ANALYTICAL METHOD DEVELOPMENT
A MATHEMATICAL APPROACH

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ABSTRACT

Drug development relies strongly on the construction of optimal analytical methods in an abbreviated timeframe in order to identify a final formulation and get to market quickly. Simplification of the analytical development process for multiple active products potentially provides a company with higher revenue as well as general cost reductions. This research strongly demonstrates the benefits of using systematic and mathematical approaches when developing analytical methods for a complex mixture.

By performing two gradient runs having different gradient slopes, and using a mathematical relationship between retention times of each solute for each gradient and each solute's characteristic values, s_j and $k'_{j,w,}$ the optimum gradient method for the separation of diclofenac, propoxyphene, and their respective impurities was developed. The method was further optimized by adjustment of the pH.

Using a mixture design approach, an isocratic method for a ternary system was developed for the aforementioned separation, which required three experiments to find the optimum mobile phase composition. A binary system for an isocratic separation was also developed. Development of this method was optimized by investigating the variations of characteristic values of each solute as a function of column temperature.

Additionally, a dissolution method was designed to mimic the release of diclofenac and propoxyphene once the drug product is ingested into the human body. A rapid isocratic HPLC method was developed for the determination of the amount of diclofenac and propoxyphene that is dissolved in dissolution samples.

ACKNOWLEDGMENTS

I would like to give sincere thanks to Dr. Dean Shirazi for his help, guidance, enthusiasm and encouragement. His strong knowledge of mathematics and chromatography was a tremendous asset throughout the course of this research and I am privileged and honored to have worked with him. I am grateful for Dr. Tania Toney-Parker who brain-stormed the idea of this particular combination product. She has thoughtfully given encouragement and support. I would also like to express my gratitude to Dr. John Tyrell, who has graciously given guidance throughout the Master's program. I would like to thank Dr. Ned Martin whose opinions I value and respect. I would like to acknowledge Chuck Bon and Lisa Olsen who processed results in SAS so quickly. Your help was invaluable and contributed to the success of my research. I would like to thank Mrs. Sherry Layton who always knew exactly what I was going through each day. Her encouragement and support has meant so much and I have found a friend for life. I am forever grateful to my husband, Michael, who sacrificed our time so that I could succeed. Special thanks go to my parents, William and Debbie, who gave endless loving support. Finally, I would like to acknowledge aaiPharma for the support both financially and professionally. I cannot express how grateful I am for this fantastic opportunity.

DEDICATION

I would like to dedicate this thesis to my husband, Michael, who has always believed in me.

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INTRODUCTION

As part of the formulation development of new pharmaceutical product lines, it is necessary to develop, optimize and validate reliable and meaningful analytical methods to support the integrity of a new product. Analytical methods are utilized for the characterization of the active pharmaceutical ingredient and its degradation profile, as well as a tool for the assessment, selection and optimization of prototypes. Analytical methodology is mandated by the FDA for determining the efficacy and safety of the final product and as a means of establishing a commercial shelf life. Analytical methods are the foundation for the success of any drug development (Green, 1996).

The safety and efficacy of a drug product is related to the purity of the drug and the formation of impurities that may potentially induce toxicological side effects. Therefore, efforts are made in the pharmaceutical industry to minimize impurities in active pharmaceutical ingredients and to control the degradation pathways of final formulations. Specific analytical methods are used to monitor the potency, process impurities, and any degradation impurities of both the drug substance and drug product during stability to assure the drug's safety and therapeutic activity.

High performance liquid chromatography (HPLC) is the preferred method for the quantitation of actives and impurities in pharmaceutical products because of its sensitivity, accuracy, specificity, separation capacity and widespread applicability (Snyder, 1997). Thus, development of a single reliable method for these actives and their impurities is necessary to save time and costs during prototype development, release of the new product, and lengthy stability studies.

Not only are analytical methods used to characterize the drug substance and drug product on the shelf but they may also serve as predictive tools to establish *in-vivo* response. For example, discriminating dissolution tests are developed to simulate the *in-vivo* environment. Based upon the defined marketed product label, a release profile may be adjusted using the dissolution rate. An optimal dissolution method not only can be used for prototype selection, it can also demonstrate batch to batch uniformity to assure product performance and can also be used to assess bioavailability which may replace the need for multiple bioequivalence clinical studies (Crison). It is also necessary to establish an *in-vitro* to *in-vivo* correlation where the dissolution will simulate the drug disintegration, dissolution, and solubilization in the aqueous environment of the gastrointestinal (GI) tract. Appropriate dissolution methods are also used for quality control purposes; product dissolution needs to be consistent with a minimal relative standard deviation. Dissolution testing serves a variety of purposes and can be a meaningful tool for assessing potential changes in bioavailability over the duration of stability.

It is very common for analysts in industry to begin HPLC method development using a non-systematic and non-mathematical approach when developing an analytical method. For example, an analyst will usually initiate development of an isocratic run with a certain percentage of organic solvent based on the analyst's previous experience or based on a literature method for a similar analyte and then make stepwise adjustments. The retention time of an analyte in reversed phase chromatography decreases when the percentage of organic is increased. Thus, the desired composition of mobile phase can be estimated using this technique. However, this approach is based on trial-and-error and

does not necessarily produce optimum separation especially for a multiple component system, which has several actives, related substances and impurities. The trial-and-error approach, although simple and easy to apply, suffers from serious drawbacks as it is laborious and contains many uncertainties.

Unlike the trial-and-error approach, the method development of this research began with a logical systematic mathematical approach. The characteristic constants of each solute using linear gradient experiments were first determined. In gradient elution chromatography, which was introduced by Alm, et al. about 50 years ago, the elutropic strength of the mobile phase is progressively increased during the separation (Alm, 1952). In principle, any type of gradient profile can be applied. Linear composition gradient and step gradient are the simplest and most popular composition variations. A linear gradient can be expressed as shown in Equation 1;

$$\varphi(t,z)=\varphi_i+b\left(t-\tau-\frac{z}{u}\right) \tag{1}$$

where φ_i is the initial modifier concentration, b is the gradient slope, t is the time, τ is the gradient delay time, u is the linear velocity, and z is the distance along the column. At the end of the column, z equals the length of the column. When gradient systems are used, especially in low pressure gradient systems, there is a delay in the delivery of the gradient to the head of the column which is called the gradient delay time, τ. This is due to the extra volume from the solvent delivery system to the column inlet which is called the dwell volume.

Extensive studies report the dependence of the capacity factor of compounds on the organic modifier concentration of mobile phase in reversed phase chromatography

Synder, 1980; Jandera, 1985). These studies indicate that, within a reasonable range, the dependence can be expressed as shown in Equation 2 which can be written as Equation 3;

$$k'_j(\varphi) = k'_{j,w} e^{s_j \varphi} \quad (2)$$

$$\ln k'_j(\varphi) = \ln k'_{j,w} + s_j \varphi \quad (3)$$

where s_j is the slope of the logarithmic plot, and $k'_{j,w} = k'_j(\varphi = 0)$ is the capacity factor of the solute in pure weak eluent (such as water or buffer). $k'_{j,w}$ and s_j are the characteristic constants of each solute. In a wider range of modifier concentration, a quadratic relationship may give a better account for the dependence of $\ln k'_j$ (Equation 4).

$$\ln k'_j(\varphi) = \ln k'_{j,w} - a\varphi + b\varphi^2 \quad (4)$$

In order to predict the retention time of each solute (j) at any specific composition of organic modifier (φ), the value of $\ln k'_{j,w}$ and s_j should be determined. There are two procedures for their determination. The first involves injection of a solution of each solute at various isocratic conditions (various φ) and determining k'_j from the retention times using Equation 5 then plotting $\ln k'_j$ versus φ for each solute. This procedure is cumbersome and time consuming.

$$k'_j = \frac{t_{rj} - t_0}{t_0} \quad (5)$$

A simpler and more accurate procedure is to run two linear gradients of a mixture of solutes with different slopes and record the retention time of each solute in each gradient run.

