EXPERIMENTAL VERIFICATION AND THERMODYNAMIC STUDY OF THE REVERSAL OF ELUTION ORDER IN CHROMATOGRAPHY

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A Thesis Submitted to the
University of North Carolina Wilmington in Partial Fulfillment
Of the Requirements for the Degree of
Master of Science

Department of Chemistry and Biochemistry

University of North Carolina Wilmington

2007

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ABSTRACT

The demand for high purity compounds in the pharmaceutical industry has led to a greater interest in purification techniques. Preparative chromatography has become a useful purification technique due to its broad range of applications. Preparative scale separations employ a high concentration of analytes which can affect both peak shape and separation of the components. Peak shape is dependant upon the adsorption isotherm of the component, which is linear at the analytical scale and nonlinear at the preparative scale. Knowledge of the adsorption characteristics of the components over a wide concentration range could lead to more efficient separation methods.

Due to the peak shape of components in nonlinear chromatography, reversal of elution order of the major and minor peaks is preferred in some cases. In this research, a chiral compound was studied to determine if a reversal of elution order could be observed by changing the chromatographic conditions. A non-chiral compound, diclofenac, and one of its process impurities, 2-chloro-N-(2,6-dichlorophenyl)-N-phenylacetamide (hereinafter referred to as "2-chloro"), was also studied to see if a reversal in elution order could be observed by changing the chromatographic conditions. The adsorption isotherms for this diclofenac and 2-chloro were measured and fitted to isotherm models. A possible mechanism to explain the retention behavior for these compounds is also discussed.

ACKNOWLEDGMENTS

I would like to extend a most sincere thank you to Dr. Dean Shirazi for his guidance, enthusiasm, and encouragement throughout this research. His knowledge of chromatography and thermodynamics was a tremendous asset to this project and I was privileged to have worked with him. I would also like to thank Dr. John Tyrell for his guidance and enthusiasm throughout the Master's program and this project. I would also like to express my gratitude to Dr. Ned Martin whose opinions I value and respect. I would like to acknowledge Caroline Pearson who processed results in SAS so quickly. Your assistance was invaluable and contributed to the success of my research project. I am grateful to AAIPharma for the support and encouragement extended to me. Thank you for this tremendous opportunity. I would like to extend special thanks to my parents, my grandmother, and my brothers for their constant love and support. Your encouragement has meant the world to me.

DEDICATION

I would like to dedicate this thesis to my husband, Jason, who has always believed in me; and to my son, Ryne, who brings constant joy to my life.

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INTRODUCTION

The separation of impurities from the active pharmaceutical ingredient (API) of a drug product is an essential step in the development of new pharmaceutical product lines. In the formulation of a drug product, it is important to study its process and degradation impurities as some may have toxicological side effects. The FDA requires all impurities that may be present in a drug product to be studied for harmful side effects. Many of the drugs produced today are chiral molecules and the pharmacological properties of the enantiomers may differ significantly from each other. Even in racemic mixtures, the enantiomer of the API is considered to be an impurity by the FDA. Limits on the level of these impurities are set and monitored to establish both safety of the drug substance and the commercial shelf life of the drug product. High purity components are therefore necessary in the manufacture of drug products. In order to monitor the levels of impurities in drug substances and drug products, analytical methods are needed to effectively separate the components of the drug product/related substance mixture. In the pharmaceutical industry, the separation method preferred for the quantitation of impurities is chromatography. This is due to the versatility, ease of use, and reproducibility of chromatographic methods as well as the commercial availability of a wide variety of stationary phases (Wang, 2003).

In this research, compounds were separated using high performance liquid chromatography (HPLC) with varying chromatographic parameters. These parameters were changed in an attempt to reverse the order of elution of the analytes. The equilibrium isotherms of the analytes were measured and isotherm models were used in order to determine a possible mechanism to explain the differences in retention behavior.

Separation by chromatography is a complex phenomenon, resulting from the combined effects of several factors. Fluid dynamics, the kinetics of mass transfer, and equilibrium thermodynamics all play important roles in the separation of compounds. Although the mobile and stationary phases of a chromatographic system can never truly be in equilibrium, in most cases it can be assumed that the two phases are close to equilibrium. The relationship between local concentrations of a compound in the mobile and stationary phases at equilibrium is given by its equilibrium isotherm (equation 1),

$$qs_i = f(c_1, c_2, \ldots, c_i) \quad (1)$$

where qsi is the concentration of the component in the stationary phase and $c_1, c_2, \ldots, c_i$ are the concentrations of component 1, 2, …, i in the mobile phase. For the single component, equation 1 is reduced to equation 2.

$$qs = f(c) \quad (2)$$

Studying the relationship of the components at equilibrium provides important information about the mechanisms of retention for the phase system (Lindholm, 2005). Information regarding the adsorption characteristics of the system over a wide range of concentrations can lead to more efficient separation methods.

A growing need for high purity compounds in the pharmaceutical industry has led to greater interest in preparative chromatographic separations. Preparative chromatography employs large volumes (volume overload) or high concentration (concentration overload) of the API/impurity or racemic mixture. There are two areas of preparative chromatography, semi-preparative and large scale. In semi-preparative scale chromatography, a small amount of pure minor compound is needed for characterization, structural identification, toxicological or pharmacological data, or for use as a standard. In large scale preparative chromatography a large

amount of the purified compound is isolated and used as the final product. Purifications and production of enantiomers, peptides and proteins are examples of large scale chromatography (Guiochon, 2006).

In preparative chromatography, unlike analytical chromatography, the adsorption isotherms are no longer linear due to the high concentration of solutes. The shape of a chromatographic peak in preparative chromatography depends on the adsorption isotherm of the component. A convex upward isotherm occurs when the capacity of the stationary phase to bind the sample component becomes limited. This results in peaks appearing with a sharp front and a broad tail (Lucy, 1991). When the isotherm is convex upward, the impurity eluting before the active results in a sharpening of its peak, which is a result of the displacement effect. This results in better separation of the compounds. When an impurity elutes after the active, its peak will be broadened and separation will be deteriorated, as a result of the tag-along effect. This occurs as a result of the competition of solutes for access to the stationary phase. Therefore, in the case of convex upward isotherms it is preferable for the impurity to elute before the active. Observed less frequently than the convex upward isotherm, a convex downward isotherm occurs when the capacity of the mobile phase to bind the sample component becomes limited. This results in peaks appearing with a broad front and a sharp rear boundary (Lucy, 1991). In this case, it is preferable for the impurity to elute second. In some cases when an analytical separation is scaled up to preparative scale, better separation could be achieved if the elution order of the analytes are reversed. Reversal of elution order, although rare, could be observed during separation in HPLC.

Adsorption Isotherm Measurement

The adsorption isotherm is needed for the design and optimization of preparative chromatography. It also could explain chromatographic behavior such as peak shape and reversal of elution order. There are several methods used to measure the adsorption isotherm of a single component. Non-chromatographic methods such as the Static method (Guiochon, 2006) can be used to determine the isotherm, however chromatographic methods are more accurate. Commonly used chromatographic methods include frontal analysis by characteristic point, elution by characteristic point, the retention time method, and frontal analysis (Guiochon, 2006).

The static method uses a closed vessel containing a known concentration of the compound being tested. A known amount of adsorbent is added to the vessel and the system is allowed to reach equilibrium. The solution is analyzed after equilibrium is reached, and the amount of the compound adsorbed is calculated using mass balance. A known amount of compound is added to the system and equilibrium is re-established. This solution is then analyzed as before, and the amount adsorbed is calculated. This process is repeated until adsorbent saturation is reached. A plot of the amount of compound adsorbed (q) versus concentration (c) yields the adsorption isotherm. The static method is a less accurate way to measure the adsorption isotherm compared to chromatographic methods. It also requires a significant amount of time, materials, and reagents (Guiochon, 2006).

Frontal analysis by characteristic point (FACP) is a less commonly used chromatographic method for determining adsorption isotherms. This method uses the rear boundary of the elution profile of a steep front to determine q and c. A large concentration step is pumped onto the column. After the column has equilibrated, the pump is switched so that pure mobile phase is being pumped through the column. FACP can also be calculated using the rear boundary

obtained during Frontal Analysis experiment. The adsorption isotherm is determined from the points on the rear profile using equation 3, where V_a is the volume of adsorbent in the column, V_0 is the void volume of the column, F is the phase ratio ($F = V_a/V_0$), and V is the retention volume of the diffuse profile at concentration c.

$$q = \frac{1}{V_a}\int_0^c (V - V_0)dc = \frac{V_0}{V_a}\int_0^c \left(\frac{t_r - t_0}{t_0}\right)dc = \frac{1}{F}\int_0^c \frac{t_r - t_0}{t_r}dc \qquad (3)$$

The area under the curve is determined from a point in the diffuse profile to the tail end, where concentration is equal to zero (Guiochon, 2006). This equation is derived assuming the column has infinite efficiency (infinite number of theoretical plates). The main disadvantage of this method is the high number of theoretical plates needed to obtain precise data. Low column efficiency introduces error to the isotherm data at low concentrations. Since the area is calculated from zero concentration of solute, data points at low concentrations cannot be disregarded and thus error is inherent in the isotherm.

The elution by characteristic point (ECP) method of isotherm determination is closely related to the FACP method. In ECP analysis, the isotherm is determined from the rear boundary of an overloaded band profile, instead of the rear boundary of a step front (FACP). Equation 3 is used to calculate the isotherm from data collected during ECP analysis (Guiochon, 2006). Isotherms measured by ECP are subject to the same limitations as those measured by FACP.

If a system follows the Langmuir model, the retention time method can be used to measure the adsorption isotherm. The elution profile of a compound with a Langmuirian isotherm exhibits a sharp front and its retention time depends only on the loading factor, a ratio of the sample size to the column saturation capacity. Using the retention time method, the adsorption isotherm is determined by one injection of a small amount of the compound and one injection of a large amount of the compound. Langmuir adsorption isotherms are linear at low

concentrations. When a small amount is injected, the retention time of the peak is obtained under linear conditions. This retention time is proportional to the initial slope of the isotherm. The retention time of the injection of the large amount is used to calculate the loading factor, which is then used to determine the column saturation capacity (Guiochon, 2006). This method is useful for a fast determination of a Langmuir isotherm using a small amount of material, but it cannot be applied to the determination of any other type of isotherm.

Frontal analysis (FA) is the most widely used chromatographic method for measuring adsorption isotherms. The column (adsorbent) is allowed to equilibrate with pure mobile phase. A constant concentration of the compound is then introduced onto the column, until the front is formed and the breakthrough curve is stabilized. This analysis results in a breakthrough curve with a front that is self-sharpening (Golshan-Shirazi, 1988). The retention time of the breakthrough curve is used to calculate the amount of the compound adsorbed q at concentration c using equation 4, where t_r is the retention time of the breakthrough curve (corrected for column hold-up time), t_0 is the void time of the column, and F is the phase ratio. The phase ratio defined by equation 5, where ε is the porosity of the column.

$$\frac{\Delta q}{\Delta c} = \frac{V_r - V_0}{V_a} = \frac{V_0}{V_a} \times \frac{t_r - t_0}{t_0} = \frac{1}{F} \times \frac{t_r - t_0}{t_0} \tag{4}$$

$$F = \frac{V_a}{V_0} = \frac{1-\varepsilon}{\varepsilon} \tag{5}$$

The concentration of the compound is then increased in a stepwise manner, allowing new front formation and a new breakthrough curve at each step. The plot of the concentration of analyte in the stationary phase versus the concentration of analyte in the mobile phase gives the adsorption isotherm. Since the retention time of the front is independent of column efficiency (number of theoretical plates), the major advantage of Frontal Analysis is that the isotherm measurement can

be made using any column (Guiochon, 2006). Unlike FACP and ECP where column efficiency is assumed to be infinite, isotherm measurement is independent of column efficiency in FA. Therefore, FA does not incur the same error as FACP and ECP. Frontal analysis is the most accurate chromatographic method for the determination adsorption isotherms and was used to determine the adsorption isotherms in this project.

The data obtained from any of these methods can be fitted to an isotherm model in order to study the factors influencing the separation such as the column saturation capacity, solute-solute interactions, and the adsorption/desorption rate constant.

Isotherm Models

The most common equilibrium isotherm model is the Langmuir isotherm (equation 6) where q is the adsorbed amount of solute in the stationary phase in equilibrium with the concentration of solute in the mobile phase.

$$\frac{q}{q_S} = \frac{bc}{1+bc} \tag{6}$$

This equation is derived assuming the solution is ideal, the solute gives monolayer coverage, there are no solute-solute interactions in the monolayer, there are no solvent-solute interactions, and the adsorbed layer is ideal. Although most of these assumptions may not be completely valid in liquid-solid adsorptions, experimental data show that the Langmuir isotherm is an excellent approximation for single component adsorption equilibriums in liquid-solid chromatography. Numerous examples can be found in scientific literature illustrating the validity of this model for accounting the equilibrium isotherm measurement (Guiochon, 2006).

The competitive Langmuir isotherm does not satisfy the Gibbs-Duhem equation if the column saturation capacities are different for the solutes (Kemball, 1948). Therefore, the

competitive Langmuir isotherm is thermodynamically valid only when the column saturation capacities of all the solutes are the same. As a result, as long as the Langmuir isotherm remains valid for the two solutes, it is impossible for the isotherms of the two solutes to cross. Therefore, it is impossible to see reversal of elution order of the two solutes. Since most of solutes in chromatography follow Langmuir-like isotherms, reversal of elution order is seldom. When a reversal of elution order takes place, it is certain that the adsorption isotherm for at least one solute does not follow Langmuir isotherm model.

The Moreau isotherm model accounts for adsorption behavior on a homogenous surface with interactions between molecules of the adsorbate (equation 7). This model assumes that the surface is homogenous, surface coverage is limited to the monolayer, and the adsorbate-adsorbate interactions are limited to interactions between adjacent molecules,

$$q = q_s \times \left(\frac{bc + Ib^2c^2}{1 + 2bc + Ib^2c^2} \right) \tag{7}$$

where $q_{s,}$ is the monolayer saturation capacity, b is the equilibrium constant at low concentrations, and I is the adsorbate-adsorbate interaction parameter (Gritti, 2003). The adsorbate-adsorbate interaction parameter is dependant on the interaction energy between two adsorbed molecules of A $(\in_{AA})$ and can be written as equation 8,

$$I = \exp\left(\frac{\in_{AA}}{RT} \right) \tag{8}$$

where R is the universal gas constant and T is the absolute temperature (Guiochon, 2006).

Other models of adsorption isotherm behavior are less commonly seen in liquid-solid chromatography than Langmuir-like isotherms. S-shaped isotherms occur frequently in gas-solid chromatography and have an initial concave upward shape which changes as concentration increases. S-shaped isotherms are described by equation 9.

$$q = qs \times \frac{b_1 c + 2b_2 c^2}{1 + b_1 c + b_2 c^2} \tag{9}$$

The BET adsorption isotherm model is a special case of S-shaped isotherms used most often in gas-solid chromatography. This model accounts for second and subsequent layers of adsorbate forming at pressures below that required for formation of the first layer. It assumes that each molecule adsorbed in the first layer provides a site for adsorption of a molecule in the second layer. Molecules in the second layer provide adsorption sites for molecules in the third layer, and so on. Equation 10 describes the BET model adapted for liquid-solid chromatography, where b_S is the adsorption-desorption equilibrium constant for the first layer and b_L is the adsorption-desorption equilibrium constant for the second and subsequent layers (Gritti, 2002).

$$q = \frac{q_S b_S c}{(1 - b_L c)(1 - b_L c + b_S c)} = \frac{q_S b c}{1 + (b_S - 2b_L)c + (b_L^2 - b_L b_S)c^2} \tag{10}$$

The BET equation is a special case of a more general empirical isotherm (equation 11), where B and D are constants.

$$q = \frac{ac}{1 + Bc + Dc^2} \tag{11}$$

Factors Influencing Separation

Several factors have been shown to influence the order of separation of compounds. These include changing the stationary phase (Wang, 1999), changing the temperature (Guillaume, 1995), altering the sample amount (Roussel, 1989), changing the pH of the mobile phase (Wu, 2003), and changing of composition of the mobile phase by varying the ratio of solvents (Schlauch, 2001) or replacing the type of organic modifier (Aboul-Enein, 2001). Several models have been proposed to explain this behavior in different systems. For example, N. Wu and associates found that changes in the mobile phase pH resulted in the reversal of

elution order of the piperazine diastereomers of an HIV protease inhibitor (Wu, 2003). Through temperature studies they were able to determine that the reversal in elution order was most likely due to the change in the ionization of the compound and not due to a change in the stationary phase. The change in ionization causes the compound to alter its hydrogen bonding with the stationary phase and the organic modifier. This causes the enantiomers to reverse in elution order at altered pH (Wu, 2003).

Separation mechanisms vary among the different types of chiral stationary phases. The retention factors of enantiomers can change from one column to another, and can reverse the elution order. Wang and coworkers studied the elution order of several enantiomer pairs on two types of chiral stationary phases: Chiralpak AD (amylose tris(3,5-dimethylphenylcarbamate)) and Chiralcel OD (cellulose tris(3,5-dimethylphenylcarbamate)). Reversal of elution order of all enantiomer pairs was observed when the chiral stationary phase was changed from the amylose-based Chiralpak AD to the cellulose-based Chiralcel OD. Because these columns have the same derivatization group, it was concluded that the retention of solutes is dependent upon the conformation of the stationary phase as well as the derivatization groups interacting with the components (Wang, 2003).

Thermodynamic properties are estimated by altering chromatographic conditions and observing the effects on the separation of the solutes. Retention factors are dependent on temperature and can be calculated using equation 12,

$$k_0' = \frac{t_r - t_0}{t_0} \tag{12}$$

where t_r is the retention time of the compound and t_0 is the column void time. The separation factor is calculated using equation 13,

$$\alpha = \frac{k'_{0,2}}{k'_{0,1}} \tag{13}$$

where $k'_{0,1}$ is the retention factor of the first peak and $k'_{0,2}$ is the retention factor of the second peak. The retention factor depends on temperature according to van't Hoff's equation. Van't Hoff plots are made by plotting the logarithm of the separation factor versus the inverse temperature. Enthalpic and entropic contributions can be estimated from the van't Hoff plots to determine which has more influence on binding and retention (Guillaume, 1995). Change in temperature could affect the retention factor of each compound in opposite directions, resulting in the inverse of the retention order.

