hydrogen bonds with the solute, because both acetonitrile and Compound A tend to function only as hydrogen acceptors in this ionization state. As the pH is decreased, Compound A partakes in hydrogen bonding as a hydrogen donor, and acetonitrile as the acceptor. As in the aqueous methanol/acetonitrile system, selectivity increases to a maximum (pH 3.8), when this hydrogen bond is at its strongest, and begins to decrease below this pH. The selectivity decrease may be attributed to a competition between acetonitrile and water for hydrogen bonds with the solute. When acetonitrile coordinates with the solute, it is better able to enter and interact with the stationary phase.

A similar rationale can be applied to the aqueous methanol mobile phase, although the shift in retention order is observed at a lower pH compared to the acetonitrile/methanol system. As the aqueous pH decreases from 4.0 to 2.0, the elution order is reversed as the first hydrogen bond (methanol as the donor) weakens and the second one forms (methanol as the acceptor). Because methanol is a strong hydrogen acceptor, it retains some ability to coordinate with the ionized solute and, consequently, engage in selective interactions with the stationary phase at pH 2.0. Selectivity was not investigated below pH 2.0 because of practical considerations.

As calculated titrimetrically, the pK_a of Compound A in 19.1 M aqueous methanol is ~4.5. In both aqueous methanol/acetonitrile and in aqueous acetonitrile, the pK_a changes to ~4.7. The differences between these pK_as is likely a function of the variability of solute pK_a in different solvent compositions. The predicted solute pK_a using the retention vs. aqueous pH data in Fig. 4(A), as described by Horvath et al.,[20] is close to these titrimetric pK_a values. It has been shown that the pK_a of basic compounds is shifted to lower values with increasing organic composition.[21–23]

Effect of Methanol Strength on Selectivity

The effect of methanol concentration on the diastereomeric selectivity at aqueous pH 6.4 and 3.4, in the presence of a constant concentration of acetonitrile, is shown in Fig. 5. At pH 3.4, there is a rectilinear relationship between ln α and the percentage of methanol, which is different from that at pH 6.4.

At pH 3.4, the piperazine is protonated and the compound is an acid. The selectivity is dominated by an equilibrium between the solute and alcohol modifier, and subsequent equilibria between the solute–alcohol complex and the stationary phase. The selectivity behavior follows the linear solvent strength model as the proportion of water in the mobile phase decreases.

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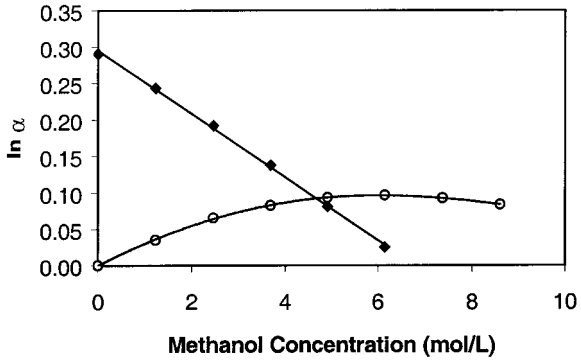

Figure 5. Effect of methanol concentration on selectivity at (◆) pH 3.4 and (○) pH 6.4. Other conditions are the same as in Fig. 2.

At pH 6.4, the compound is effectively neutral. One can explain the selectivity variation with methanol concentration by considering competing equilibria. Methanol competes with both water and acetonitrile to solvate the piperazine moiety of the solute. The organically solvated complex has a greater interaction potential with the stationary phase and enhanced selectivity is observed. Beyond a certain methanol concentration (~6 M), however, the selectivity is dictated by the solvent strength model, and the selectivity diminishes.

The observed behavior at both of these pHs is consistent with our contention that interactions between compound A and hydrogen bonding constituents of the mobile phase fosters chromatographic selectivity. The strength and location of these bonds is dictated by the pH environment.

Applications

A typical separation of two samples is shown in Fig. 6. In the analysis of drug substance, the major component and all individual impurities are of interest; this requires high resolution and often long analysis times. Figure 6(A) is a chromatogram of a degraded sample where ~50 impurities were baseline separated within 35–40 min. Often, when monitoring a single reaction during drug substance synthesis, only the major component and perhaps one or two reaction mixture components are of interest. It was shown [Fig. 6(B)], that with careful selection of the mobile phase (combined with small particles and a short column) only 2 min are required to separate the piperazine diastereomers of Compound A.

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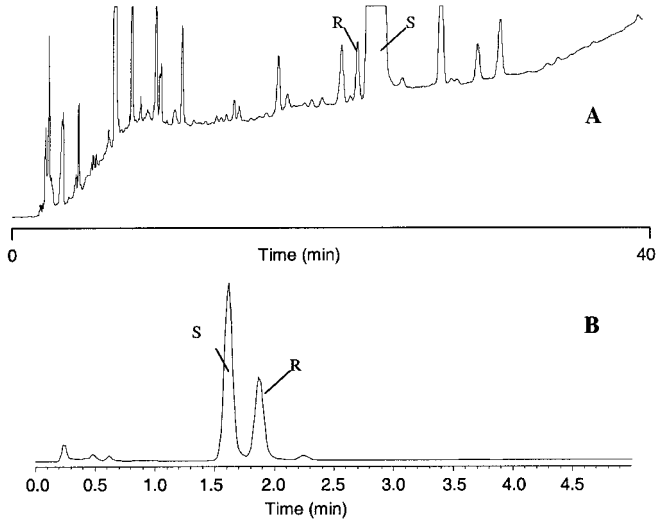

Figure 6. Chromatograms of two typical samples containing piperazine diastereomers. (A) Impurity profile of a degraded sample; (B) fast separation of diastereomers. Conditions: (A) the following gradient was used:

Time (min)	pH 6.4 buffer (2 mM K_2HPO_4)[a]	Acetonitrile[a]	Methanol[a]
0	65	20	15
5	45	40	15
30	35	50	15
40	20	65	15

[a]Volume percent. Other conditions are the same as in Fig. 2. (B) 50 × 4.6 mm YMC Pro C_{18} column packed with 3 μm porous (120 Å, C_{18}) particles, 40°C; flow rate: 3.5 mL/min; 35% (v/v) acetonitrile, 15% (v/v) methanol, 50% (v/v) pH 3.5 buffer, 5 μL injection.

CONCLUSIONS

The ability of the mobile phase to coordinate with the piperazine diastereomers of Compound A through solvation and/or hydrogen bonding is instrumental to the selectivity. The diastereomers, thus coordinated with the solvent, then interact with the C_{18} stationary phase selectively. It appears that the hydrophobicity afforded by the octadecyl silane is important to the separation. The aqueous pH of the mobile phase affects the ionic state of

the diastereomers and, thus, affects its ability to hydrogen bond as a hydrogen donor or hydrogen acceptor.

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