Method Development Theory of a Binary System

It has been shown that the retention time of each solute in a linear gradient, provided that the solute elutes before the completion of the gradient, is given by Equation 6 (Snyder, 1980; Schoenmakers, 1978 and 1991).

$$t_{r_j} = -\frac{1}{s_j \times b} \times \ln\left\{1 - \left[s_j \times b \left(t_0 - \frac{\tau}{k'_{j_{\varphi i}}} \right) \times k'_{j_{\varphi i}} \right]\right\} + \tau + t_0 \qquad (6)$$

where t_{rj}, is the retention time of a solute (j) in each gradient run, s_j is the slope of the graph of ln k_j' vs. φ, b is the gradient slope, t_0 is the column hold-up time, τ is the gradient delay time, and $k'_{j\varphi\, i}$, is the capacity factor of a solute (j) at the start of the gradient ($\varphi = \varphi_i$). In many cases, $k'_{j\varphi\, i}$ is very large so that $t_0 >> \tau / k'_{j\varphi\, i}$ and $s_j\, b\, k'_{j\varphi\, i}\, t_0 >> 1$. Therefore, Equation 6 can be simplified to Equation 7.

$$t_{r_j} = -\frac{1}{s_j \times b} \times \ln\left(s_j b t_0 k'_{j\varphi i}\right) + \tau + t_0 \qquad (7)$$

Equation 6 is valid only for solutes that elute before the completion of the gradient ($\varphi = \varphi_f$) (Schoenmakers, 1978). For solutes that elute after the completion of the gradient, Schoenmakers defines the following analytical solution (Equation 8 and 9) to predict the retention times in the gradient run (Schoenmakers, 1978).

$$t_{r_j} = k'_{j\varphi f}\left(t_0 - \frac{\tau}{k'_{j\varphi i}} \right) + \frac{k'_{j\varphi f}}{s_j \times b}\left(k'_{j\varphi f} - k'_{j\varphi i}\right) - \frac{\varphi_i - \varphi_f}{b} + \tau + t_0 \qquad (8)$$

$$\ln\left(k'_{j\varphi f}\right) = \ln\left(k'_{j\varphi i}\right) + s_j\left(\varphi_f - \varphi_i\right) \qquad (9)$$

Where $k'_{j\varphi i}$ is the capacity factor at the beginning of the gradient and $k'_{j\varphi f}$ is the capacity actor at the end of the gradient. Equation 8 was investigated when the predicted retention time results obtained using this equation were very different than actual measured retention times. By re-deriving this equation as shown in Appendix A, a mathematical error was found in Equation 8 and the following equation was derived (Equation 10);

$$t_{r_j} = k'_{j\varphi f}\left(t_0 - \frac{\tau}{k'_{j\varphi i}}\right) + \frac{1}{s_j \times b}\left(\frac{k'_{j\varphi f} - k'_{j\varphi i}}{k'_{j\varphi i}}\right) - \frac{\varphi_i - \varphi_f}{b} + \tau + t_0 \qquad (10)$$

where $k'_{j\varphi f}$ is related to $k'_{j\varphi i}$ through Equation 9.

Column Hold-Up Volume and Column Hold-Up Time

In order to use Equation 6 or 10, the value of the column hold-up time must be determined. The volume of the mobile phase required to elute a non-retained component is called the column hold-up volume and the corresponding time is called the column hold-up time. They are related according to Equation 11;

$$V_0 = F_v \times t_0 \qquad (11)$$

where V_0 and t_0 are column hold-up volume (mL) and column hold-up time (minutes) and F_v is the mobile phase flow rate (mL per minute). The column hold-up volume, V_0, and hold-up time, t_0, are characteristics of a column. Different methods for the determination of the hold-up time have been reviewed in detail (Grushka, 1982). It is usually measured by injecting an inert or non-retained tracer. Unlike gas chromatography, the definition and determination of the hold-up volume is not straight forward. The density of the mobile phase in the bulk mobile phase and that in the monolayer in contact with the surface of the stationary phase is not usually the same. The situation is more complex in

reversed-phase HPLC because the bonded layer swells when the proportion of the organic modifier in the mobile phase increases (McCormick, 1982). The organic modifier dissolves in the bonded layer and when its concentration in this layer is sufficient, some molecules of water may also penetrate into it. The value obtained for the hold-up time depends on the selection of the tracer. The best tracer is an isotopically labeled compound but that is impractical (McCormick, 1982). Therefore, most often an unretained solute is used for the hold-up measurement. In reverse-phase HPLC, thiourea or uracil is commonly used.

Method Development of a Binary System

The retention time of a component at any composition of mobile phase, φ, in an isocratic separation can be predicted by running two gradient methods at two different gradient slopes. It can also be determined whether better separation is achieved by either isocratic or gradient methods. By rewriting Equation 6 for the retention times of each solute as it elutes before the completion of the gradient or Equation 10 for each solute as it elutes after the completion of the gradient, the values of $k'_{j\varphi i}$ and s_j can be calculated.

Once $k'_{j\varphi i}$ and s_j values are known, the capacity factor at 0% organic ($k'_{j,w}$) can be calculated for each solute by means of Equation 12.

$$\ln k'_{j,w} = \ln k'_{j_{\varphi i}} - s_j \varphi_i \qquad (12)$$

Using a plot of $\ln k'_j$ vs. φ for each solute, an isocratic separation and optimum solvent composition for the mobile phase can potentially be identified. In addition, the capacity factor, (k'_j), of each solute at any percentage of organic can also be calculated using the following relationship shown in Equation 13.

$$\ln k'_{j,\varphi} = \ln k'_{j,w} + s_j \varphi \tag{13}$$

The retention time can then be calculated from the $k'_{j,\varphi}$ for any given φ with Equation 14.

$$t_{r_j} = t_0 \times (1 + k'_{j,\varphi}) \tag{14}$$

Isoelutropic Binary Expression

When the optimum separation with a certain organic modifier (such as acetonitrile) is determined using the aforementioned approach and still the separation of all peaks of interest is not achieved, the next step in method development is to change the organic modifier to a different solvent. The concentration of the new organic modifier (such as methanol or tetrahydrofuran) in the new binary system, which is predicted to yield a chromatogram exhibiting the same range of *k*' values, can be calculated using the "transfer rule" equation (Schoenmakers, 1981).