Retention factor also depends on the solvent composition. The most widely used relationship is the linear solvent strength theory (LSST), developed by Snyder and Stadalius (Snyder, 1980, 1986). According to the LSST models, the retention factor, k', of a solution is related to the solvent composition by equation 14,

$$\ln k' = \ln k'_w - S\Phi \tag{14}$$

where k'_w is the retention factor of the solute in water, S is the solvent strength parameter and Φ is the volume fraction of the stronger solvent. According to this equation, the retention factor of each solute could be affected by the organic modifier type and composition, which could result in the reversal of elution order. Although band broadening results from both kinetic mass transfer and equilibrium thermodynamics, for most separations the retention time of the peak and the general peak shape are determined exclusively from thermodynamic factors (equilibrium isotherm). The isotherm parameters depend on the composition of mobile phase. Therefore when mobile phase composition is changed, the equilibrium isotherms of each solute will be affected. This could affect both the retention time and peak shape of each solute. Since the dependence of isotherm parameters on the mobile phase composition is different for each solute,

changing the mobile phase could affect the separation. The change could either improve or deteriorate the separation of solutes.

In studies performed by Schlauch and coworkers, the enantiomers of amino acids were separated using a copper (II)-D-penicillamine chiral stationary phase. During these studies, increasing the amount of acetonitrile in the mobile phase resulted in reversal of elution order for some of the enantiomer pairs. This group concluded that in order for reversal of elution order to occur the stationary phase must have at least two binding sites which are affected differently according to the mobile phase. Because the penicillamine molecule has a simple structure, they concluded that the molecule forms bidentate copper(II) complexes which are affected by the acetonitrile concentration. The stability of these complexes in various mobile phase compositions determines the retention order of the enantiomers (Schlauch, 2001).

The type of organic modifier used in the mobile phase can also change the retention order of compounds. In studies performed by Aboul-Enein and Ali, reversal of elution order was observed when changing the organic modifier using a Chiralpak AD (amylose tris(3,5-dimethylphenylcarbamate)) chiral stationary phase. Through their studies they concluded that the cavities on the stationary phase fit one enantiomer, (-), better than the other, (+), giving it more opportunities for hydrogen bonding and π-π interactions. When 2-propanol or 1-butanol were used in the mobile phase, the conformation of the stationary phase was reversed, and the (+) enantiomer fit better into the cavity. Aboul-Enein and Ali hypothesized the 3,5-dimethylphenyl carbamate molecules were initially in an equatorial position on the amylose stationary phase and the conformation was changed to the axial position when the organic modifier was changed (Aboul-Enein, 2001).

Retention factor is also dependent on sample concentration. In most cases, the relative affinity of the two solutes for the stationary phase remains the same by increasing the concentration. In rare cases, however, the adsorption isotherms of two solutes could cross, which results in the reversal of affinity of the solute after the cross point. This has adverse effect on separation in displacement chromatography. This phenomenon has been demonstrated experimentally in displacement chromatography by several authors (Vigh, 1990; Subramanian, 1989). In studies performed by Roussel and coworkers, enantiomers were separated using a microcrystalline triacetylcellulose chiral stationary phase. When the amount of sample injected was increased from an analytical amount (3 mg) to a preparative amount (50 mg) a reversal in elution order was observed. A study of the isotherms of the enantiomers showed the capacity factor (k') increased with increasing sample amount for the (+) enantiomer and decreased with increasing sample amount for the (-) enantiomer. Through studying the effects of the sample amount of similar compounds, Roussel and coworkers concluded the concentration dependence of the capacity factors was due to the presence of a lipophilic propyl group. Chiral recognition was thought to be affected by the amount of steric confinement of the sites (Roussel, 1989).

For this research project, the chiral compound BRX-345 was used in an attempt to find conditions under which a reversal of elution order is observed. At the initial conditions, the enantiomers are well resolved with the major enantiomer eluting first. The chromatographic conditions were altered (including temperature, the pH of buffer solution in the mobile phase, the type of organic modifier in the mobile phase, the concentration of organic in the mobile phase, the amount of solute, and stationary phase) in an attempt to determine conditions at which the retention times of the enantiomers were reversed.

The non-chiral compound diclofenac and one of its process impurities, 2-chloro-N-(2,6-dichlorophenyl)-N-phenylacetamide (hereinafter referred to as "2-chloro"), were also used in an attempt to find conditions under which a reversal of elution order is observed. At the initial conditions, the peaks are well resolved with the process impurity eluting first. The chromatographic conditions were altered (including temperature, the type of organic modifier in the mobile phase, and the concentration of organic in the mobile phase) in an attempt to determine conditions at which the retention times of the peaks were reversed.

Once conditions were determined at which the retention times are reversed, the adsorption isotherms were determined and a possible mechanism for the retention of the compounds is discussed.

EXPERIMENTAL

Equipment

The high performance liquid chromatography (HPLC) equipment used in this research was a Hewlett-Packard 1100, equipped with a gradient pump, temperature controlled column compartment, temperature controlled autosampler, and ultraviolet (UV) variable wavelength detector. The following columns were used for the chiral separation: Chiral AGP, 100 x 4.6 mm 5µm particle size column supplied by ChromTech; Chiral TBB, 250 x 4.6 mm 5µm particle size column supplied by Kromasil; Chiralcel OD, 250 x 4.6 mm 10µm particle size column supplied by Daicel Chemical Industries. The following column was used for the reversed phase separation of Diclofenac and its related substances: XTerra RP C18, 150 x 4.6 mm 5µm particle size column supplied by Waters. All columns were selected because of their availability.

Chemical Information

The following chemicals were used during this research:

BRX-345 active pharmaceutical ingredient

diclofenac potassium active pharmaceutical ingredient

2-chloro-N-(2,6-dichlorophenyl)-N-phenylacetamide (Mikromol, 99.95%)

Phosphate buffer (Potassium Phosphate Monobasic, Mallinckrodt) was prepared at various pH values for the aqueous portion of some mobile phases used. The following organic solvents were used in mobile phase preparations:

2-propanol (Fisher, 99.9%)

methanol (Fisher, HPLC grade)

ethanol (Aaper, 200 proof)

1-propanol (Sigma-Aldrich, 99.5%)

acetonitrile (Mallinckrodt, HPLC grade)

hexane (Mallinckrodt, 99.8%)

MTBE (Fluka, 99%)

Computer Software

Chromatographic data was collected by Waters Millennium acquisition software, version 4.0. The Millennium program was used to measure retention times of compounds tested and the retention times of breakthrough fronts during frontal analysis. Microsoft Excel 2003 was used to calculate q using equations 4-6 and experimental data. Excel was also used to generate plots of q versus c (adsorption isotherms). For equation 11 (BET-like empirical isotherm model) and equation 7 (Moreau isotherm model), a non-linear modeling program in SAS PC version 8.2 was used to determine unknown parameters using data determined experimentally (q and c values).

Procedure

The objective of this research was to find conditions at which a reversal in elution order was observed. Starting conditions for the chiral separation were taken from an AAIPharma test procedure. Alternate column temperatures, pH values, organic modifiers, and chiral sorbants (columns) were used in an attempt to reverse the elution order of the enantiomers of the chiral compound. Starting conditions for the non-chiral separation were chosen based on an AAIPharma test procedure. Alternate column temperatures and organic modifiers were used in an attempt to reverse the elution order. Reversal of elution order was observed in the reversed phase separation of diclofenac and its related compound (non-chiral system).

The adsorption isotherm was measured for each compound at different conditions using frontal analysis. Frontal analysis was performed using the following procedure for both high and low acetonitrile mobile phase conditions. Line A of the gradient pump of the HPLC instrument was connected to a reservoir of mobile phase. Line B of the gradient pump was connected to a solution of the compound (diclofenac or 2-chloro) dissolved in the mobile phase. The column was equilibrated with the pure mobile phase prior to the start of the analysis. Using the gradient program of the HPLC instrument, a constant stream a solutions from lines A and B were pumped through the column at varying concentrations (ratios were not changed until column had re-equilibrated), creating a step chromatogram. The retention times of the breakthrough curves were estimated from this chromatogram. Theoretical models were used to fit experimental data using Microsoft Excel and SAS statistical software.

Column Void Time Determination

The void time, t_0, of each column was required to determine the separation factors at each condition and in isotherm measurement by frontal analysis. The void time of the column is the

length of time required for an unretained component to pass through the column void volume and elute after it is injected onto the column. Column void time can be estimated from the solvent front of the chromatogram or it can be measured by injecting a non-retained material onto the column. For the chiral columns, void time was measured by injecting a solution with a low concentration of sodium nitrate (a non-retained solute) onto the column. For the reversed phase column, void time was measured by injecting a solution containing uracil (a non-retained solute) onto the column. The retention time of the peak was equal to the void time of the column (t_0). See Figures 1 and 2 for example chromatograms of solutions used to determine column void time.

System Delay Time Determination

In order to accurately determine the retention time of the breakthrough curve during frontal analysis, the system delay time, τ, was required. This time represented the amount of time required for the compound to travel through the HPLC system to the beginning of the column. The system delay time was measured by replacing the column with a zero-volume union and pumping pure methanol through the detector. At the start time of the determination, the pump was switched to a mixture of acetone and methanol so that a breakthrough curve was produced (see Figure 3). The retention time of the breakthrough front was used to determine the system delay time.

Porosity and Phase Ratio Determination

The porosity and phase ratio of the column are required in order to calculate the amount of compound adsorbed by the stationary phase given the concentration in the mobile phase. The porosity is defined as the ratio of the volume of the column not occupied by the stationary phase (*Vvoid*) to the total volume of the column (*Vcolumn*), shown in equation 15.

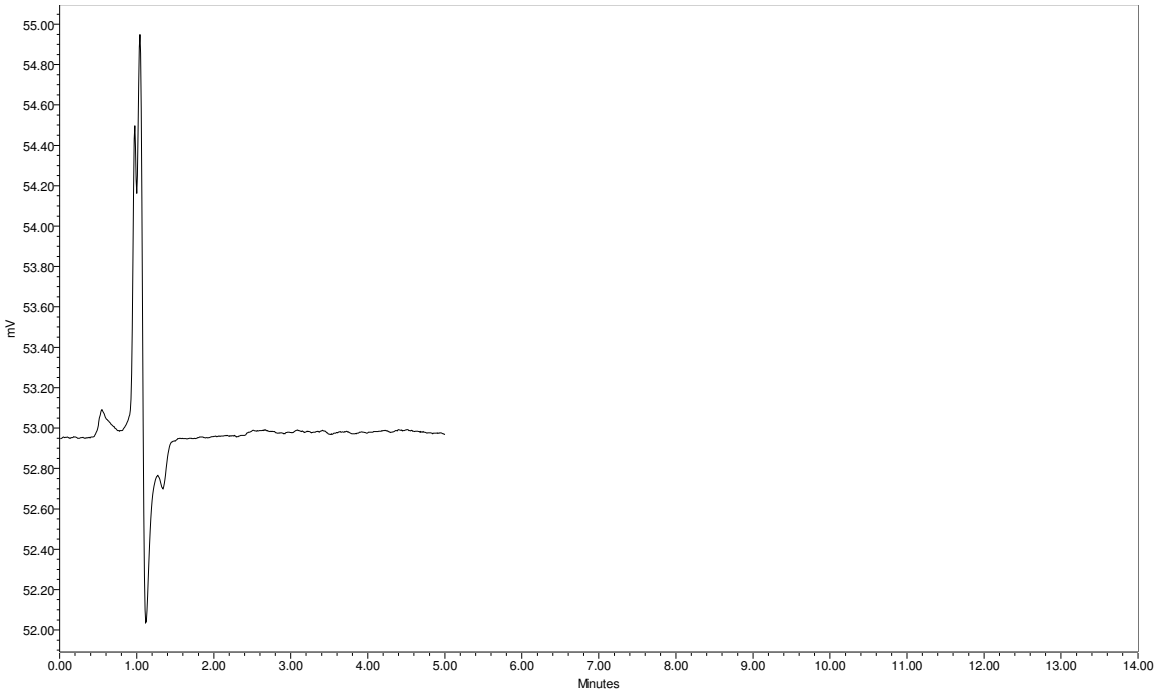


Figure 1. Example chromatogram of sodium nitrate used to determine column void time

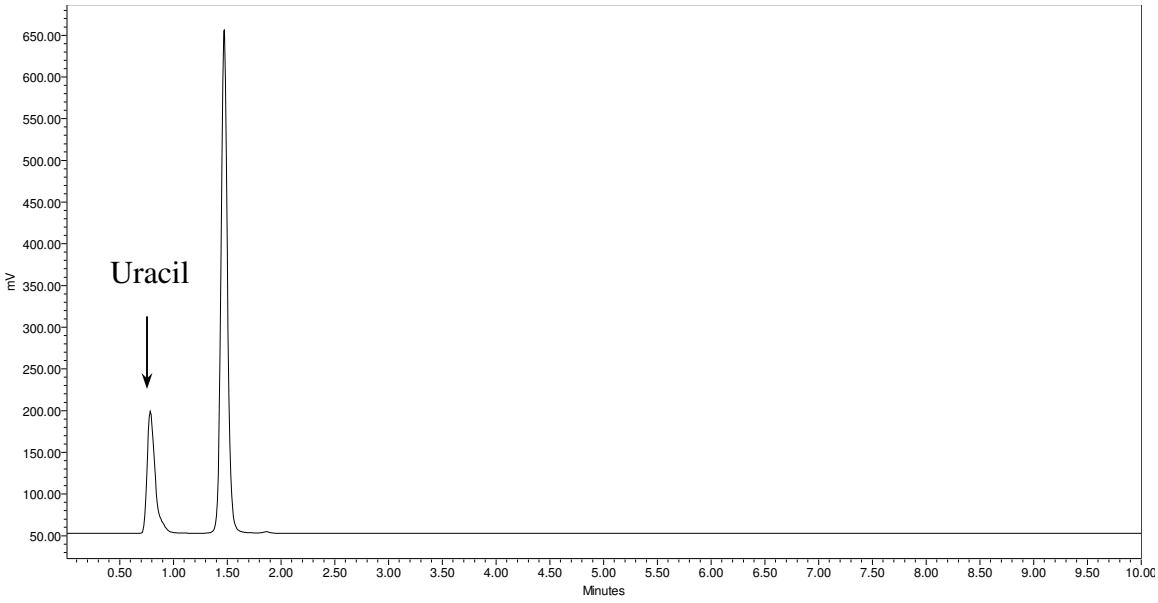


Figure 2. Example chromatogram of uracil used to determine column void time

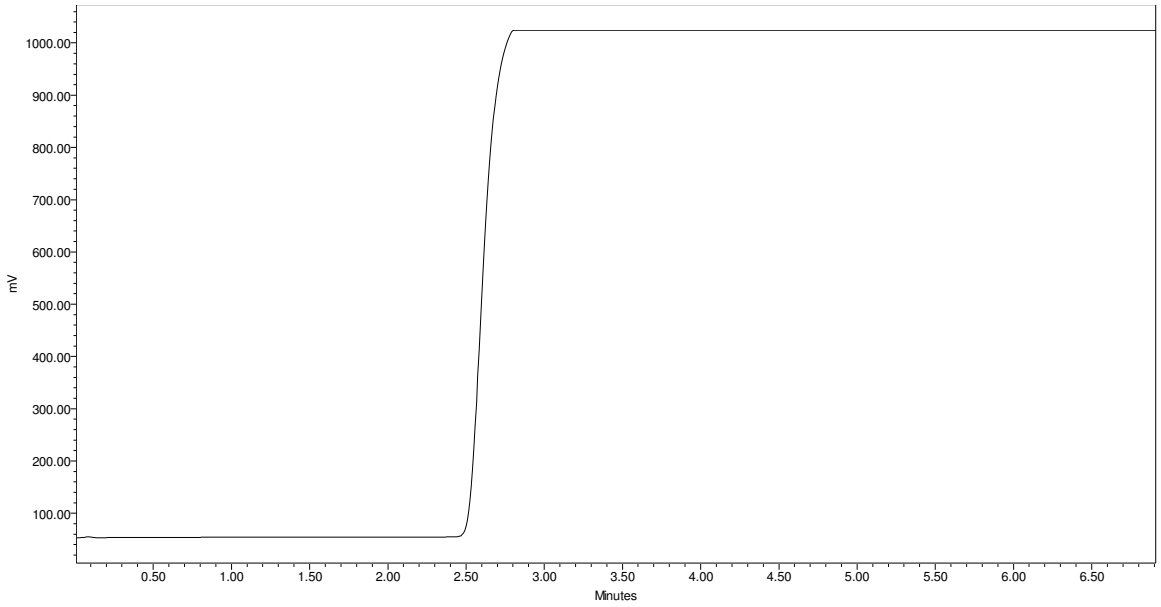


Figure 3. Chromatogram of breakthrough curve used to determine system void time

$$\varepsilon = \frac{Vvoid}{Vcolumn} \tag{15}$$

The volume of the column not occupied by the stationary phase can be calculated using equation 16, where t_0 is the void time of the column.

$$Vvoid = t_0 \times flow\ rate \tag{16}$$

The total volume of the column can be calculated from the column dimensions using equation 17, where D is the diameter of the column and L is the length of the column.

$$Vcolumn = \frac{\pi \times D \times 2L}{4} \tag{17}$$

The phase ratio (F) is calculated using the value obtained for porosity (ε) according to equation 5.

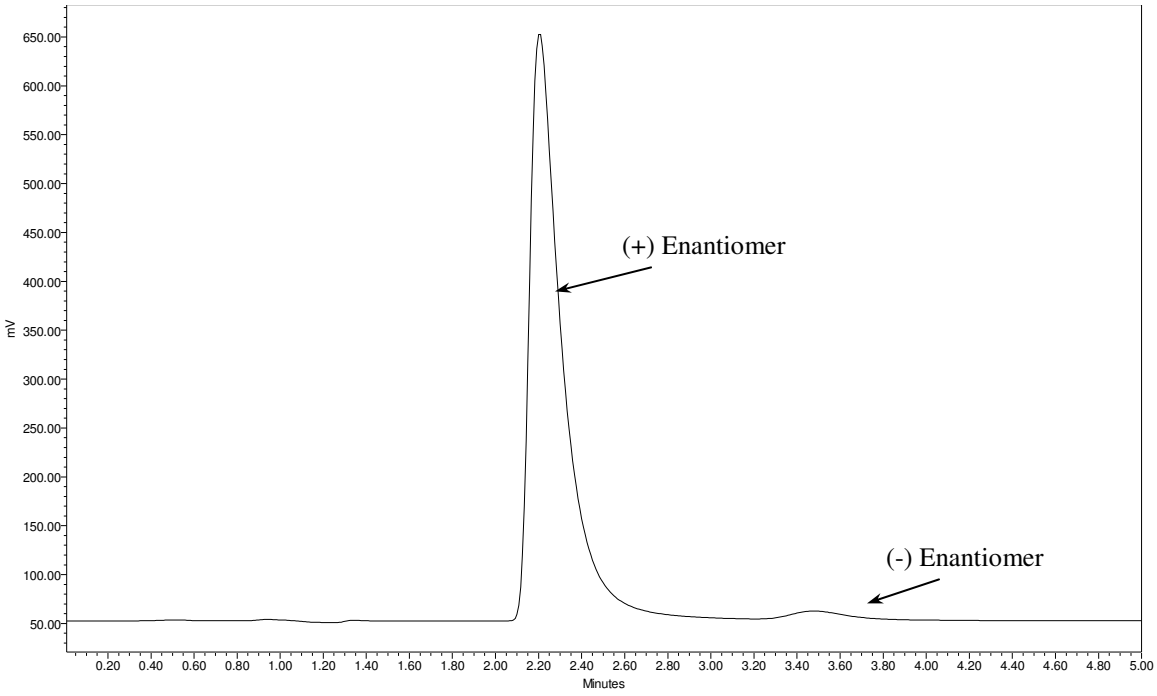
RESULTS AND DISCUSSION – CHIRAL SYSTEM

Effects of Altered Chromatographic Conditions

Initial conditions for the separation of the enantiomeric mixture of BRX-345 were the following:

Column: Chiral-AGP, 100 mm by 4.0 mm, 5 µm particle size
Column Temperature: 25°C
Injection Volume: 5 µL
Flow Rate: 1.0 mL/min
Mobile Phase: 50mM phosphate buffer, pH 7.0:2-propanol (98:2)
Detection: UV at 260 nm

At the initial conditions, the major isomer eluted first and the peak shape of the major isomer exhibited high tailing (see Figure 4). Therefore, better separation would be achieved if the minor isomer eluted first. Attempts were made to reverse the elution order of BRX-345(+) and its enantiomer by changing the chromatographic conditions.



Column: Chiral-AGP, 100 mm by 4.0 mm, 5 µm particle size
Column Temperature: 25°C
Flow Rate: 1.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 7.0, 2-propanol (98:2)
Detection: UV at 260 nm

Figure 4. Chromatogram of breakthrough curve used to determine system void time

Results of these studies are discussed below. Reversal of elution order of the enantiomers was not achieved.

Effect of Stationary Phase

Separation of enantiomers using chiral stationary phases is achieved through various retention mechanisms specific to the type of stationary phase. For the chiral separation in this research, a Chiral α-1 glycoprotein (AGP) column was used. AGP retains compounds through a combination of hydrogen bonding and hydrophobic interactions, although the exact selection mechanism is unknown. In order for the compound to bind to the AGP, at least one binding group must be present near the chiral center. How close the binding group is to the chiral center plays a role in the degree of selectivity for the compound. Some of the structures that act as binding groups are alcohols, amides, and carboxylic acid groups. Also, the compound must contain a ring structure near the chiral center in order for the enantiomers to be resolved and resolution is better for the hydrogen bonding groups to be near the chiral center (Narayanan, 1992). The results of injections at various chromatographic conditions using the Chiral AGP column are shown in Table 1. Reversal of elution order was not achieved using the Chiral AGP column. Other types of chiral stationary phases have different separation mechanisms that may or may not result in the separation of the enantiomers studied. In this research, two alternate chiral stationary phases were used: a polymeric-type CSP (Kromasil CHI-TBB) and a polysaccharide CSP (Chiralcel OD).

The Kromasil CHI-TBB stationary phase consists of O,O'-bis (4-tert-butylbenzoyl)-N,N'-diallyl-L-tartar diamide monomers covalently bonded to a silica support to form a network polymer with a bifunctional C2-symmetric chiral selector.

Type, Percent Organic in Mobile Phase	pH of Phosphate Buffer	Column Temp (°C)	Sample Amount (µg)	Elution Order	Separation Factor (α)
2-propanol, 2%	7	25	2.5	(+),(-)	2.07
2-propanol, 2%	7	5	2.5	(+),(-)	2.04
2-propanol, 1%	7	25	2.5	(+),(-)	3.31
2-propanol, 3%	7	25	2.5	(+),(-)	1.62
2-propanol, 2%	3	25	2.5	--	--
2-propanol, 10%	7	25	0.5, 2.5, 12.5, 25	--	--
2-propanol, 15%	7	25	0.5, 2.5, 12.5, 25	--	--
methanol, 0.5%	7	25	2.5	--	--
methanol, 2%	7	25	2.5	--	--
methanol, 10%	7	25	2.5	(+), (-)	3.66
methanol,15%	7	25	2.5	(+), (-)	2.69
methanol, 20%	7	25	2.5	(+), (-)	2.13
methanol, 20%	7	5	2.5	(+), (-)	2.17
methanol, 20%	7	45	2.5	(+), (-)	1.91
ethanol, 0.5%	7	25	2.5	(+), (-)	6.10
ethanol, 2%	7	25	2.5	(+), (-)	3.51
ethanol, 10%	7	25	2.5	(+), (-)	1.28
ethanol, 15%	7	25	2.5	--	--
ethanol, 20%	7	25	2.5	--	--
ethanol, 20%	7	5	2.5	--	--
ethanol, 20%	7	45	2.5	--	--
acetonitrile, 0.5%	7	25	2.5	(+), (-)	6.43
acetonitrile, 2%	7	25	2.5	(+), (-)	3.50
acetonitrile, 10%	7	25	2.5	--	--
acetonitrile, 15%	7	5, 25, 45	2.5	--	--
acetonitrile, 20%	7	5, 25, 45	2.5	--	--
1-propanol, 0.5%	7	25	2.5	(+), (-)	3.69
1-propanol, 2%	7	25	2.5	(+), (-)	1.50
1-propanol, 10%	7	25	2.5	--	--
1-propanol, 15%	7	5, 25, 45	2.5	--	--
1-propanol, 15%	7	25	0.25, 6.25	--	--
1-propanol, 20%	7	5, 25, 45	2.5	--	--
1-propanol, 20%	7	25	0.25, 6.25	--	--

Table 1. Separation of enantiomers at various chromatographic conditions – Chiral AGP column

Kromasil chiral columns are stable in all organic solvents due to the nature of the bonding of the network polymer to the silica support. The enantioselectivity of the Kromasil CHI-TBB is due mainly to hydrogen bonding of the isomer to the stationary phase. This hydrogen bonding is influenced by the type of organic modifier, with esters and ethers yielding higher enantioselectivity than alcohols and ketones. Steric interactions and π-π interactions between the isomers and the stationary phase also affect the enantioselectivity of the Kromasil CHI-TBB column (Kromasil). The results of injections at various chromatographic conditions using the Kromasil CHI-TBB CSP are shown in Table 2; separation of enantiomers was not achieved using this column.

The most widely used polysaccharide chiral stationary phases are derivatized cellulose and amylose. These columns have been shown to separate a wide variety of chiral compounds. The Chiralcel OD column contains cellulose tris (3,5-dimethyl-phenylcarbamate) monomers. These monomers are bound with carbamate linkages between the side chain and polysaccharide backbone. Compounds interact with the stationary phase through hydrogen bonding, pi-pi interactions, dipole interactions, and induced dipole interactions. Steric constraints yield enantioselectivity due to the helical conformation of the backbone. Coated polysaccharide chiral stationary phases are limited by the solvents that can be used. Solvents such as methylene chloride, chloroform, and DMSO solubilize the polysaccharide polymer and can be harmful to the column even in very small amounts. Normal phase, polar organic, and reversed phase mobile phases may be used with this type of chiral stationary phase (Chiral Technologies, 2006). The results of injections at various chromatographic conditions using the Chiralcel OD CSP are

Type, Percent Organic Modifier in Mobile Phase	Column Temp (°C)	Sample Amount (µg)	Elution Order	Separation Factor (α)
2-propanol, 8%	25	2.5	--	--
2-propanol, 4%	5	2.5	--	--
2-propanol, 16%	25	2.5	--	--
ethanol, 10%	25	2.5	--	--
ethanol, 15%	25	2.5	--	--
ethanol, 20%	25	2.5	--	--
ethanol, 7%	25	2.5	--	--
MTBE, 20%	25	2.5	--	--
MTBE, 30%	5	2.5	--	--
MTBE, 35%	45	2.5	--	--
MTBE, 40%	25	2.5	--	--

Table 2. Separation of enantiomers at various chromatographic conditions – Kromasil CHI-TBB column

shown in Table 3. Separation of enantiomers was not achieved using this column.

Effect of pH

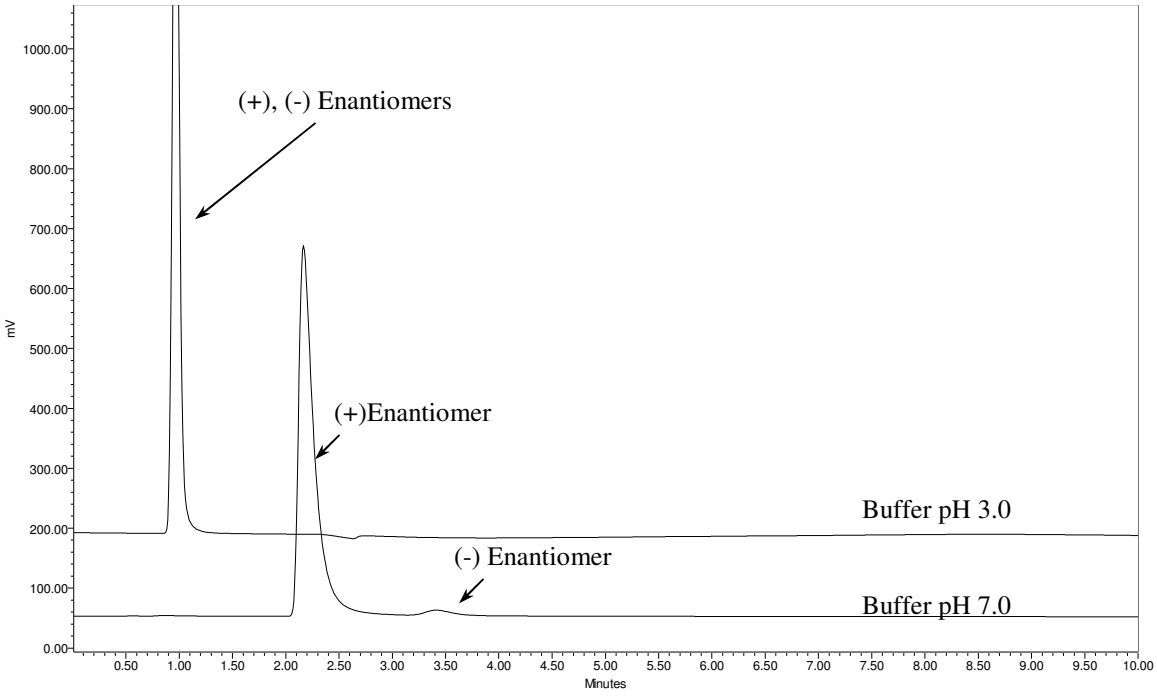
In chiral separations, the pH of the mobile phase can affect the hydrogen binding ability of each enantiomer differently. The effect of pH was evaluated using the Chiral α-1 glycoprotein (AGP) column by altering the pH of the aqueous portion of the mobile phase and injecting a sample solution containing both enantiomers. The nominal pH of the phosphate buffer was 7.0 and the altered pH was 3.0. At the nominal condition, separation was achieved with the major enantiomer eluting first. At the altered condition, neither component was retained by the column. Both enantiomers eluted in the solvent front and separation was not achieved. See Figure 5 for example chromatograms of the separation achieved at buffer pH 7 and buffer pH 3. Due to the incompatibility of the Chiral AGP column with high pH mobile phases, the pH of the phosphate buffer was not increased. The other columns used in this research were normal phase columns, with no aqueous portion in the mobile phase. Therefore, the pH of the mobile phases used with the Kromasil CHI-TBB and Chiralcel OD columns were not altered.

Effect of Temperature

The effect of temperature was evaluated at various mobile phase compositions using the Chiral AGP column, the Kromasil CHI-TBB column, and the Chiralcel OD column. Separation was only achieved using the Chiral AGP column, the peaks coeluted on the Kromasil CHI-TBB and Chiralcel OD columns. In cases where separation was achieved (for example, samples injected using a mobile phase of pH 7 phosphate buffer and methanol (80:20) on the Chiral AGP column), neither increasing nor decreasing the column temperature resulted in a reversal of elution order.

Type, Percent Organic Modifier in Mobile Phase	Column Temp (°C)	Sample Amount (µg)	Elution Order	Separation Factor (α)
isopropanol, 1%	25	2.5	--	--
isopropanol, 5%	25	2.5	--	--
isopropanol, 7%	25	2.5	--	--
isopropanol, 10%	5, 25, 45	2.5	--	--
isopropanol, 10%	25	0.25, 5, 12.5	--	--
isopropanol, 15%	5, 25, 45	2.5	--	--
isopropanol, 15%	25	0.25, 5, 12.5	--	--
isopropanol, 20%	5, 25, 45	2.5	--	--
isopropanol, 20%	25	0.25, 5, 12.5	--	--
ethanol, 2%	25	5	--	--
ethanol, 5%	5, 25, 40	5	--	--
ethanol, 10%	5, 25, 40	5	--	--
ethanol, 15%	5, 25, 40	5	--	--
ethanol, 15%	25	0.25, 5, 12.5	--	--
ethanol, 20%	5, 25, 40	5	--	--
ethanol, 20%	25	0.25, 5, 12.5	--	--
ethanol, 25%	5, 25, 40	5	--	--
ethanol, 25%	25	0.25, 5, 12.5	--	--

Table 3. Separation of enantiomers at various chromatographic conditions – Chiralcel OD column



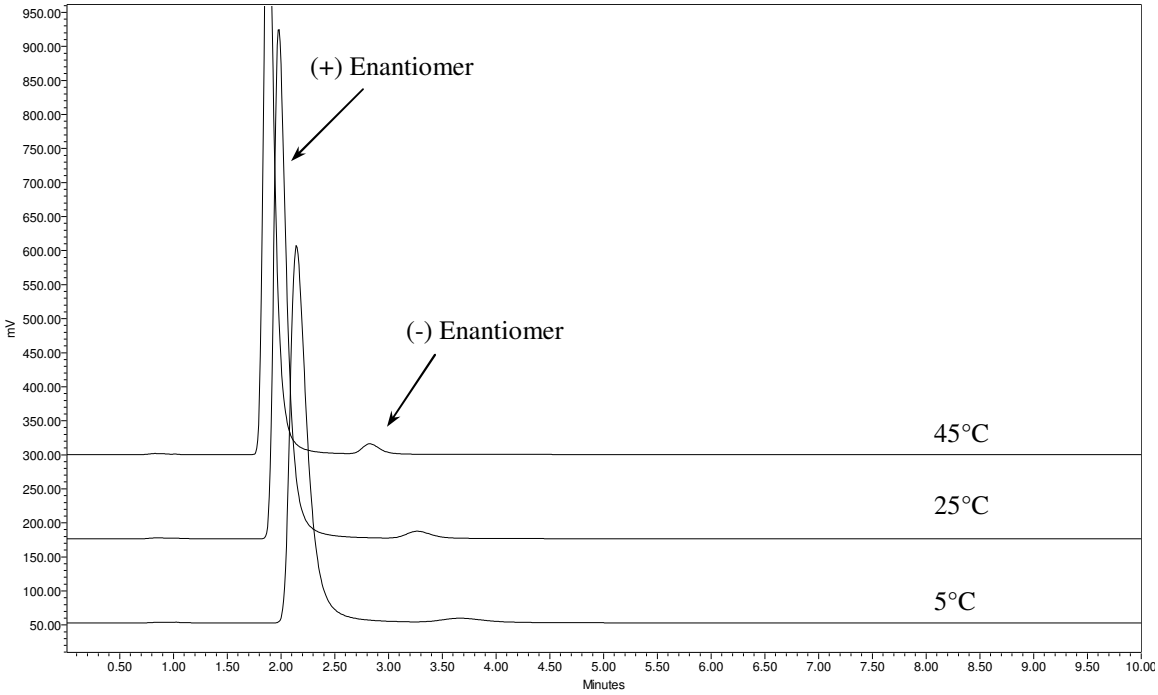
Column: Chiral-AGP, 100 mm by 4.0 mm, 5 µm particle size
Column Temperature: 25°C
Flow Rate: 1.0 mL/min
Mobile Phase: 50mM phosphate buffer (various pH), 2-propanol (98:2)
Detection: UV at 260 nm

Figure 5. Example chromatogram of effect of buffer pH

In cases where the two enantiomers co eluted (for example, samples injected using a mobile phase composed of pH 7 phosphate buffer and 1-propanol (85:15) on the Chiral AGP column), neither increasing nor decreasing the column temperature resulted in separation of enantiomers. Reversal of elution order due to change in column temperature was not achieved for any of the conditions tried. See Figures 6 and 7 for example chromatograms of the separation achieved at various temperatures.