After selecting the acetonitrile-buffer elutropic strength, $\varphi_{ACN,}$ using the aforementioned approach, which produces an optimum chromatogram in which all solutes of the mixture elute with a suitable range of retention times, calculation of equivalent elutropic strength relative to $\varphi_{ACN,}$ for methanol-buffer and THF-buffer can be performed using the solvent polarity scale first described by Snyder in 1974. All binary eluents with equivalent solvent polarity are, to a first approximation, assumed to be isoelutropic. The expression given by Snyder for calculating the solvent polarity is shown in Equation 15;

$$P'_{mixture} = \varphi_A P'_A + \varphi_B P'_B \tag{15}$$

where φ_A and φ_B are the volume fraction of solvents A and B and P'_A and P'_B are the polarity index values of the pure solvents A and B. The *P'* value for water, methanol, acetonitrile, and tetrahydrofuran (THF) are 9.0, 6.6, 6.2, and 4.2 respectively. An alternative approach for obtaining the composition of isoelutropic binary solvents is derived by Schoenmakers (1981). Based on the isocratic retention behavior of a set of 32 solutes in all three binary eluents (acetonitrile-water, methanol-water, and THF-water), "transfer rule" equations relating isoelutropic volume fractions were expressed in Equations 16 and 17.

$$\varphi_{ACN} = 0.32\varphi_{MeOH}^2 + 0.57\varphi_{MeOH} \tag{16}$$

$$\varphi_{THF} = 0.66\varphi_{MeOH} \tag{17}$$

Equations 16 and 17 represent an average of the eluent transfer behaviors of each of the solutes of the data set considered. The scatter of these average predicted values is fairly large such that a large deviation between predicted and actual isoelutropic volume fractions is observed in practice. Therefore, these equations provide only a first approximation prediction of equivalent elutropic strengths among the three binary eluents. A re-evaluation of these "transfer rule" equations are described by Herman, et al. who proposed Equations 18 and 19 for isoelutropic volume correlation (Herman,1989).

$$\varphi_{ACN} = -0.49\varphi_{MeOH}^3 + 0.953\varphi_{MeOH}^2 + 0.447\varphi_{MeOH} \tag{18}$$

$$\varphi_{THF} = -0.42\varphi_{MeOH}^3 + 0.702\varphi_{MeOH}^2 + 0.423\varphi_{MeOH} \tag{19}$$

Applying Mixtures of Organic Solvents

When an isocratic separation is not successful using any of the binary systems, it may be necessary to use a ternary or quaternary system. In recent years, many practical

examples of the advantages to use of ternary mobile phase in reversed phase liquid chromatography have been published (Bakalyar, 1977; Glajch, 1980). Bakalyar, et al. (1977) performed the first systematic investigation of a ternary mobile phase. Glajch, et al. (1980) studied the behavior of ternary and quaternary mixtures of water, acetonitrile, methanol, and THF. They also describe a procedure for an optimization of a multi-component mobile phase. Schoenmakers, et al (1981). reported a systematic study of the retention behavior of two ternary mobile phase systems (methanol, acetonitrile, water and methanol, THF, water). They showed that the relationship of the logarithm of the capacity factor to the volume fraction of the two organic modifiers can be expressed by a quadratic equation (Schoenmakers, 1981).

$$\ln k' = A_1\varphi_1^2 + A_2\varphi_2^2 + B_1\varphi_1 + B_2\varphi_2 + D_1\varphi_1\varphi_2 + \text{constant} \qquad (20)$$

There are a number of alternative methods in the literature for optimizing ternary and quaternary separations and these include the simplex approach and the mixture design approach.

Simplex Approach

One approach for optimizing ternary and quaternary separations is the sequential simplex method which was first proposed by Spendly, et al. in 1962 (Berridge, 1988). The simplex procedure is a hill-climbing method in which the direction of advance is dependent solely on the ranking of responses (Berridge, 1988). The great advantage of the simplex procedure in the optimization of liquid chromatography separations is that it is able to optimize many inter-dependent variables without prior knowledge about the mode of separation or the complexity of the samples. It also does not require any pre-

conceived model for the retention behavior of solutes. There are however, significant disadvantages associated with simplex optimization. Most notable is the problem of locating a local rather than a global optimum. An additional disadvantage is the large number of experiments required.

Mixture Design Approach

Another approach for optimizing ternary and quaternary separations is the mixture design approach. This statistical approach is suitable for related variables. Related variables are those that directly affect each other. For example, the sum of the mobile phase composition must be 100% at all times, so individual mobile phase solvents are related variables. This approach is well known in statistical literature (Cornell, 1981). The mixture design statistical approach for mobile phase optimization is illustrated in Figure 1. Seven experiments are employed to fit experimental retention data (ln *k'*) to a second order polynomial equation with respect to three mobile phase modifiers. In the case of reversed-phase liquid chromatography, the mobile phase carrier, water or buffer, is modified with acetonitrile, methanol, and tetrahydrofuran (THF). In normal phase, n-hexane or n-heptane is the carrier solvent and it is modified with chloroform, methylene chloride, and methyl-tertbutyl ether (MTBE). Retention (*k'*) values are measured for each component in the mixture using the seven mobile phase solvent mixtures shown in Figure 1. These data are then fitted to a second order polynomial to obtain the constants in the following equations, from which the optimum mobile phase composition can be determined (Glajch, 1983).

For a Quaternary System:

$$\ln k_j' = a_1x_1 + a_2x_2 + a_3x_3 + a_{1,2}x_1x_2 + a_{2,3}x_2x_3 + a_{1,3}x_1x_3 + a_{1,2,3}x_1x_2x_3 \quad (21)$$

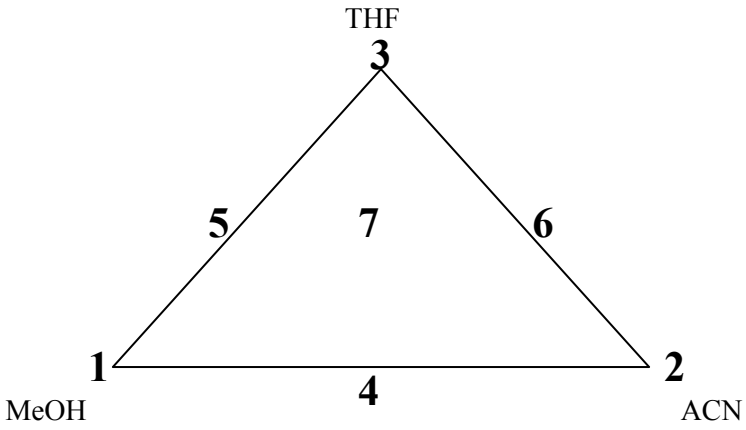


Figure 1. Overlapping mixture design model

For a Ternary System:

$$\ln k_j^{'} = a_1 x_1 + a_2 x_2 + a_{1,2} x_1 x_2 \quad (22)$$

This systematic approach can determine the optimum ratio of mobile phase with a small number of experiments. Instead of ln k', the resolution of each peak pair in the system also can be mapped using the same methodology (Glajch, 1983; Ong, 1995).

Optimization by Factorial Design

Unlike mobile phase composition, pH and temperature are discrete variables since they have little direct effects on each other or on other mobile phase or stationary phase components. For discrete variables such as temperature and pH, the factorial design is suitable. Figure 2 illustrates a factorial design for optimization of temperature (20-40 °C) and pH (3-5) effects in liquid chromatography that employs nine experiments (3 levels and two factors) to fit the retention time data (ln k') of each solute in these nine experiments to a second order polynomial to determine the optimum temperature and pH (Glajch, 1983).