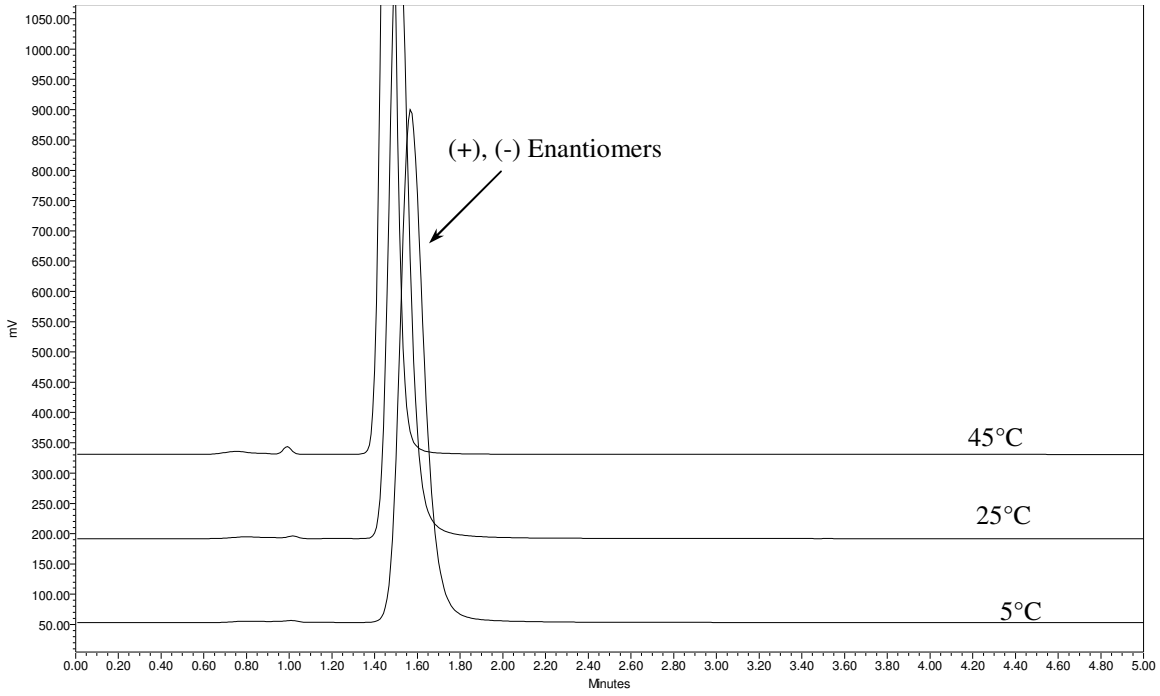
Effect of Mobile Phase Composition – Organic Concentration

The effect of mobile phase composition was evaluated using organic modifiers at varying concentrations. A Chiral AGP, a Kromasil CHI-TBB, and a Chiralcel OD CSP were used to determine the effects of altering mobile phase composition. Starting mobile phase components were selected based on available literature and the concentration of the organic modifier was varied. Tables 1-3 show the results for each concentration of organic modifier used with each column tested. Separation was achieved using the Chiral AGP CSP; however resolution was lost at higher concentrations (≥10%). Reversal of elution order was not achieved using the Chiral AGP CSP. See Figure 8 for example chromatograms of injections using various organic modifier concentrations on the Chiral AGP CSP. Mobile phases with various concentrations of organic modifiers were used to attempt to separate the enantiomers on the Kromasil CHI-TBB CSP. Separation was not achieved at any organic concentration using this column. Mobile phases with various concentrations of organic modifiers were used to attempt to separate the enantiomers on the Chiralcel OD CSP. Separation was not achieved at any organic concentration using this column.



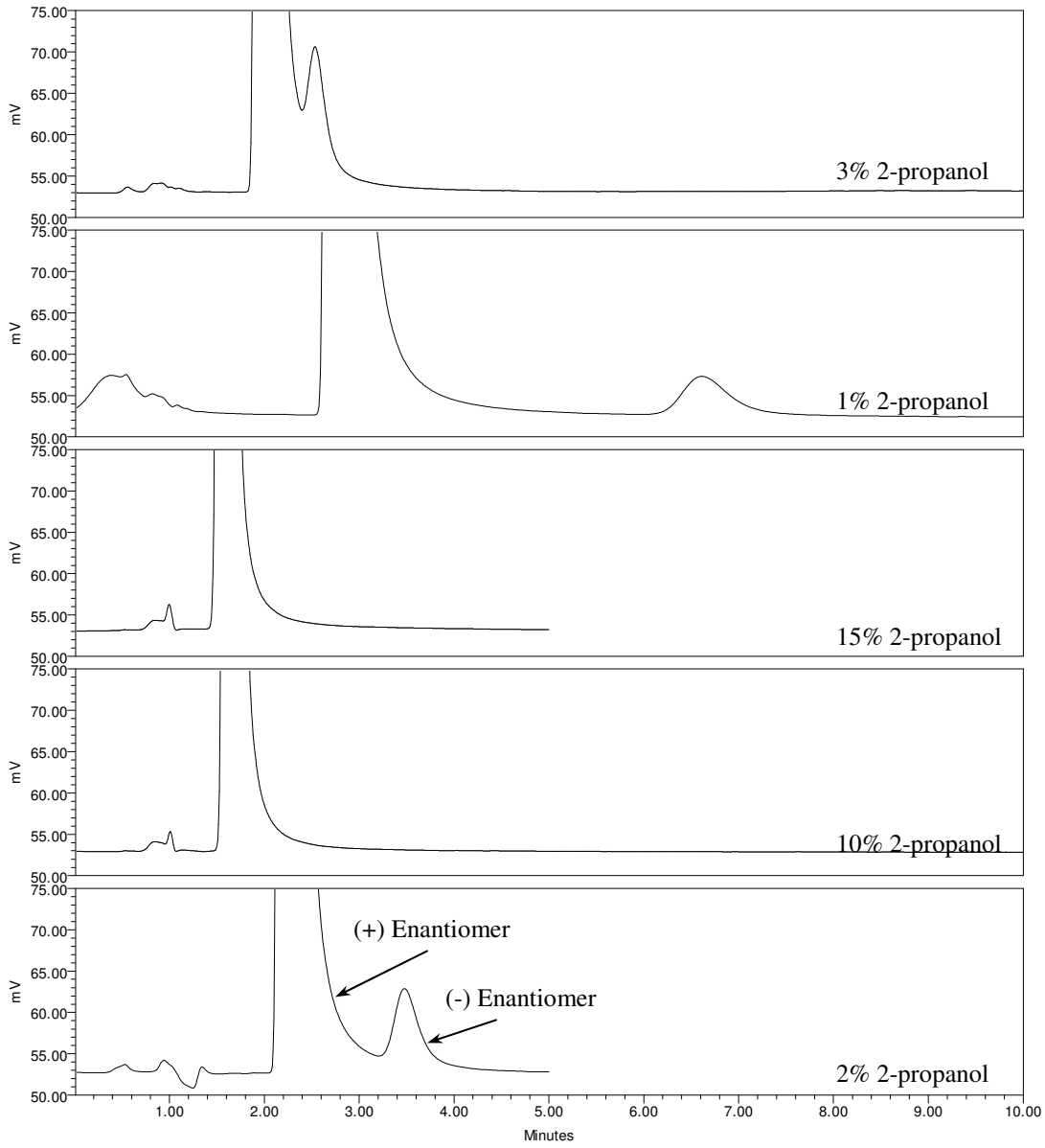
Column: Chiral-AGP, 100 mm by 4.0 mm, 5 µm particle size
Column Temperature: various
Flow Rate: 1.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 7.0, methanol (80:20)
Detection: UV at 260 nm

Figure 6. Example chromatogram of effect of temperature



Column: Chiral-AGP, 100 mm by 4.0 mm, 5 µm particle size
Column Temperature: various
Flow Rate: 1.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 7.0, 1-propanol (85:15)
Detection: UV at 260 nm

Figure 7. Example chromatogram of effect of temperature



Column: Chiral-AGP, 100 mm by 4.0 mm, 5 µm particle size
Column Temperature: 25°C
Flow Rate: 1.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 7.0, 2-propanol (various)
Detection: UV at 260 nm

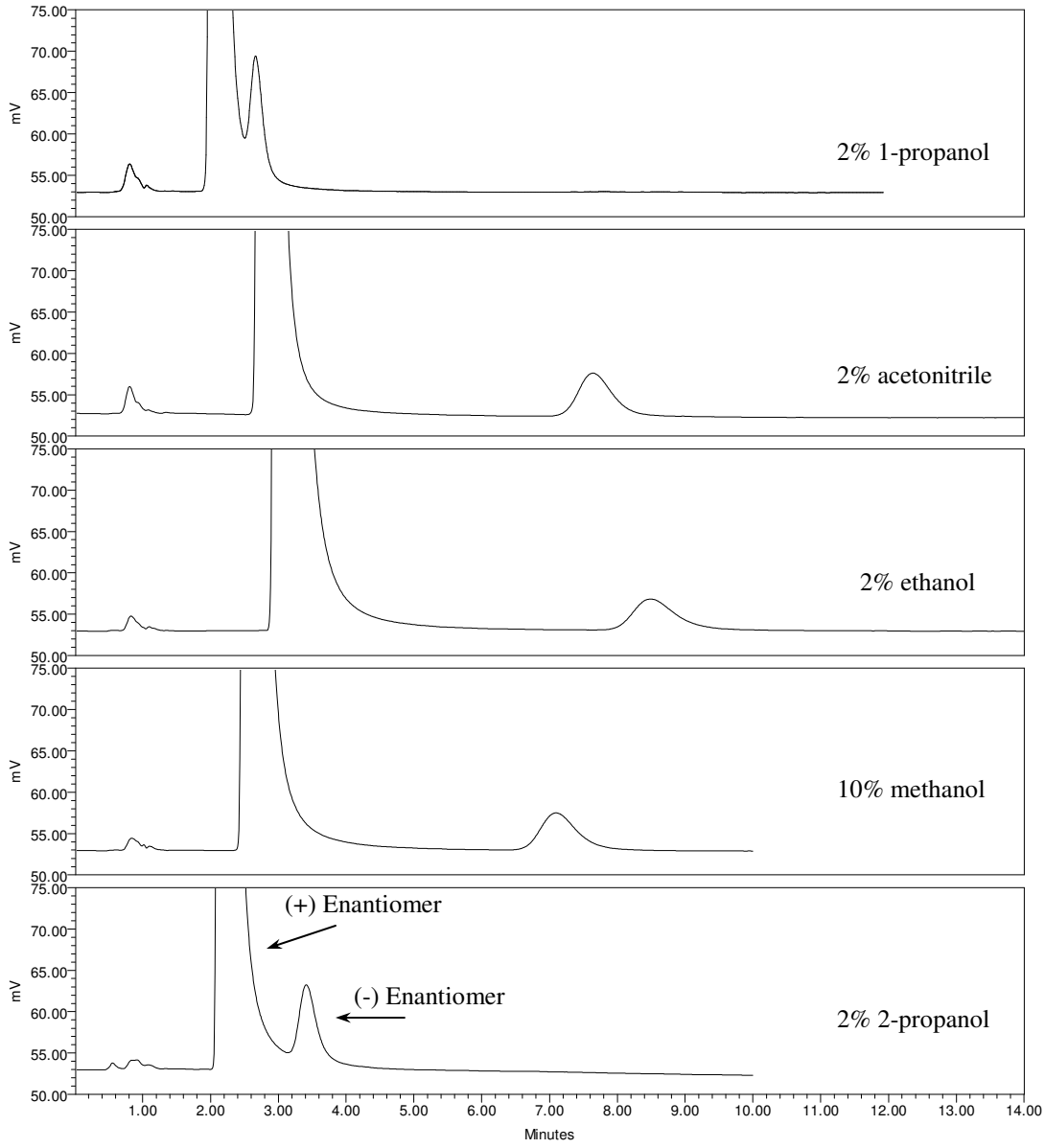
Figure 8. Example chromatogram of separation at various organic modifier concentrations (Chiral AGP CSP)

Effect of Mobile Phase Composition – Organic Modifier

The effect of mobile phase composition was evaluated using different organic modifiers for each column. Mobile phase components were varied according to compatible solvents listed for each type of CSP. Tables 1-3 show the results for each organic modifier used with each column tested. Separation was achieved using the Chiral AGP CSP with 2-propanol, methanol, ethanol, acetonitrile, and 1-propanol; however resolution reversal of elution order was not achieved using any of these organic modifiers. See Figure 9 for example chromatograms of injections using various organic modifiers on the Chiral AGP CSP. Mobile phases with various concentrations of 2-propanol, ethanol, and methyl tert-butyl ether (MTBE) were used to separate the enantiomers on the Kromasil CHI-TBB CSP. Separation was not achieved at any condition using this column. See Figure 10 for example chromatograms of injections made on the Kromasil CHI-TBB CSP using various organic modifiers. Mobile phases with various concentrations of 2-propanol and ethanol were used to separate the enantiomers on the Chiralcel OD CSP. Separation was not achieved at any condition using this column. See Figure 11 for example chromatograms of injections made on the Chiralcel OD CSP using various organic modifiers.

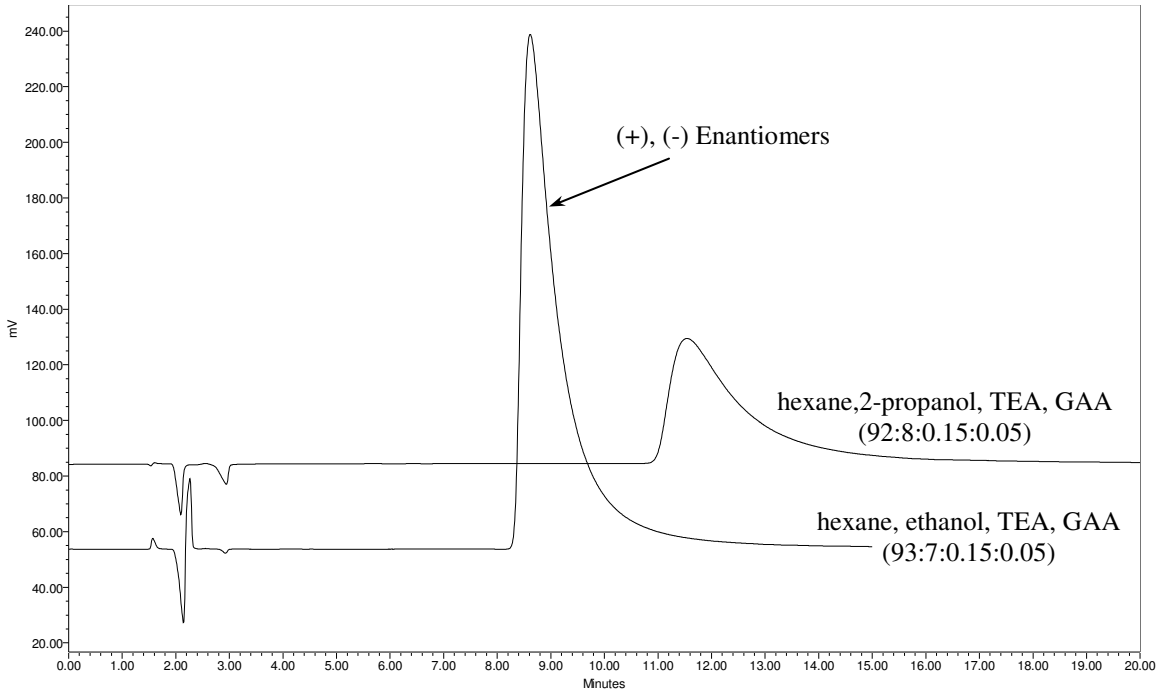
Effect of Sample Amount

The amount of sample injected onto the column can affect the retention of the analyte. With higher concentrations of analyte there is more competition for binding sites on the stationary phase. The effect of sample concentration on the retention of the enantiomers was evaluated by increasing and decreasing the injection volume, thereby changing the amount of sample introduced to the column. The effect of altered sample amount was evaluated at



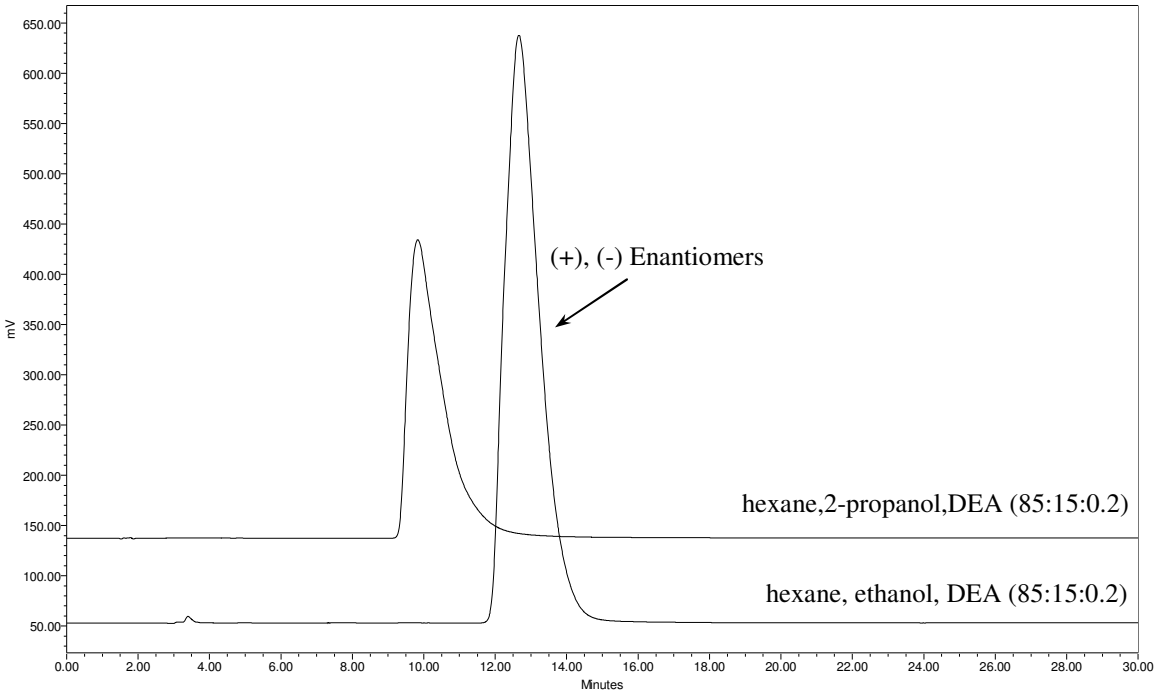
Column: Chiral-AGP, 100 mm by 4.0 mm, 5 µm particle size
Column Temperature: 25°C
Flow Rate: 1.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 7.0, various organic modifiers
Detection: UV at 260 nm

Figure 9. Example chromatogram of effect of organic modifier (Chiral AGP CSP)



Column: Kromasil CHI-TBB, 250 mm by 4.6 mm, 5 µm particle size
Column Temperature: 25°C
Flow Rate: 2.0 mL/min
Mobile Phase: Hexane: various organic modifiers: triethylamine: glacial acetic acid (various)
Detection: UV at 260 nm

Figure 10. Example chromatogram of effect of organic modifier type (Kromasil CHI-TBB CSP)



Column: Chiralcel OD, 250 mm by 4.6 mm, 10 µm particle size
Column Temperature: 25°C
Flow Rate: 1.0 mL/min
Mobile Phase: hexane, various organic modifiers, diethylamine (various)
Detection: UV at 260 nm

Figure 11. Example chromatogram of effect of organic modifier type (Chiralcel OD CSP)

conditions where the two peaks coeluted, using the Chiral AGP and Chiralcel OD columns. The results of this study are shown in Tables 1 and 3 and Figure 12. Separation was not achieved by changing the amount of sample injected onto the column.

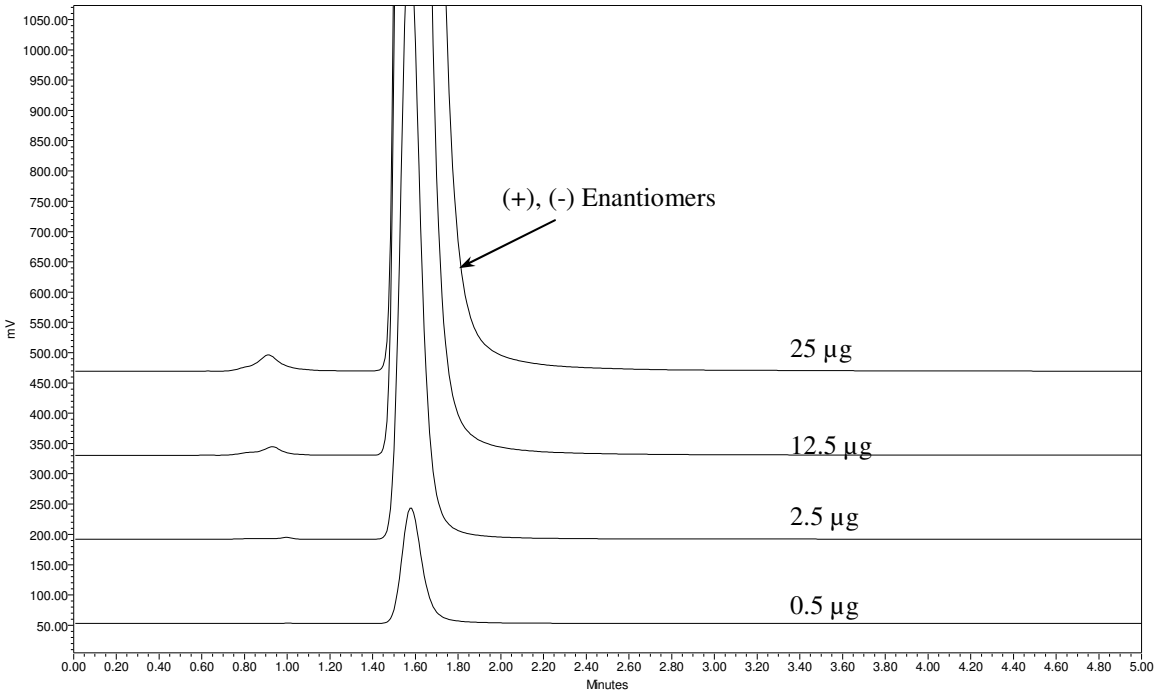
The goal of this research is to find a system exhibiting reversal of elution order under changed chromatographic conditions. Chromatographic conditions were altered in order to evaluate changes in retention. Conditions altered include the pH of the aqueous portion of the mobile phase, the column temperature, the organic modifier and its concentration in the mobile phase, the sample amount, and the chiral stationary phase. Separation was achieved using the Chiral α-1 glycoprotein chiral stationary phase; however reversal of elution order was not seen at any condition. Two other stationary phases were used, a Kromasil CHI-TBB CSP and Chiralcel OD CSP, resulting in no enantioselectivity for either column at any condition tried. Work was not continued with this compound due to the cost of acquiring additional chiral stationary phases.

RESULTS AND DISCUSSION – NON-CHIRAL SYSTEM

Effects of Altered Chromatographic Conditions

Initial conditions for the separation of diclofenac and related substances were the following:

Column: XTerra RP C18, 150 mm by 4.6 mm, 5 µm particle size
Column Temperature: 25°C
Injection Volume: 20 µL
Flow Rate: 2.0 mL/min
Mobile Phase: 50mM phosphate buffer, pH 3.5:methanol (56:44)
Detection: UV at 217 nm



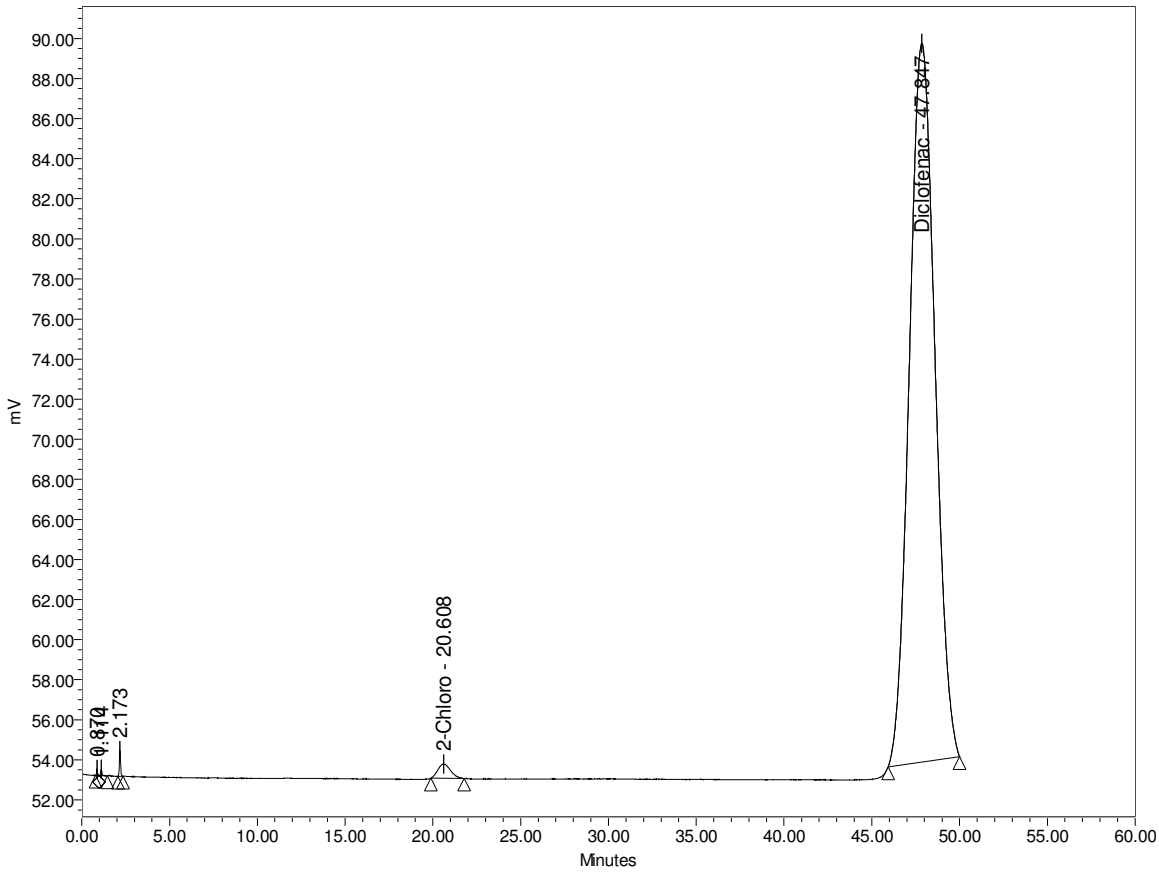
Column: Chiral-AGP, 100 mm by 4.0 mm, 5 µm particle size
Column Temperature: 25°C
Flow Rate: 1.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 7.0, 2-propanol (98:10)
Detection: UV at 260 nm

Figure 12. Example chromatogram of effect of sample amount (Chiral AGP CSP)

At the initial conditions, the diclofenac eluted second and the 2-chloro-N-(2,6-dichlorophenyl)-N-phenylacetamide (2-chloro) peak eluted first (Figure 13). In most chromatographic separations, peaks elute with a sharp front and a tailing rear boundary. In this case it is better for the impurity to elute first for purification using preparative chromatography. However, when the mobile phase was changed to acetonitrile (28%), the chromatograms for injections of diclofenac at higher concentrations showed that the peak shape of diclofenac exhibited fronting with a sharp rear boundary (Figure 14). Therefore, better separation would be achieved if the diclofenac peak eluted first and the related compound eluted second. Attempts were made to reverse the elution order of diclofenac and 2-chloro by changing the chromatographic conditions. Results of these studies are shown below.

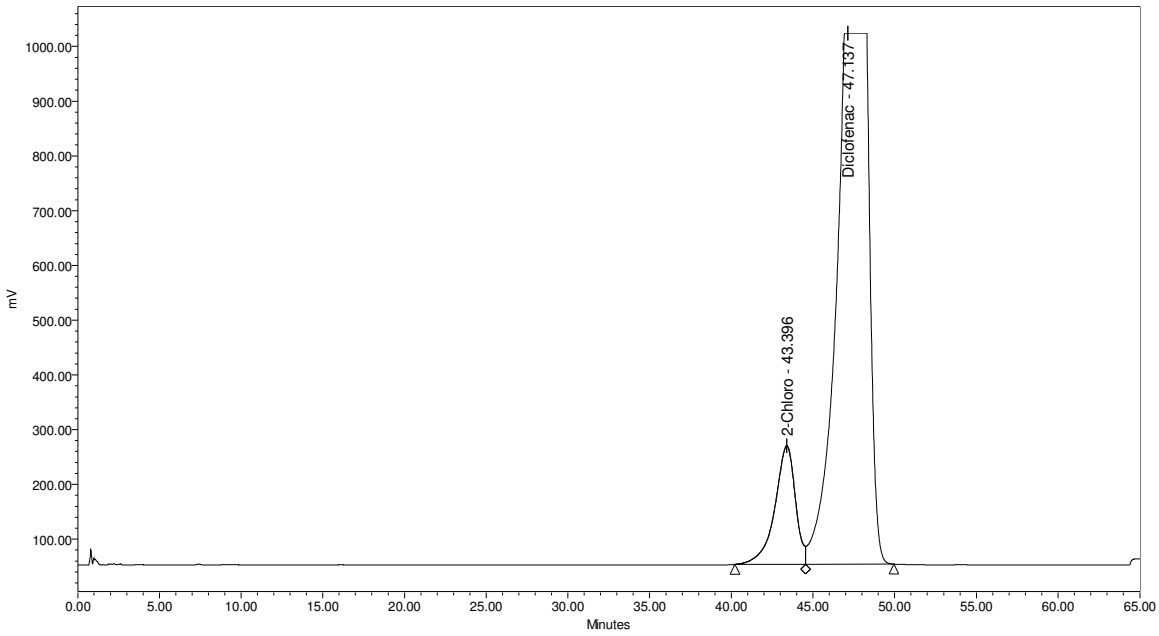
Effect of Temperature

The effect of temperature was determined by comparing the separation factor and retention order of the two compounds at higher and lower column temperature than the initial conditions. The separation factor for the initial condition, 25°C, was 2.26 with diclofenac eluting second. The temperature was increased to 45°C, resulting in a separation factor of 1.17. This indicated that temperature was affecting the separation, but reversal of elution order was not seen. The temperature was decreased to 5°C, and the flow rate was reduced to 1.8 milliliters per minute due to increased pressure on the column. The separation factor at lowered temperature was 2.96, indicating the peaks are better separated but elution order is not reversed. See Figure 15 for example chromatography of injections at various column temperatures.



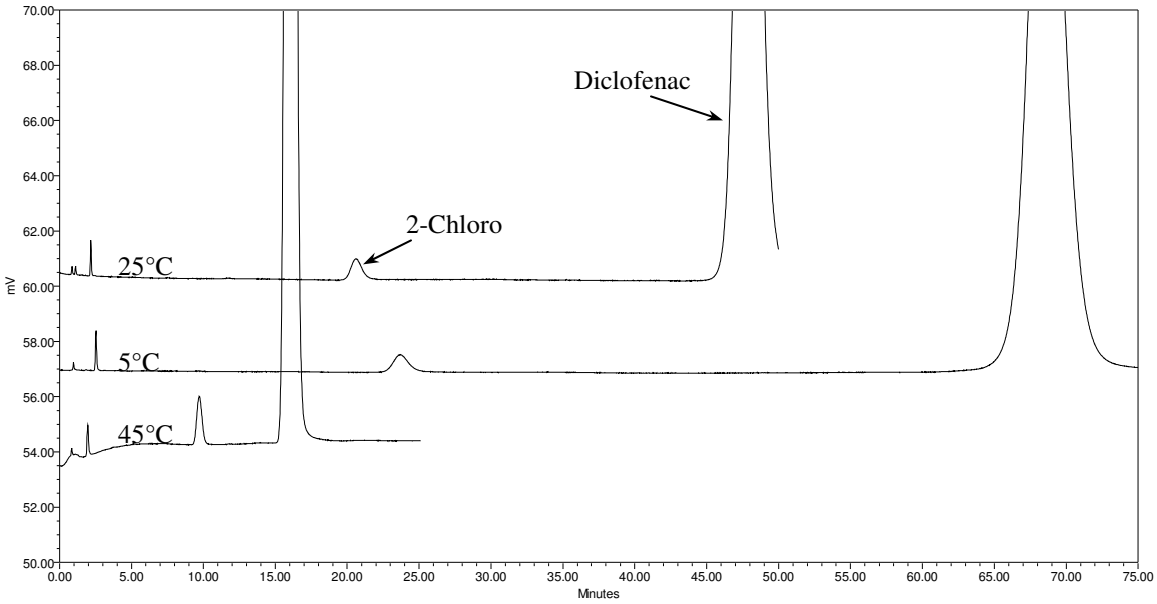
Column: XTerra RP C18, 150 mm by 4.6 mm, 5 µm particle size
Column Temperature: 25°C
Injection Volume: 20 µL
Flow Rate: 2.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 3.5, methanol (56:44)
Detection: UV at 217 nm

Figure 13. Example chromatogram of separation at nominal conditions (non-chiral system)



Column: XTerra RP C18, 150 mm by 4.6 mm, 5 µm particle size
Column Temperature: 25°C
Injection Volume: 100 µL
Flow Rate: 2.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 3.5, acetonitrile (72:28)
Detection: UV at 217 nm

Figure 14. Peak shape at high concentration



Column: XTerra RP C18, 150 mm by 4.6 mm, 5 µm particle size
Column Temperature: Various
Injection Volume: 20 µL
Flow Rate: 2.0 mL/min, 1.8mL/min
Mobile Phase: 50mM phosphate buffer pH 3.5, methanol (43:57)
Detection: UV at 217 nm

Figure 15. Example chromatogram of the effect of temperature

Effect of Mobile Phase Composition – Organic Concentration

The effect of mobile phase composition was first observed by comparing the separation factor and retention order of diclofenac and 2-chloro at altered concentrations of methanol in the mobile phase. At initial conditions, 2-chloro eluted first with a separation factor of 2.38. Increasing the concentration of methanol in the mobile phase decreased the separation factor, indicating the retention of the compounds was affected by the concentration of methanol in the mobile phase (see Table 4). However, change in elution order was not observed.