EXPERIMENTAL

Equipment

The high performance liquid chromatography (HPLC) equipment used for all experiments was a Hewlett-Packard 1100 equipped with a gradient pump, autosampler, temperature controlled column compartment and an ultraviolet wavelength detector. The columns used in the experiments were X-Terra MS C18, 150 × 4.6 mm, 3.5 μm particle size and X-Terra RP C18, 150 × 4.6 mm, 5.0 μm particle size. The X-terra columns were selected because of their ability to withstand higher pHs. Potassium phosphate

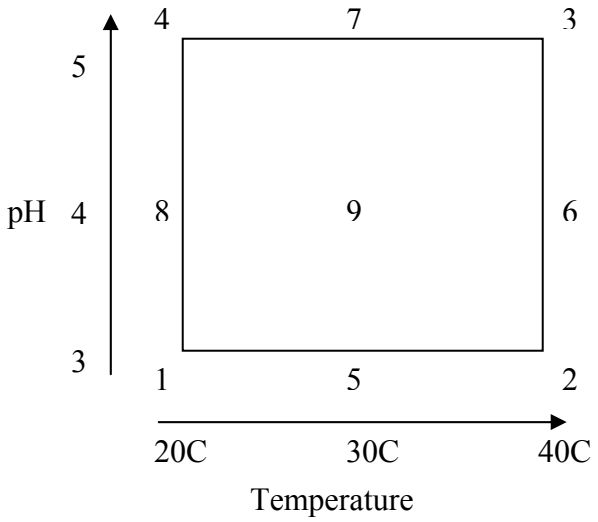


Figure 2. Factorial design

monobasic at a concentration of 50 mM was used for the aqueous portion of the mobile phases in combination with organic solvents acetonitrile, methanol or both. Injections were analyzed at 217 nm. The low wavelength enhanced the detection of most solutes.

Determination of the Column Hold-Up Time

A measurement of the column hold-up time, t_0, was needed in the calculations to predict the retention times. It was measured by an injection of a sodium nitrate solution, an inert compound at a low concentration. A small peak eluted within the solvent front (Figure 3). The retention time of this peak is equal to the column hold-up time.

Determination of the Gradient Delay Time

A measurement of the gradient delay time, τ, is necessary to predict the retention time of the solutes. To measure the delay time, τ, a step gradient was used. To perform frontal analysis, the mobile phase must contain a chromophore such as acetone to promote UV absorption. A solution of 1% acetone in methanol was prepared and used as solvent B. Pure methanol was used as solvent A. The column was disconnected and the inlet and outlet tubing were connected using a dead volume union. The HPLC system was equilibrated with solvent A (100% methanol). After 2 minutes of Solvent A, the mobile phase was switched to solvent B (1% acetone in methanol) giving a breakthrough curve as shown in Figure 4. The gradient delay time is measured using the breakthrough curve.

Computer Software and Program

Waters Millennium 4.0 was used as the data acquisition program to collect chromatograms for each injection and to measure the retention times and resolutions. For

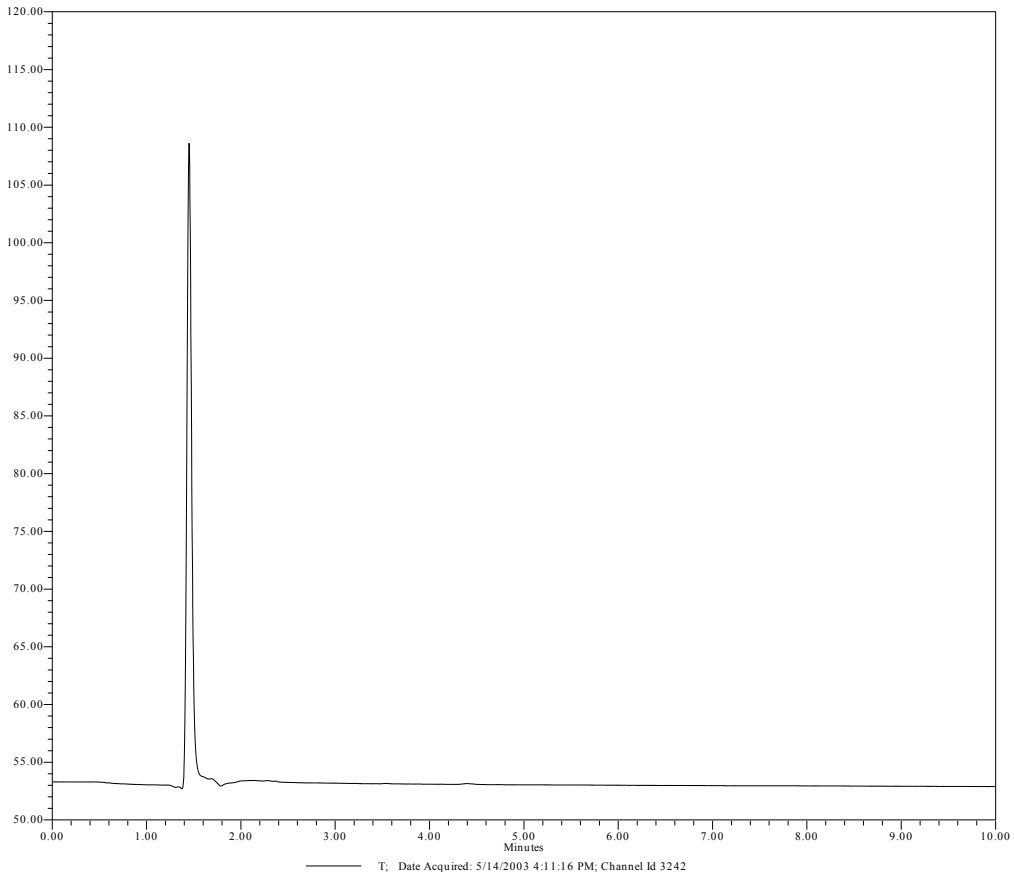


Figure 3. Chromatogram of sodium nitrate for the measurement of void time of the column