Effect of Mobile Phase Composition – Organic Modifier

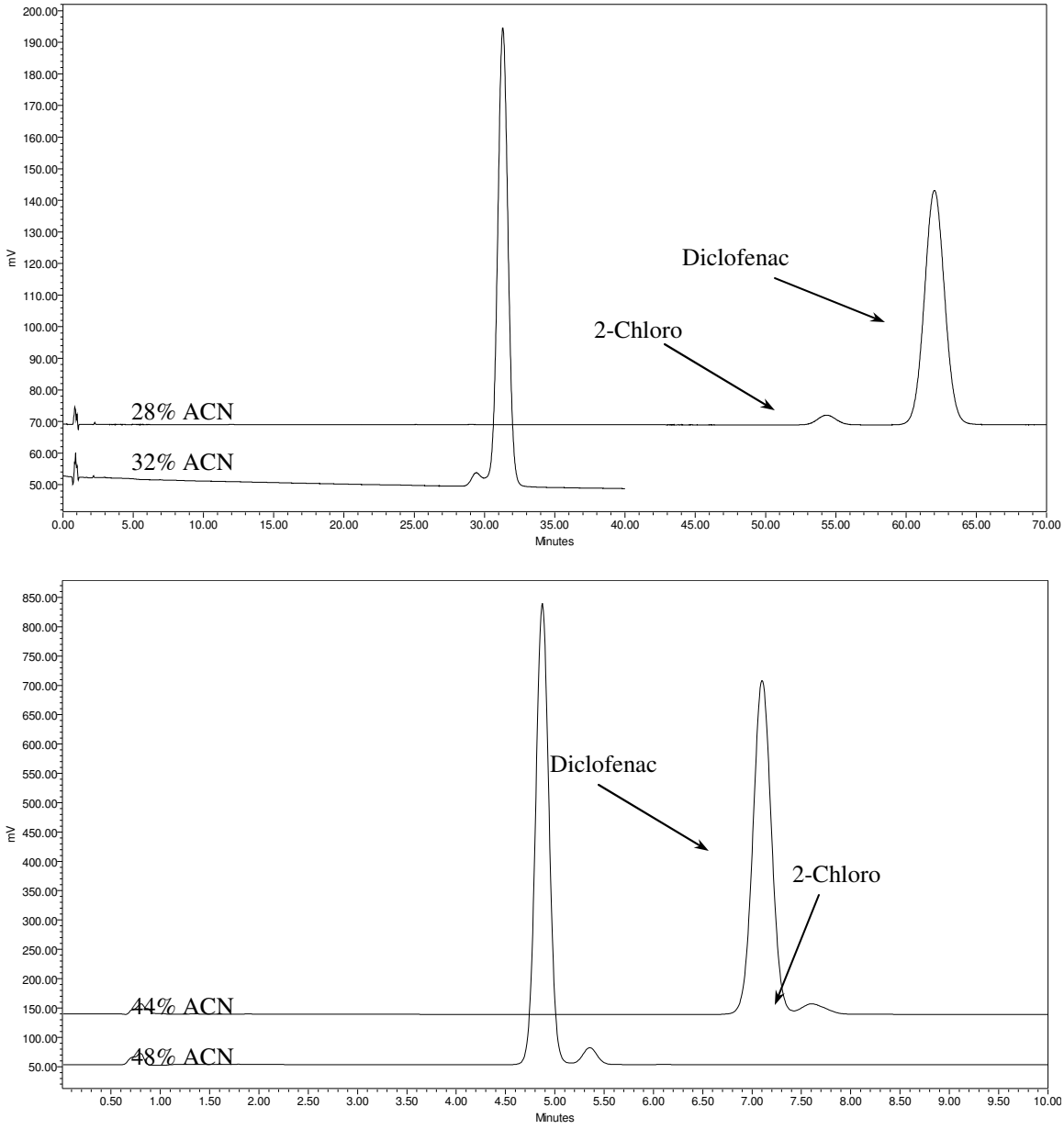
The effect of mobile phase composition was then studied by replacing the methanol in the mobile phase with acetonitrile. A solution containing both compounds was injected on an HPLC system using a series of mobile phases with acetonitrile concentrations ranging from 28% to 48%. The results of this study are shown in Table 5. At lower acetonitrile conditions (28% and 32%) the 2-chloro peak eluted first with separation factors of 1.14 and 1.07 respectively. The change in separation factor indicated that the retention of the two compounds was affected by the concentration of acetonitrile in the mobile phase. When the concentration was increased further, to 44% and 48%, the order of elution reversed and the diclofenac peak eluted first. See Figure 16 for example chromatograms of diclofenac and 2-chloro separation at various concentrations of acetonitrile in the mobile phase. Increasing the acetonitrile concentration further resulted in deterioration of the separation due to the peaks eluting close to the solvent front.

Percent Methanol in Mobile Phase	Elution Order	Separation Factor
44	2-chloro, diclofenac	2.38
52	2-chloro, diclofenac	2.08
55	2-chloro, diclofenac	1.98
57	2-chloro, diclofenac	1.91

Table 4. Separation factors and retention order at various methanol concentrations

Percent Acetonitrile in Mobile Phase	Elution Order	Separation Factor
28	2-chloro, diclofenac	1.14
32	2-chloro, diclofenac	1.07
44	diclofenac, 2-chloro	1.08
48	diclofenac, 2-chloro	1.12

Table 5. Separation factors and retention order at various acetonitrile concentrations



Column: XTerra RP C18, 150 mm by 4.6 mm, 5 µm particle size
Column Temperature: 25°C
Injection Volume: 20 µL
Flow Rate: 2.0 mL/min
Mobile Phase: 50mM phosphate buffer pH 3.5, acetonitrile (various)
Detection: UV at 217 nm

Figure 16. Example chromatography of the effect of acetonitrile concentration

The goal of this study was to find conditions at which a reversal of elution order was observed. Changing the concentration of methanol in the mobile phase did not change the elution order although separation was affected. When the concentration of the organic modifier was changed from 28% to 44% acetonitrile, a reversal of elution order was observed.

Final Chromatographic Conditions

Column: XTerra RP C18, 150 mm by 4.6 mm, 5 µm particle size
Column Temperature: 25°C
Injection Volume: 20 µL
Flow Rate: 2.0 mL/min
Mobile Phase: 50mM phosphate buffer, pH 3.5, acetonitrile (52:48)
Detection: UV at 217 nm

Adsorption Isotherm Measurement

Equilibrium isotherms are measured in order to better understand the retention mechanisms of the system. In liquid-solid chromatography, the equilibrium occurs between the liquid mobile phase and the solid stationary phase that interacts with the components. The equilibrium isotherm is a plot of the concentration of the component in the stationary phase versus the concentration of the component in the mobile phase.

Because the gas-solid interface is much simpler than the liquid-solid interface, more literature can be found describing the gas-solid equilibria. Two theories described for gas-solid equilibria provide guidance for our understanding of liquid-solid equilibria. The Gibbs equation relates the amount of the component adsorbed at the interface to the surface or interface tensions. The adsorption theory of Brunauer, Emmett, and Teller (BET) describes the phase equilibria of multilayer adsorption at the gas-solid interface. It is more difficult to determine precisely the mechanism of component retention in liquid-solid chromatography because the component has

to compete with the mobile phase and possibly other components for binding sites on the solid phase.

Isotherms are measured by using one of several techniques. The static method is simple to perform, however it is time consuming and requires a large amount of components to be used. Frontal analysis by characteristic point (FACP) and elution by characteristic point (ECP) are two chromatographic methods which are more accurate than the static method. However, since the calculation in these methods assumes the column has infinite theoretical plates, these methods are limited by the efficiency (column with a high number of theoretical plates) required in order to obtain precise data. The retention time method is the simplest and fastest chromatographic method for determining the isotherm; however it is only accurate when the component follows the Langmuir model. Frontal analysis is the most accurate way to determine the isotherm, and is used in this project to determine the equilibrium isotherms of diclofenac and 2-chloro at both high and low acetonitrile conditions.

Figure 17 shows an example of the step chromatogram obtained during frontal analysis of diclofenac. The breakthrough curves obtained experimentally for diclofenac exhibit the characteristics of a multi-component system, as evidenced by the extra steps in the chromatogram. The extra steps present in the chromatogram are most likely due to the competition of diclofenac with an impurity or with a strong solvent for the binding sites on the stationary phase. This competition is neglected in this research and it is assumed that the major breakthrough step is due to diclofenac. The retention time of the breakthrough curve can be approximated from the sigmoidal curve of the step chromatogram (Jacobson, 1987).

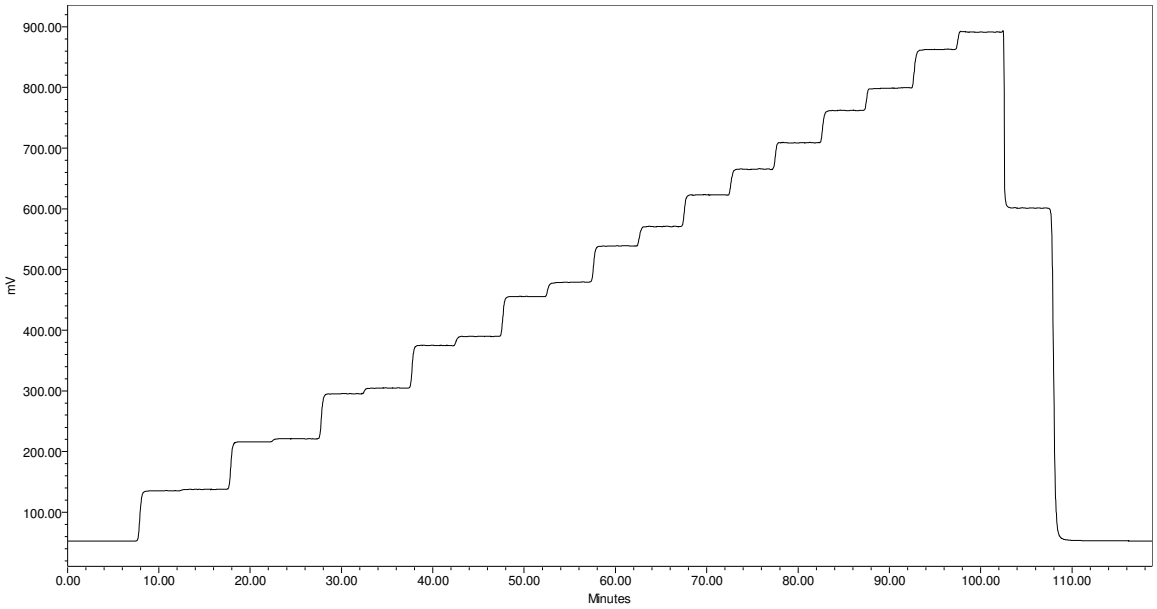


Figure 17. Step chromatogram of diclofenac at high acetonitrile condition

Figure 18 shows an example of the step chromatogram obtained during frontal analysis of 2-chloro. The breakthrough curves obtained experimentally for 2-chloro exhibit the characteristics of a single component system. The retention time of the breakthrough front for each concentration step was used to calculate the adsorption isotherm.

For each component at each mobile phase condition, the values for q calculated for each concentration step c are given in Table 6. These values were calculated using equations 4-6. A plot of q versus c was generated for each data set and these plots are shown in Figure 19. The experimental isotherms for diclofenac at both high and low acetonitrile mobile phases are convex upward, in contrast to the experimental isotherms for 2-chloro, which are convex downward at both high and low acetonitrile mobile phase conditions.

Adsorption Isotherm Modeling

The Langmuir model can be used to determine the saturation capacity (q_S) and the adsorption-desorption equilibrium constant (b) for most compounds. The adsorption-desorption equilibrium constant is described by equation 18, where k_a is the rate coefficient of adsorption and k_b is the rate coefficient of desorption.

$$b = \frac{k_a}{k_b} \tag{18}$$

The Langmuir model can be written as equation 19, where a is equal to $q_s b$, and q is the adsorbed amount of solute in the stationary phase in equilibrium with the concentration of solute in the mobile phase c.

$$q = \frac{ac}{1+bc} \tag{19}$$

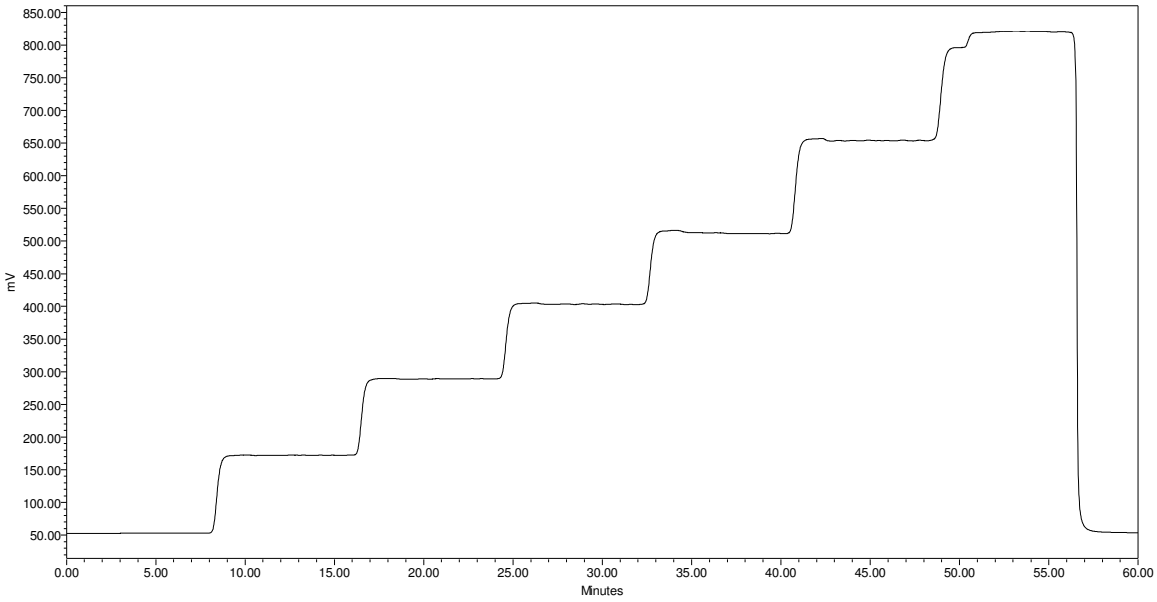


Figure 18. Step chromatogram of 2-chloro at high acetonitrile condition

Diclofenac (44% acetonitrile)

c (mMol/L)	*q* (mMol/L)
0	0
0.16	1.85
0.31	3.68
0.47	5.50
0.63	7.28
0.78	9.04
0.94	10.78
1.10	12.50
1.25	14.21
1.41	15.92
1.57	17.62

2-Chloro (44% acetonitrile)

c (mMol/L)	*q* (mMol/L)
0	0
0.24	3.04
0.47	6.12
0.71	9.25
0.94	12.42
1.25	16.73
1.57	21.13

Diclofenac (29% acetonitrile)

c (mMol/L)	*q* (mMol/L)
0	0
0.24	21.90
0.48	43.80
0.72	65.63
0.96	87.30
1.28	115.78
1.59	144.01

2-Chloro (29% acetonitrile)

c (mMol/L)	*q* (mMol/L)
0	0
0.09	7.98
0.19	16.27
0.28	24.86
0.38	33.75
0.50	46.06
0.63	58.92

Table 6. Calculated values of *q* and *c* for components at each mobile phase condition

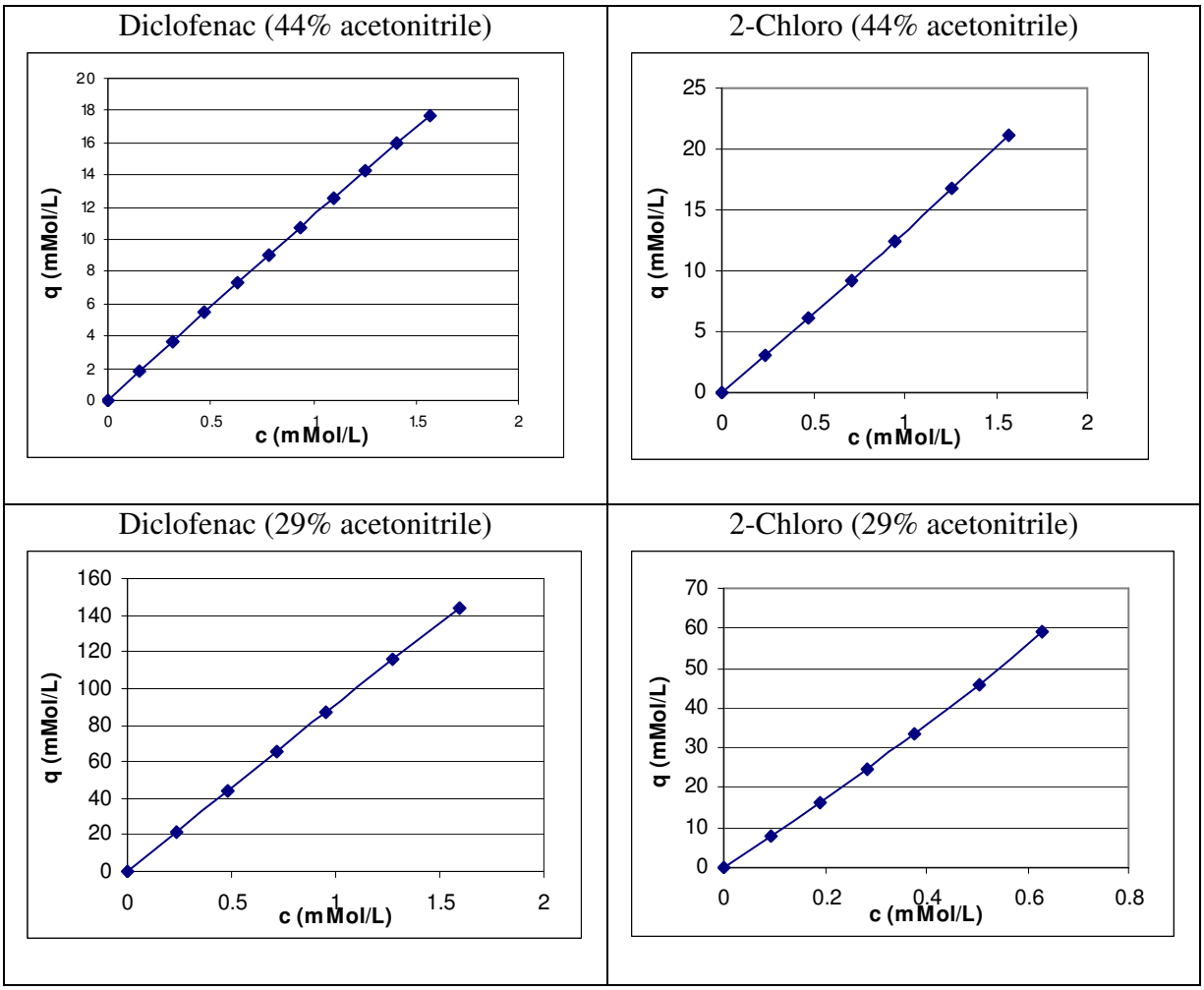


Figure 19. Plots of experimental data for components at each mobile phase condition

If the isotherms for both compounds followed the Langmuir isotherm model, the selectivity would have been constant. Because a reversal in elution order was observed, hence a change in the selectivity, both compounds are not expected to follow the Langmuir model.

Scatchard plots were made by plotting *q/c* verses *q* for each compound at each condition. If the system is Langmuirian, the graph produced should be linear with a negative slope. Any inflection points in the isotherm would be more obvious in the Scatchard plot. The slope of the line produced by the Scatchard plot is equal to *–b* and the y-intercept is equal to *a*. See Figure 20 for the Scatchard plots generated for each compound at each condition studied. Of the four plots generated, only one exhibited Langmuirian behavior (diclofenac at the high acetonitrile condition). The results which fit the Langmuir model are shown in Table 7 and the adsorption isotherm for diclofenac at the high acetonitrile condition (44%) is shown in Figure 21. In the figure, experimental data is represented by plotted points and the fitted Langmuir model is a solid line.

The Scatchard plot generated for diclofenac at the low acetonitrile condition was not fully linear; therefore it does not follow the Langmuir model. An attempt was made to fit the data to the more general Moreau model. Unlike the Langmuir model, which ignores adsorbate-adsorbate interaction, the Moreau model considers the interaction between molecules of the adsorbate. In this type of system, the surface is homogenous and there is only one layer of surface coverage. Equation 7 describes the Moreau model for a single component on a homogenous surface. When *I* is small (negligible interaction between molecules of the adsorbate) and *b* is positive, equation 7 reduces to the Langmuir isotherm model.

Solid lines represent fitted regression lines.

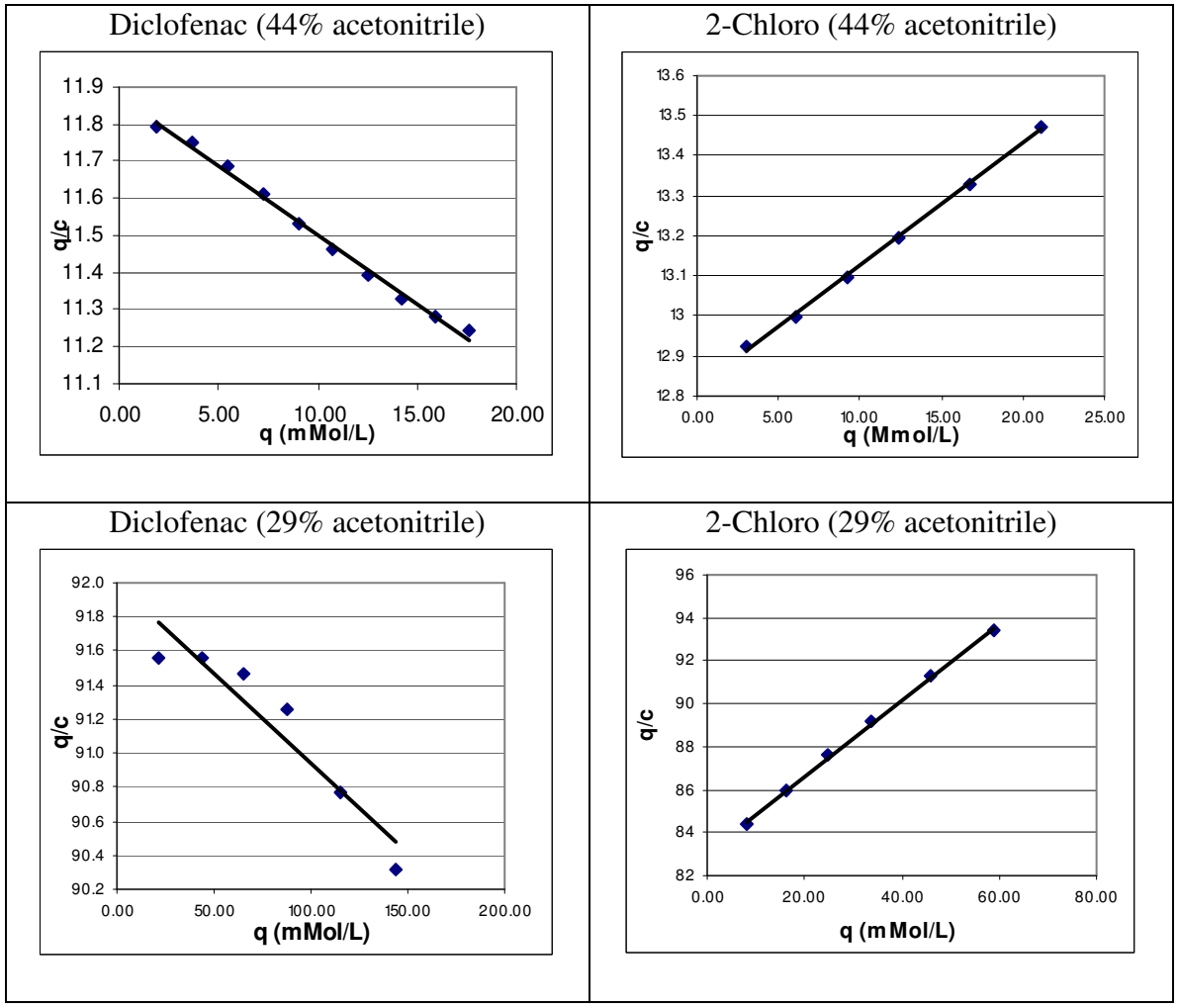


Figure 20. Scatchard plots for each component at each mobile phase condition

Compound/Condition	q_S (mMol/L)	b (L/mMol)	a
diclofenac/44% ACN	318	0.0373	11.9

Table 7. Results for component fitted to the Langmuirian isotherm model

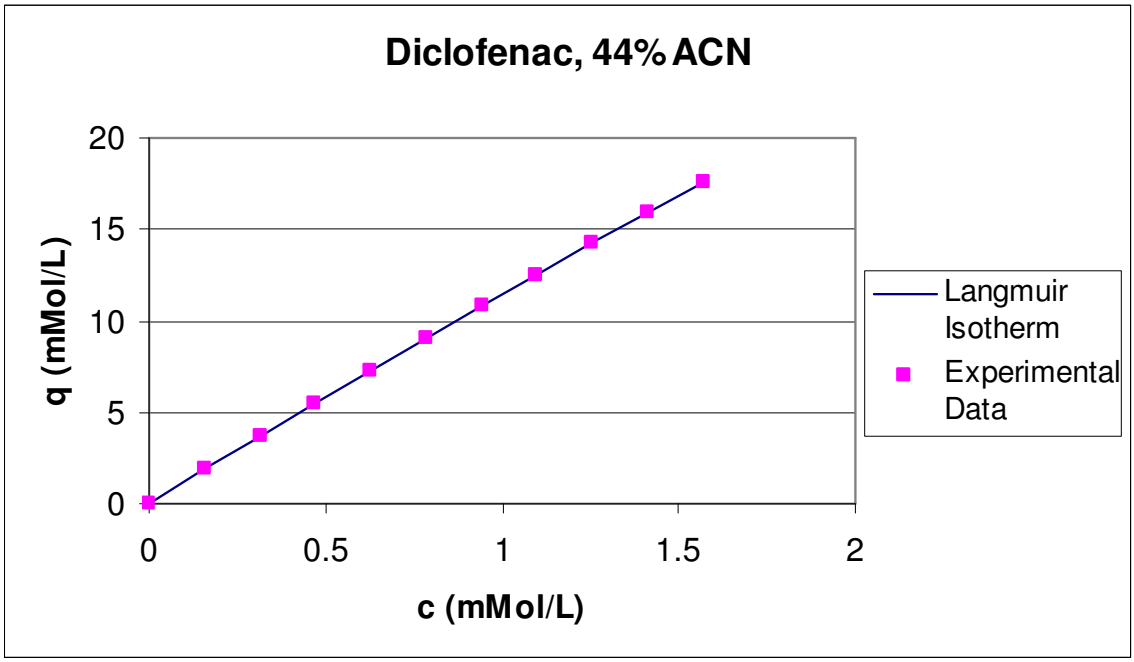


Figure 21. Adsorption isotherm for diclofenac at the high acetonitrile condition

SAS statistical software was used to find values which best fit the Moreau model using the data collected during frontal analysis of diclofenac at the low acetonitrile mobile phase condition (29%). The isotherm data determined for diclofenac at the low acetonitrile condition, shown in Table 8, indicates that diclofenac follows the Moreau isotherm. The adsorption isotherm for diclofenac low acetonitrile condition is shown in Figure 22. In the figure, experimental data is represented by plotted points and the fitted Moreau model is a solid line.

The two Scatchard plots generated for 2-chloro (one each for high and low acetonitrile conditions) yielded a negative value of *b*, however the Langmuir model with a negative value of *b* is not realistic in liquid-solid chromatography. The isotherm would have a vertical asymptote with an infinite value of *q* at $c = \frac{1}{|b|}$. The isotherm in liquid-solid chromatography should have a horizontal asymptote and a finite saturation capacity (Guiochon, 2006). The Langmuir model cannot be used to fit these isotherms. Since the isotherm data for 2-chloro did not fit the Langmuir model, an attempt was made to fit the data to the BET isotherm model. The BET adsorption isotherm model is used to account for more than one layer of adsorption. In this type of system, the first layer of molecules adsorbed provides adsorption sites for the second layer. The adsorption-desorption equilibrium constant for the first layer is different than that of the second and subsequent layers. Equation 10 describes the BET model, where b_S is the adsorption-desorption equilibrium constant for the first layer and b_L is the adsorption-desorption equilibrium constant for the second and subsequent layers. Statistical evaluation of the 2-chloro isotherm data using equation 10 did not converge. Therefore, the more general empirical form of the BET model, equation 11, was tried. In this general form, *B* and *D* are constants, *a* is equal to $q_s b$, and *q* is the adsorbed amount of solute in the stationary phase in equilibrium with the concentration

Compound/Condition	*qs*	*B*	*I*
diclofenac/29% ACN	16650.1	0.00549	0.7508

Table 8. Results for component fitted to the Moreau Model

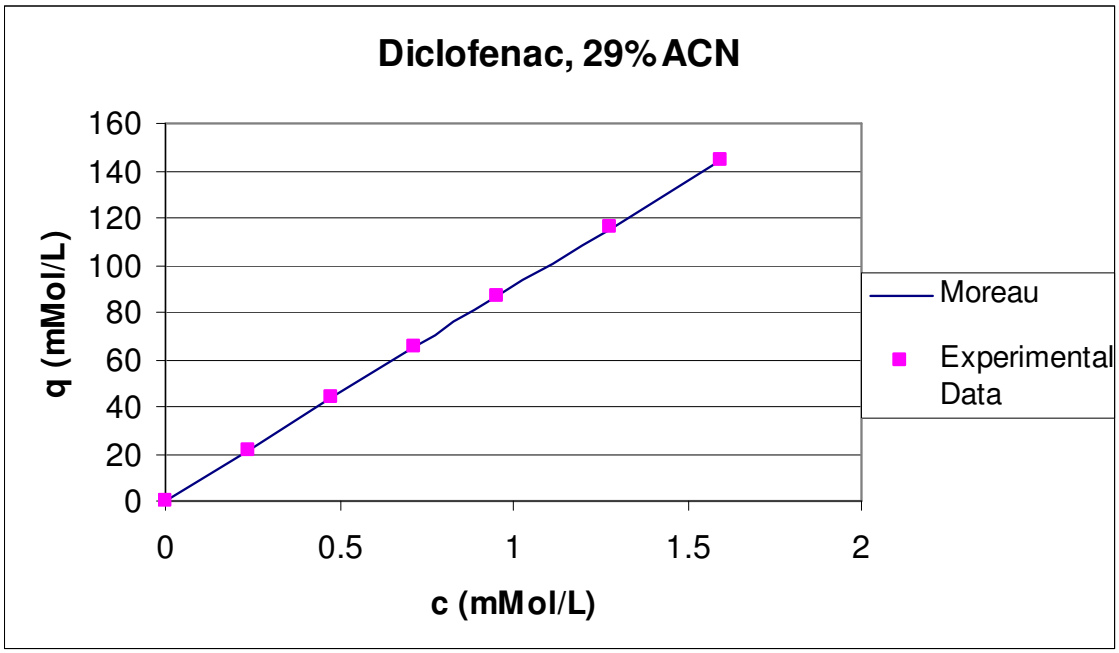


Figure 22. Adsorption isotherm for diclofenac at the low acetonitrile condition

of solute in the mobile phase *c*. When *D* is small and *B* is positive, equation 11 reduces to the Langmuir isotherm model. Statistical analysis was performed on the data sets collected during frontal analysis in order to find the best fit numbers for each condition. Table 9 contains results of the statistical analysis for the two data sets fitted to equation 11. The isotherm data for 2-chloro at both low and high acetonitrile conditions indicates that 2-chloro follows S-shaped isotherms in both mobile phase compositions. The initial slope of the 2-chloro isotherms is convex downward (slope increases with increasing concentration). Adsorption isotherms for 2-chloro at high and low acetonitrile conditions are shown in Figures 23 and 24. In the figures, experimental data is represented by plotted points and the fitted model is a solid line.

Figures 25 and 26 compare the isotherms for diclofenac and 2-chloro at high and low acetonitrile conditions respectively, with diclofenac at the 44% acetonitrile condition fitted to the Langmuir isotherm model, diclofenac at the 29% acetonitrile condition fitted to the Moreau isotherm model, and 2-chloro at both conditions fitted to the BET isotherm model. The graph in Figure 25 shows that in 44% acetonitrile mobile phase the slope of the isotherm of 2-chloro is always higher than that of diclofenac. No intersection of the isotherms occurred at the scale used in this project. The graph in Figure 26 indicates the isotherms for 2-chloro and diclofenac cross each other. At low concentrations the slope of the isotherm of 2-chloro is lower than that of diclofenac. At c = ci = 0.488 mMol/L, q = 44.55 mMol/L, the two isotherms intersect. At c > ci the slope of the isotherm of 2-chloro is higher than that of diclofenac.

Compound/Condition	*a*	*B*	*D*
2-chloro/44% ACN	12.5174	-0.0745	0.0190
2-chloro/29% ACN	83.9910	-0.1727	0.0144