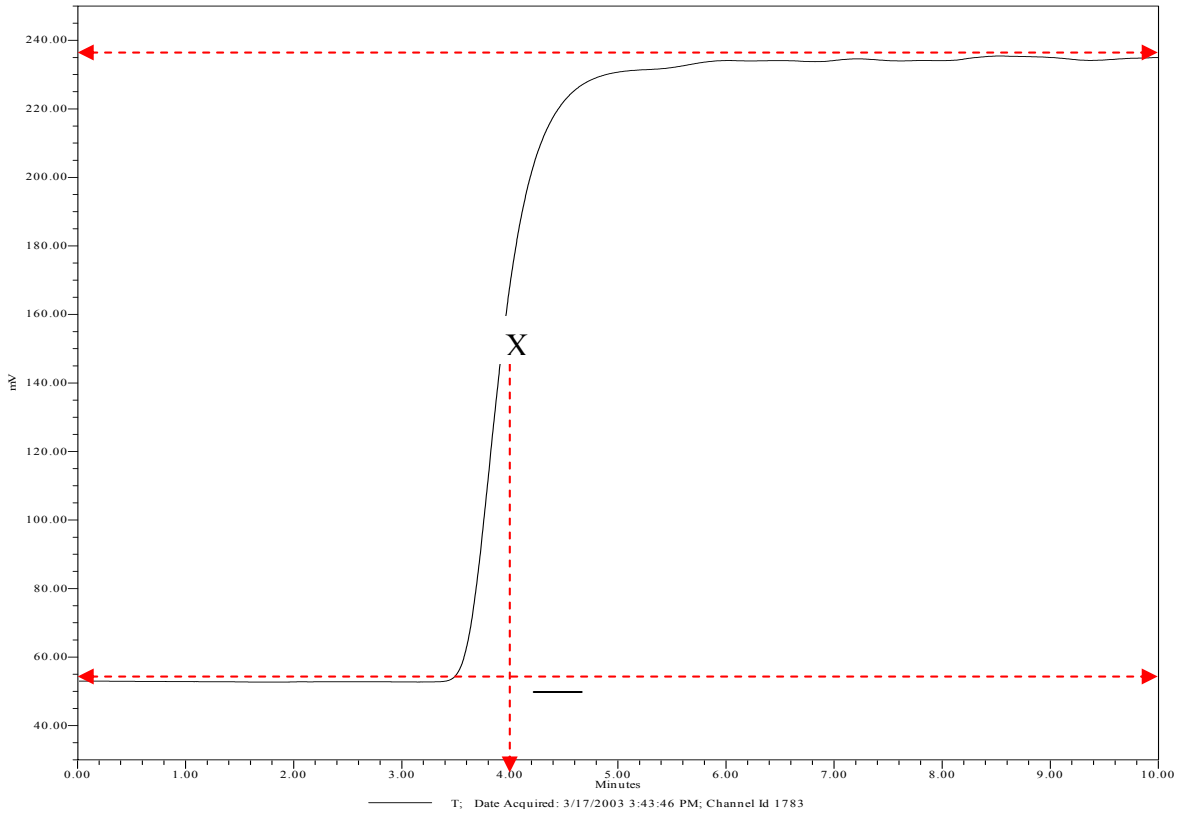


Figure 4. Chromatogram of a breakthrough curve using acetone to calculate the delay time of the gradient

the calculations of $k'_{j\varphi_i}$ and s from equation 6 and/or 10 using data input (retention times of solutes, t_{rj}, gradient delay time, τ, column hold-up time, t_0 and gradient slope, b), a non-linear modeling program in SAS® PC version 6.12 was used to solve for these parameters. Microsoft Excel 2003 was used to calculate $k'_{j,w}$ and to create the plots of ln k'_j versus organic fraction. Excel was also used during isoelutropic and mixture design experiments to calculate the optimum mobile phase composition.

Chemical Information

The following list provides the names of the actives and available related substances that were used in this research. Individual solutions were prepared at a concentration of 0.2 mg/mL for the actives and 0.002 mg/mL for the related substances and process impurities.

List of Components	Component Type
Propoxyphene Napsylate	Active
Diclofenac Potassium/Diclofenac Sodium	Active
2-Indolinone	Related Substance
Propoxyphene Related Compound A (Prop Rel Cmpd A)	Related Substance
Propoxyphene Related Compound B (Prop Rel Cmpd B)	Related Substance
Cis-4-dimethylamino-1,2-diphenyl-3-methyl-butene (CT1)	Related Substance
Diclofenac Related Compound A (Diclo Rel Cmpd A)	Related Substance
[2-[(2,6-Dichlorophenyl)amino]phenyl]methanol (Alcohol)	Related Substance
2-Chloro-n-(2,6-dichlorophenyl)acetamide (2 Chloro)	Process Impurity
2,6-Dichlorodiphenylamine (Dichloro)	Process Impurity
2-[(2.6-Dichlorophenyl)amino]benzaldehyde (Aldehyde)	Related Substance

Procedure

Schoenmakers' equations, Equations 23, 24, and 25, were implemented to predict

the retention times of each solute. Injections of solutes were performed for two different gradient runs having different gradient slopes. The retention times were measured by the data acquisition system. For each gradient, the retention time was set equal to Schoenmakers' expression for each solute.

For solutes that elute before the completion of the gradient;

$$t_{r_j} = -\frac{1}{s_j \times b} \times \ln\left\{1 - \left[s_j \times b \left(t_0 - \frac{\tau}{k'_{j_{\varphi i}}} \right) \times k'_{j_{\varphi i}} \right]\right\} + \tau + t_0 \tag{23}$$

For solutes that elute after the completion of the gradient:

$$t_{r_j} = k'_{j\varphi f}\left(t_0 - \frac{\tau}{k'_{j\varphi i}} \right) + \frac{1}{s_j \times b}\left(\frac{k'_{j\varphi f} - k'_{j\varphi i}}{k'_{j\varphi i}} \right) - \frac{\varphi_i - \varphi_f}{b} + \tau + t_0 \tag{24}$$

$$\ln\left(k'_{j\varphi f}\right) = \ln\left(k'_{j\varphi i}\right) + s_j\left(\varphi_f - \varphi_i\right) \tag{25}$$

All the variables in these equations are known except for the capacity factor at the beginning of the gradient run for each solute, $k'_{j\,\varphi\,i,}$ and the slope for each solute, s. Once $k'_{j\varphi\,i}$ was determined, the capacity factor of the solute in pure weak eluent, $k'_{j,w}$ (y-intercept) was calculated using the following linear relationship.

$$\ln k'_{j_w} = \ln k'_{j_{\varphi i}} + s_j \varphi_i \tag{26}$$

Once $k'_{j,w}$ and s were calculated, a plot of ln $k'_{j\varphi}$ versus. s was established to predict the retention time of each solute at any organic fraction. The trendlines for each

solute indicated the expected separation of each solute. The closer the trendlines appeared in the plot, the less separation was expected in the chromatography.

RESULTS AND DISCUSSION

Effect of pH

For basic solutes, generally the retention time increases with increasing pH as long as the pH does not exceed the log of the ionization constant for a base, pKb. Typically, basic solutes are protonated at a pH lower than their pKb. As pH increases, the ionization of the base decreases, the solute becomes hydrophobic, preferring the non-polar stationary phase which increases the retention time. However, the decrease in retention time at a pH higher than pKb cannot be explained in this way. The degree of protonation of the solute and the residual silanol group must also be considered. By increasing the pH of the mobile phase, more residual silanol groups are negatively charged and these groups behave as a weak cation exchanger. When the pH of the mobile phase is higher than the pKb of the solute, the protonation of the basic solute is suppressed causing less interaction with residual silanol groups and therefore decreasing the retention time. The influence of pH on the retention of acidic solutes is the opposite of that observed with basic solutes. The retention times of the acidic solutes decreases with increasing pH. Acids are negatively charged at pH higher than the log of the ionization constant for an acid, pKa, preferring the polar mobile phase. A second reason for the decreased retention time might be that at higher pH the residual silanol groups and the acidic solute are negatively charged so the solute is excluded from the stationary phase. The decrease in retention time is more pronounced for stronger acids than for weaker acids.

In order to determine the behavior of the solutes based on their sensitivity to pH of the mobile phase, two gradient runs, each with different gradient slopes were performed for pH 2.5, 6.8, and 9. The experiments employed the X-Terra MS 150 x 4.6 mm, 3.5 μm particle size column with a flow rate of 1.0 mL/minute. Mobile phase A was buffer and acetonitrile in a volumetric ratio of 90:10 and mobile phase B was buffer and acetonitrile in a volumetric ratio of 35:65. The first gradient slope was 1.6% per minute or 2% to 98% mobile phase B in 37 minutes and the second gradient slope was 1.1% per minute or 2% to 98% mobile phase B in 55 minutes. After the completion of the gradient, the mobile phase remained at the final composition for at least 10 minutes to allow any late eluting solutes to elute during the run time. The retention time of each solute was measured for each gradient run at each pH. The slopes, *s*, and capacity factors in pure weak solvent ($k_{j,w}$) were calculated by SAS (Tables 1-3). Plots of ln k'_j versus the fraction of organic (φ) were established for each pH (Figure 5-7).

Because most of the solutes are basic, the retention time increased as the pH increased. Generally, a low pH of about 2.5 to 3.0 is a good starting point for the separation of the mixture of acids and bases. At this pH range, the basic solutes are less retained as they are protonated while the acidic solutes exhibit greater retention as they are mainly non-ionic. Therefore, both acidic and basic solutes can be separated within a reasonable analysis time. The results of this study confirmed the general rule that at higher pH, generally, acidic solutes are less retained and basic solutes are more retained resulting in a long impractical analysis time. As shown from the plots in Figures 5-7, the run time is increased with increasing pH at a certain percentage of organic modifier. The run time can be decreased by increasing the percent of organic in the mobile phase;

Table 1. Calculated characteristic values of s and $k'_{j,w}$ at pH 2.5 using SAS, based on Schoenmakers' equations

Components	**s**	$\boldsymbol{k'_{j,w}}$
Napsylate	-19.464	4.581
2-Indolinone	-11.927	3.511
Propoxyphene Related Compound A	-14.109	5.166
Propoxyphene Related Compound B	-14.252	5.444
CT1	-13.838	5.665
Propoxyphene	-14.879	6.007
Diclofenac Related Compound A	-10.033	5.824
Alcohol	-13.987	7.515
Diclofenac	-9.941	5.988
2 Chloro	-9.634	6.054
Dichloro	-8.847	6.141
Aldehyde	-10.431	6.844

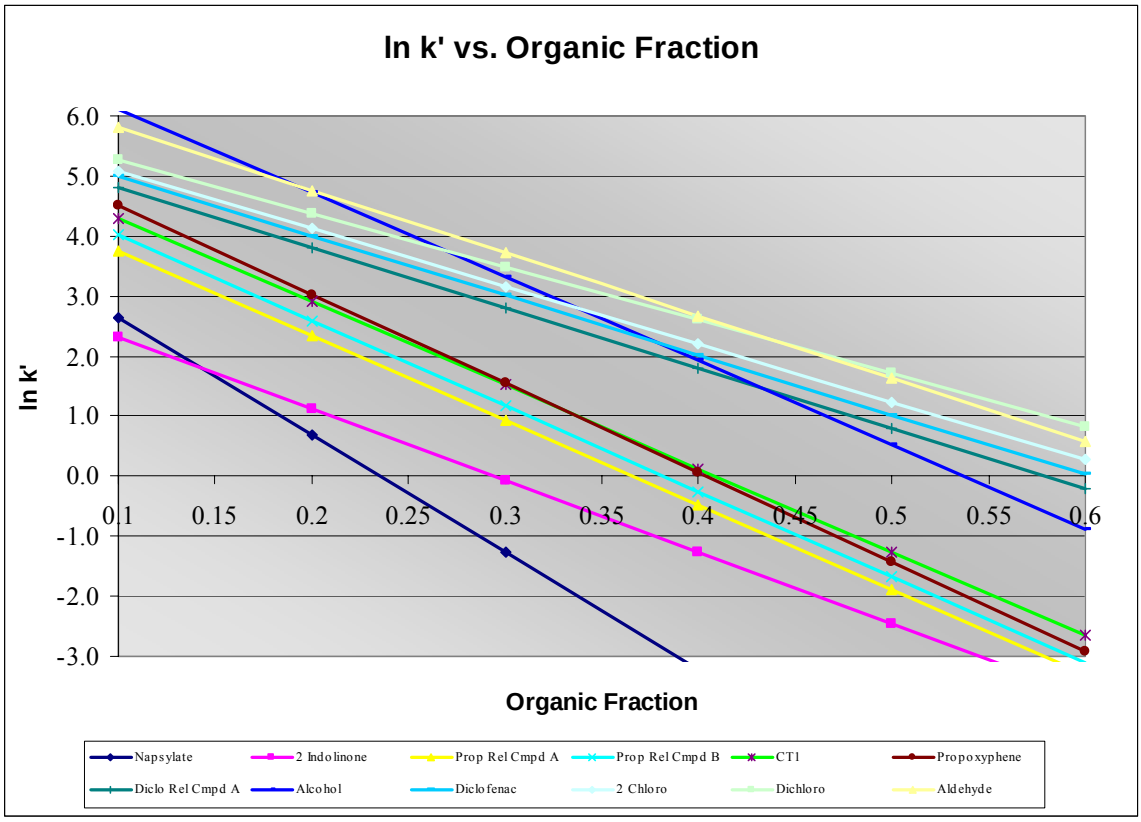


Figure 5. Plot of ln *k*' vs. organic fraction at pH 2.5 based on Schoenmakers' equations

Table 2. Calculated characteristic values of s and $k'_{j,w}$ at pH 6.8 using SAS, based on Schoenmakers' equations

Components	**s**	$\boldsymbol{k'_{j,w}}$
Napsylate	-16.230	4.152
2-Indolinone	-13.321	3.563
Propoxyphene Related Compound A	-10.688	5.357
Propoxyphene Related Compound B	-12.283	5.936
CT1	-10.423	6.055
Propoxyphene	-11.033	6.239
Diclofenac Related Compound A	-10.918	6.716
Alcohol	-11.012	7.057
Diclofenac	-17.881	7.041
2 Chloro	-10.612	7.097
Dichloro	-10.505	7.574
Aldehyde	-10.162	7.447