Table 9. Results for components fitted to the BET-like isotherm model

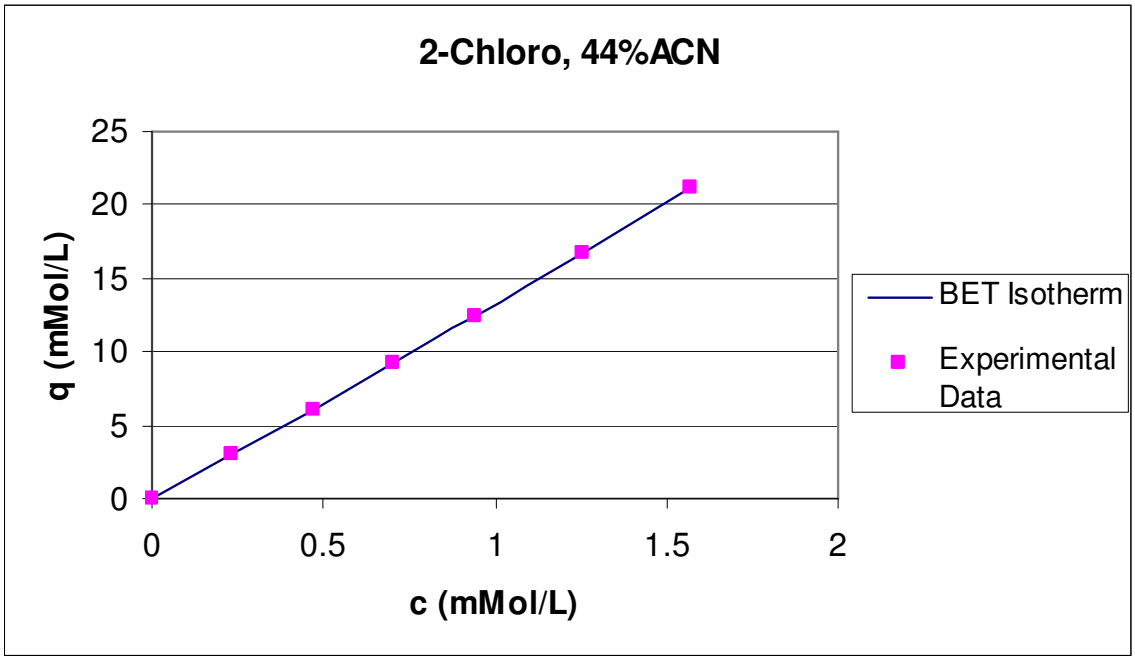


Figure 23. Adsorption isotherm for 2-chloro at the high acetonitrile condition

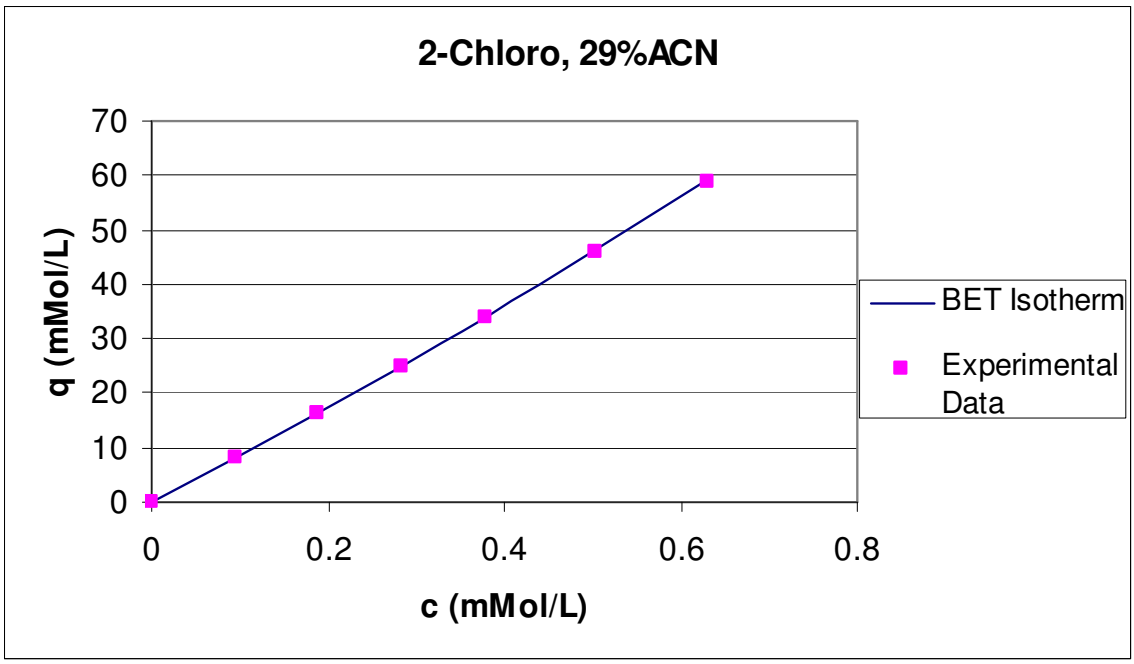


Figure 24. Adsorption isotherm for 2-chloro at the low acetonitrile condition

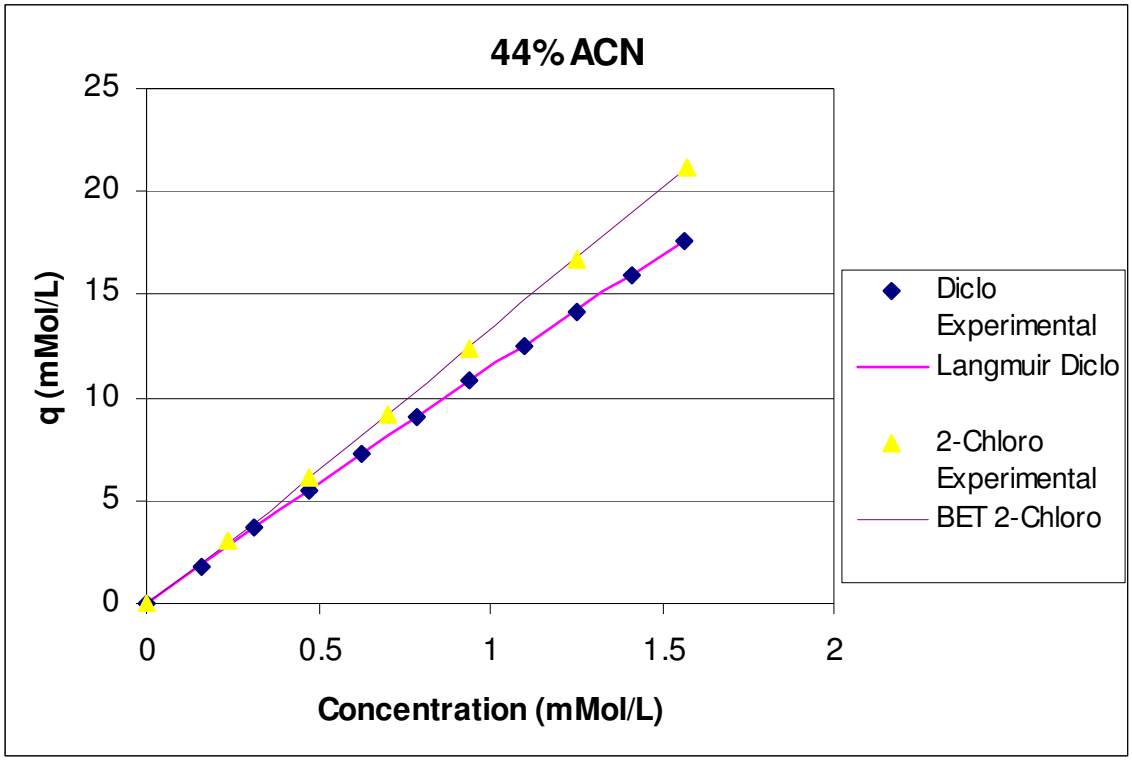


Figure 25. Comparison of adsorption isotherms at high acetonitrile condition

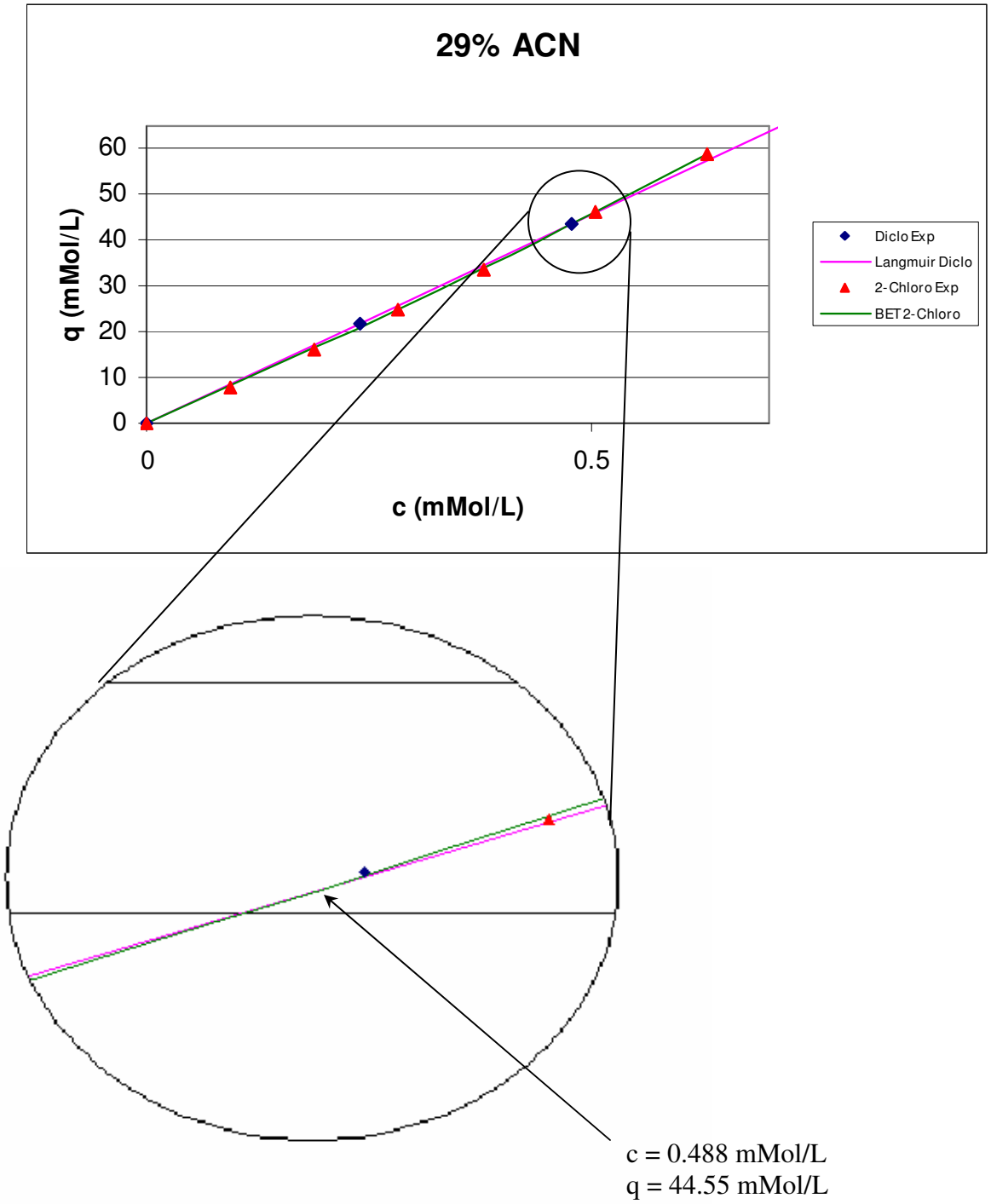


Figure 26. Comparison of adsorption isotherms at low acetonitrile condition

At very low concentrations of solutes (where *c* is close to zero) the isotherms are reduced to the linear isotherms as follows. At 44% acetonitrile, the isotherm for diclofenac is reduced to:

$$q_{(D)} = ac = 11.9c_{(D)}$$

The isotherm for 2-chloro at 44% acetonitrile reduces to:

$$q_{(C)} = 12.5\ c_{(C)}$$

The theoretical value of k_0' can be calculated using equation 20,

$$k_0' = a \times F \tag{20}$$

where *a* is equal to $q_s \times b$ and *F* is the phase ratio. Table 10 displays the phase ratios and theoretical k_0' results. At the high acetonitrile condition $k'_{0(D)}$ is equal to 7.0 and $k'_{0(C)}$ is equal to 7.3. This indicates that diclofenac should elute first and 2-chloro should elute last. At very low concentrations of solutes, the isotherms at 29% acetonitrile reduce to the following.

$$q_{(D)} = ac = 92.3c_{(D)} \text{ and}$$

$$q_{(C)} = 84.0\ c_{(C)}$$

At this condition $k'_{0(D)}$ is equal to 43.1 and $k'_{0(C)}$ is equal to 39.6. This indicates that 2-chloro should elute first and diclofenac should elute last. This shows a reversal of elution order when the mobile phase is changed from 29% acetonitrile to 44% acetonitrile.

These results indicate the reversal of elution order is due to different nature of the adsorption isotherms for each compound. The adsorption isotherm of diclofenac at the 44% acetonitrile condition follows the typical Langmuirian behavior and at the 29% acetonitrile condition it follows the Moreau isotherm model, while the adsorption isotherm of 2-chloro follows the rare case of S-shaped isotherm. If the isotherms of both solutes followed the

Condition	Compound	a	F	k_0'
High ACN	diclofenac	11.9	0.587	7.0
	2-chloro	12.5	0.587	7.3
Low ACN	diclofenac	91.6	0.471	43.1
	2-chloro	84.0	0.471	39.6

Table 10. Theoretical values for retention factor

Langmuir isotherm, reversal of elution order would have been impossible. The experimental data supports the theoretical prediction.

The value of k_0' was determined experimentally to confirm the theoretical values obtained from the isotherm measurement. A solution containing very low concentrations of both diclofenac and 2-chloro was injected at both high and low acetonitrile conditions. At very low concentrations the isotherms for the components are linear, and the value of k_0' can be determined using equation 21,

$$k_0^{'} = \frac{t_r - t_0}{t_0} \tag{21}$$

where t_r is the retention time of the peak and t_0 is the void time of the column. The results of this calculation are shown in Table 11. The experimentally determined values of k_0' support the values determined theoretically.

Mechanism Discussion

The retention of an analyte in reversed-phase chromatography is based on the polarity of the analyte compared to that of the mobile phase and the stationary phase. In reversed-phase chromatography the stationary phase is non-polar. The column used in this project was an XTerra RP C18 which contains a straight chain alkyl (C18) stationary phase. The mobile phase used in reversed phase chromatography is more polar than the stationary phase. The less polar components are more retained by the stationary phase than the more polar components, causing the less polar components to have longer retention times. Increasing the polarity of the solvent increases the retention times whereas decreasing the polarity of the solvent decreases the retention times. The solvent polarity is usually altered by changing the composition of a binary solvent mixture.

Condition	Compound	t_r (min)	t_0 (min)	k_0'
High ACN	diclofenac	6.35	0.79	7.0
	2-chloro	6.76	0.79	7.6
Low ACN	diclofenac	38.90	0.85	44.8
	2-chloro	34.53	0.85	39.6

Table 11. Experimentally determined values for retention factor

The isotherm data collected indicated that the reversal in elution order is due to the difference in the isotherms and the amount of adsorbate-adsorbate interactions. At the high acetonitrile condition, the isotherm data for diclofenac fits the Langmuirian model. At low concentrations, the isotherm data for diclofenac fits the Moreau model. The Moreau model takes adsorbate-adsorbate interactions into account, where the Langmuir model does not. The pKa of diclofenac is about 4.5 (Diclofenac Sodium, 2004) (see structure in Figure 26) and the pH of the mobile phase used in this research was 3.5. At this pH, diclofenac does not have a charge, has low solubility, and is of low polarity. When the concentration of acetonitrile in the mobile phase decreases from 44% to 29%, the mobile phase becomes more polar. As a result, the diclofenac is more attracted to the non-polar stationary phase (C18). This causes the retention time of diclofenac to increase significantly. The column saturation capacity becomes about fifty times larger in 29% acetonitrile mobile phase than in the 44% acetonitrile mobile phase (16650 mMol/L and 318 mMol/L, respectively). The adsorbate-adsorbate interactions (the interactions between adsorbed molecules of diclofenac in the stationary phase) become more pronounced at 29% acetonitrile mobile phase, due to the increased attraction of diclofenac molecules to each other and to the stationary phase relative to the mobile phase.

In contrast, 2-chloro is less polar than diclofenac (see structure in Figure 27) and has low water solubility. Molecules of 2-chloro are more attracted than diclofenac is to the non-polar stationary phase and to other non-polar molecules. As a result, even at the high acetonitrile condition (44%) the adsorbate-adsorbate interactions are significant. These strong adsorbate-adsorbate interactions cause multilayer adsorption, as evidenced by the isotherm data for 2-chloro at both high and low acetonitrile conditions. The isotherm data at both conditions fit a BET-like isotherm model, which accounts for multilayer adsorption. Decreasing the mobile

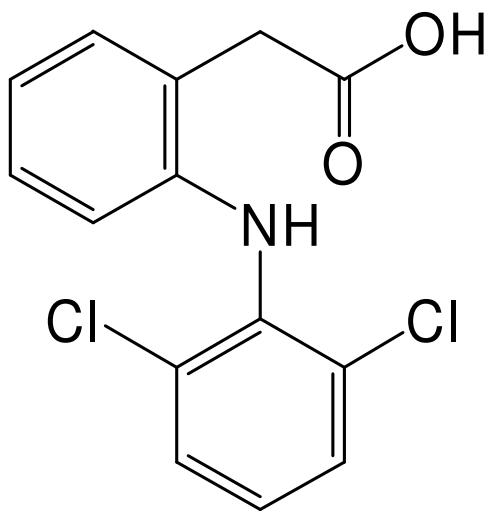


Figure 27. Structure of diclofenac

phase to 29% acetonitrile causes the mobile phase to become more polar, increasing the attraction of 2-chloro molecules to each other and to the stationary phase compared to the mobile phase. This increases the adsorbate-adsorbate interactions in 29% acetonitrile relative to 44% acetonitrile (larger b_L in the BET equation). The larger value of b_L (adsorption-desorption coefficient of 2-chloro in second and subsequent layers) in 29% acetonitrile is seen in the general equation (equation 11) as a larger value of B (0.1717 in 29% acetonitrile versus 0.0745 in 44% acetonitrile).

The mechanism described above can not be applied to the separation of the enantiomers of the chiral product studied. The enantiomers have the same polarity, and would not be affected differently by a change in the polarity of the mobile phase. Due to the complex nature of chiral recognition mechanisms on chiral stationary phases (CSPs), the failure of the enantiomers to reverse elution order could not have been predicted. Possible reversal of elution order using alternate CSPs would have to be determined experimentally.

CONCLUSIONS

In the development of new pharmaceutical products, the separation and purification of all components is an essential process. Preparative scale chromatography has been useful for these separations in the pharmaceutical industry due to its ability to produce highly pure compounds. An increase in the number of developed analytical methods using preparative scale chromatography has led to an increased interest in the fundamentals of non-linear chromatography. An understanding of the thermodynamics and the kinetics involved in the chromatographic separation process is required for optimization of the experimental conditions in preparative chromatography. In preparative chromatography, the shape of the peak is

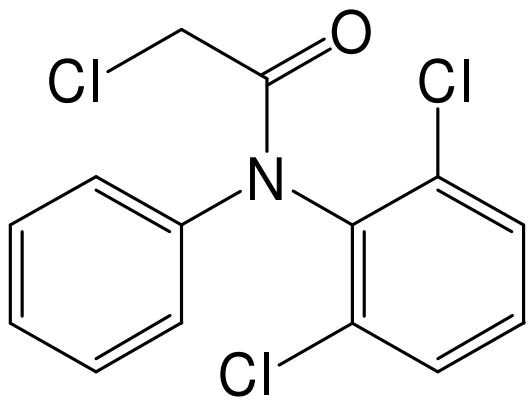


Figure 28. Structure of 2-chloro

determined largely by the equilibrium thermodynamics of the components. Strong non-linear behavior of the isotherms of the components can have a negative impact on the resolution at high concentrations. It is therefore beneficial to reverse the elution order of components in cases where the minor component needing to be isolated elutes after a major component.

A chiral compound was studied to determine if conditions could be found where a reversal in the elution order of the enantiomers was observed. Chromatographic conditions were altered, including the concentration of analyte injected, the pH of the mobile phase, the temperature of the column, the stationary phase, and the composition of the mobile phase (both concentration of organic and type of organic modifier). Separation was only achieved on a chiral AGP column using mobile phases with a pH of 3. Separation was achieved using various organic modifiers at various concentrations; however reversal of elution order was not observed at any condition.

A commercially available non steroid anti inflammatory drug (NSAID), diclofenac, and one of its process impurities, 2-chloro, was studied in order to determine if conditions could be found where a reversal in the elution order was observed. A method for the reversed phase chromatographic separation of diclofenac and 2-chloro was altered in order to observe how the changes affected the separation. During this study, it was determined that increasing the amount of acetonitrile in the mobile phase from 29% to 44% caused a reversal in the elution order of the compounds.

The equilibrium isotherms for diclofenac and 2-chloro at both high and low acetonitrile conditions were measured experimentally using frontal analysis and SAS statistical software was used to fit the experimental data to equilibrium isotherm models. diclofenac at the high acetonitrile condition fits the Langmuir isotherm model, diclofenac at the low acetonitrile

condition fits the Moreau isotherm model, and 2-chloro at both high and low acetonitrile conditions fit a BET-like isotherm model. Theoretical retention factors were confirmed by retention factors determined experimentally under linear conditions.

The reversal in elution order observed is most likely due to the difference in the equilibrium isotherms and the amount of adsorbate-adsorbate interactions occurring in the system at different conditions. The Moreau and BET isotherm models, unlike the Langmuir isotherm model, take into account interactions between adsorbed molecules in the stationary phase. Adsorbate-adsorbate interactions between molecules of 2-chloro in the stationary phase probably cause multi-layer adsorption, which is accounted for in BET-like isotherm models.

This research experimentally verified a reversal of elution order for diclofenac and its process impurity, 2-chloro. The thermodynamic and kinetic properties were studied by determination of equilibrium isotherms for each compound at both high and low acetonitrile conditions. Since a reversal in elution order was observed, both compounds were not expected to follow Langmuir isotherms. This research showed that the isotherms for diclofenac followed Langmuir-like isotherms and 2-chloro followed BET-like isotherms. The reversal in elution order was caused by a change in the amount of adsorbate-adsorbate interactions, which was increased when the polarity of the mobile phase was increased. Both 2-chloro and diclofenac are non-ionized and have low solubility at low mobile phase pH. 2-chloro is more polar than diclofenac, and this difference in polarity caused it to exhibit a higher degree of adsorbate-adsorbate interactions than diclofenac. This research has shown that in reversed phase chromatography, given two compounds with similar polarity, reversal of elution order will not be seen when the polarity of the mobile phase is changed. If the compounds are both non-ionized, have low solubility at low mobile phase pH, and have polarity differences, the isotherms of the

compounds are expected to be different. A reversal of elution order in reversed phase chromatography can be predicted qualitatively, however it is not possible to predict the reversal quantitatively (the exact amount of organic modifier needed to reverse the elution order must be determined experimentally).

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