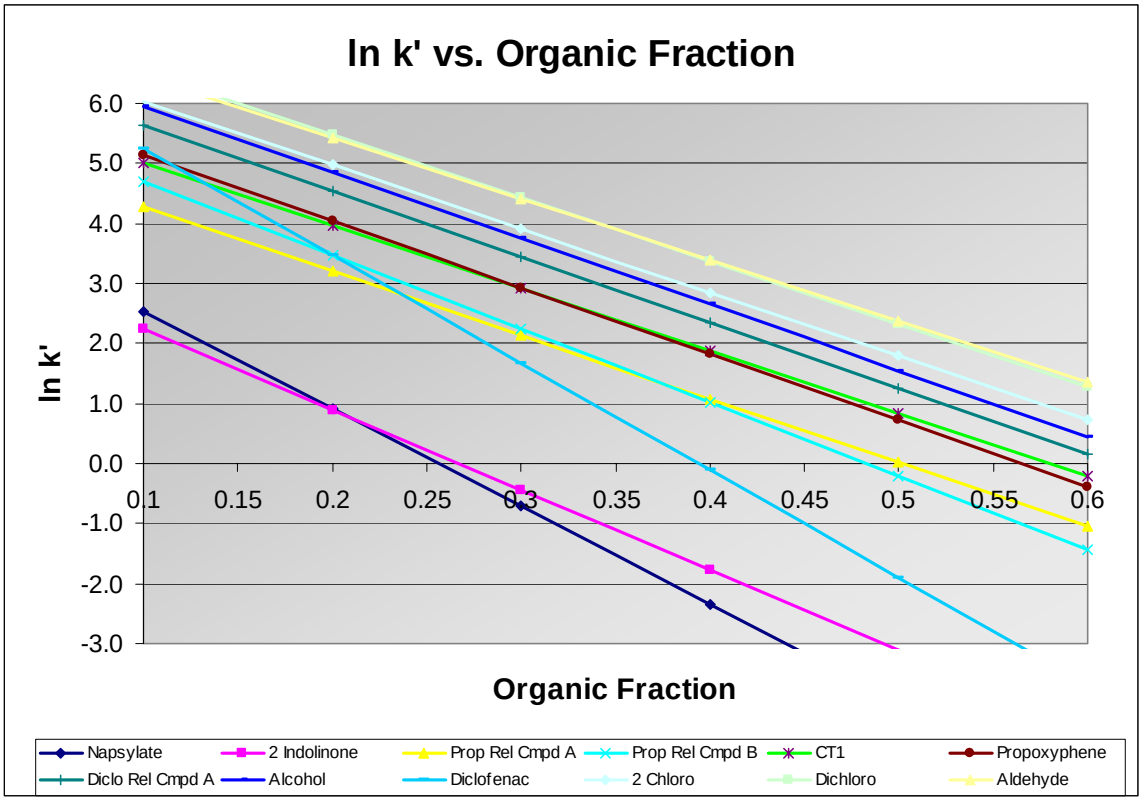


Figure 6. Plot of ln *k*' vs. organic fraction at pH 6.8 based on Schoenmakers' equations

Table 3. Calculated characteristic values of s and $k'_{j,w}$ at pH 9.0 using SAS, based on Schoenmakers' equations

Components	**s**	**$k'_{j,w}$**
Napsylate	-16.819	39.635
2-Indolinone	-12.815	22.292
Propoxyphene Related Compound A	-8.706	883.918
Propoxyphene Related Compound B	-9.841	793.108
CT1	-7.227	581.281
Propoxyphene	-9.429	1074.221
Diclofenac Related Compound A	-10.978	590.021
Alcohol	-10.807	754.045
Diclofenac	-18.888	730.689
2 Chloro	-10.551	830.537
Dichloro	-10.183	1186.919
Aldehyde	-10.133	1223.991

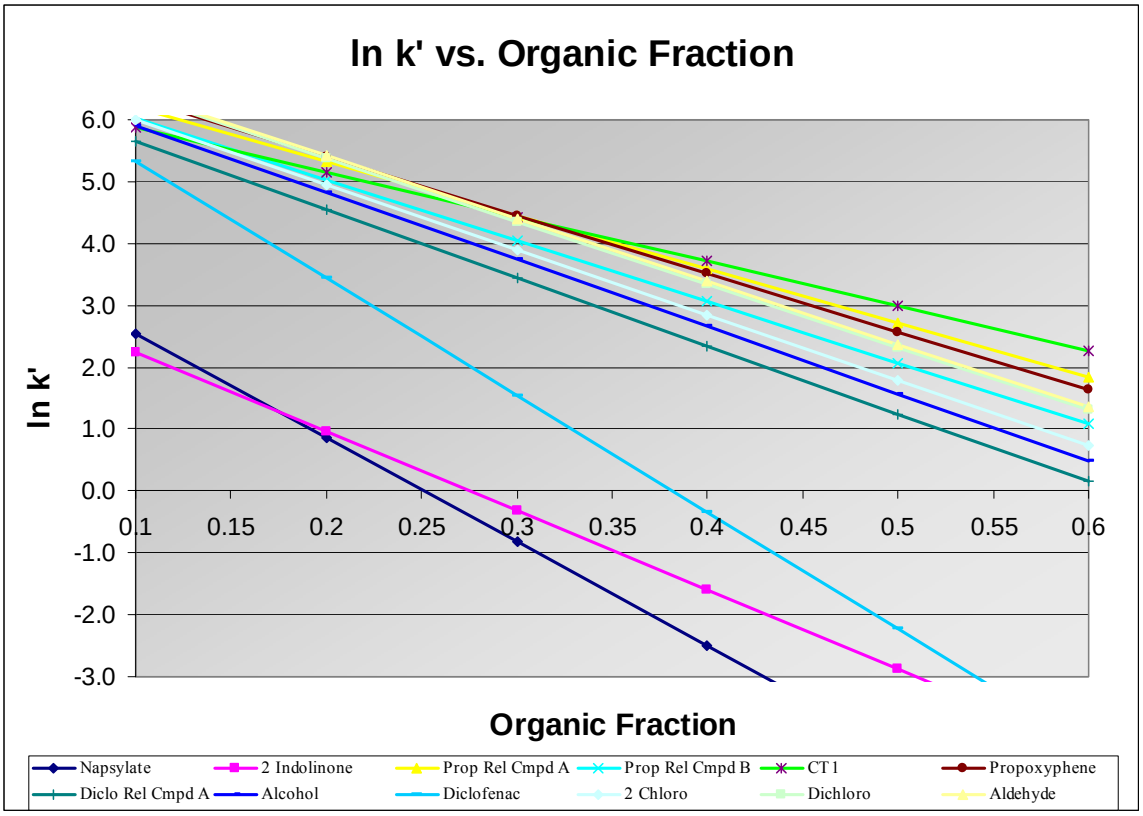


Figure 7. Plot of ln *k*' vs. organic fraction at pH 9.0 based on Schoenmakers' equations.

however, higher organic will cause some solutes to elute in the solvent front. Therefore, the lower pH would be more suitable to give a shorter run time. The pH of 2.5 was identified to be the optimal pH to initiate development due to both a reasonable retention time and better resolution of the solutes relative to that of the other pHs. The pH also may be optimized, if required, to provide better resolution of critical pairs.

Method Development of a Binary System

After selecting the starting pH of 2.5, two gradient runs, each with different gradient slopes were performed. The experiments utilized the X-Terra MS 150 x 4.6 mm, 3.5 μm particle size column with a flow rate of 1.0 mL/minute. Mobile phase A was buffer, pH 2.5 and acetonitrile in a volumetric ratio of 90:10 and mobile phase B was buffer, pH 2.5 and acetonitrile in a volumetric ratio of 35:65. The first gradient slope was 2.5% per minute or 5% to 95% mobile phase B in 20 minutes and the second gradient slope was 1.2% per minute or 5% to 95% mobile phase B in 40 minutes. After the completion of the gradient, the mobile phase remained at the final composition for at least 10 minutes to allow any late eluting solutes to elute during the run time. All solutes eluted before the completion of the gradient with the exception of dichloro and aldehyde in the 2.5% gradient slope. The slope, s and capacity factor in pure weak solvent ($k_{j,w}$) were calculated by SAS (Table 4) and a plot of ln k'_j versus the fraction of organic (φ) was established (Figure 8). This plot provided important information that can aid in the development of an optimal isocratic method such as run time and coelution of solutes, demonstrated by the overlapping of trendlines. A reasonable run time for this complex separation is about 60 minutes or shorter. Based on Figure 8, a run time of less than 60

Table 4. Calculated characteristic values of s and $k'_{j,w}$ at pH 2.5 using SAS, based on Schoenmakers' equations

Components	**s**	k'_w
Napsylate	-14.761	3.903
2-Indolinone	-10.154	3.021
Propoxyphene Related Compound A	-15.376	5.834
Propoxyphene Related Compound B	-15.835	6.331
CT1	-15.943	6.837
Propoxyphene	-15.747	6.888
Diclofenac Related Compound A	-10.300	6.501
Alcohol	-10.214	6.746
Diclofenac	-11.204	7.143
2 Chloro	-10.131	6.909
Dichloro	-9.858	7.287
Aldehyde	-9.543	7.172

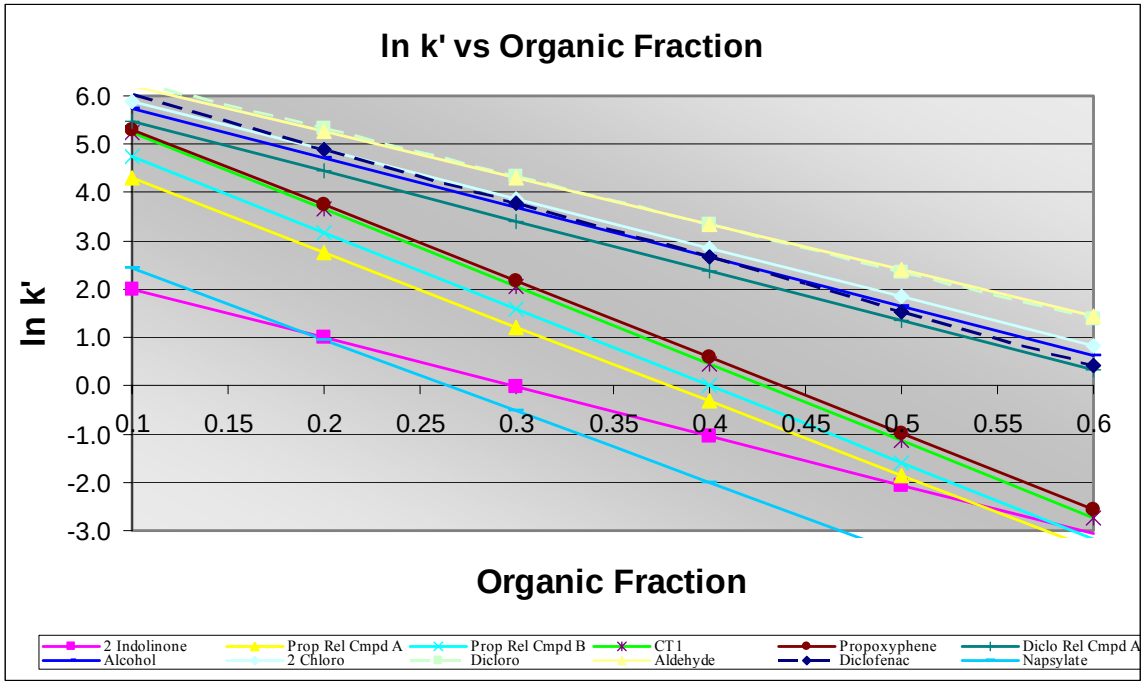


Figure 8. Plot of ln *k*' vs. organic fraction at pH 2.5 based on Schoenmakers' equations

minutes required a percent organic of greater than 32%. However, if the organic composition is too high, some peaks may elute with or too close to the solvent front. Based upon Figure 8, there are several solutes that may coelute at most organic compositions. By examining the chromatogram of the gradient run, it was determined whether isocratic separation was feasible or not. When the ratio of the absolute difference of the retention time of the most retained solute and least retained solute to the linear gradient time is less than 0.25, an isocratic method is the method of choice and there is no need for the development of a gradient method. When this ratio is greater than 0.25, but less than 0.40, development of an isocratic method may be feasible. If the ratio is greater than 0.4, an isocratic separation is impossible and the development of a gradient method will be required. A gradient run with 1.2% per minute slope in which the ratio is 0.74 can be observed in Figure 9. From this data, it can be predicted that an isocratic method, using acetonitrile in the mobile phase with the X-Terra 3.5 μm particle size column, cannot separate all components.

In order to demonstrate the reliability of these predictive methodologies, an isocratic run was performed. Based on Figure 8, the best isocratic run was determined to be 40% acetonitrile. An isocratic run was performed using an X-Terra MS 150 x 4.6 mm, 3.5 μm particle size column with mobile phase composed of 50 mM potassium phosphate buffer, pH 2.5:acetonitrile in a volumetric ratio of 60:40. The flow rate was 1.0 mL/minute and 50 μL of a mixture solution and all independent solutions were injected and collected at 217 nm. Figure 10 shows the resulting chromatogram. The predicted retention times were compared to the experimental retention times (Table 5) and were observed to be similar. However, as predicted by the plot in Figure 8, the

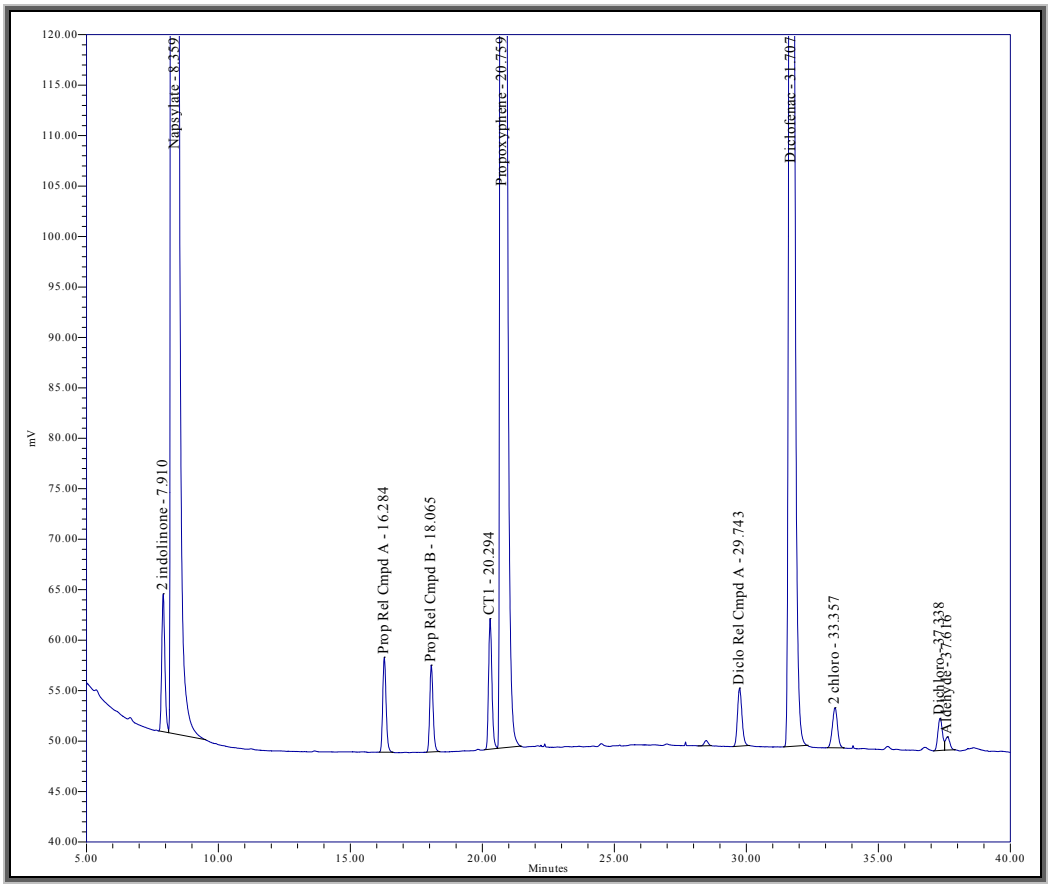


Figure 9. Chromatogram of a mixture solution, 1.2% per minute gradient slope using acetonitrile as organic modifier

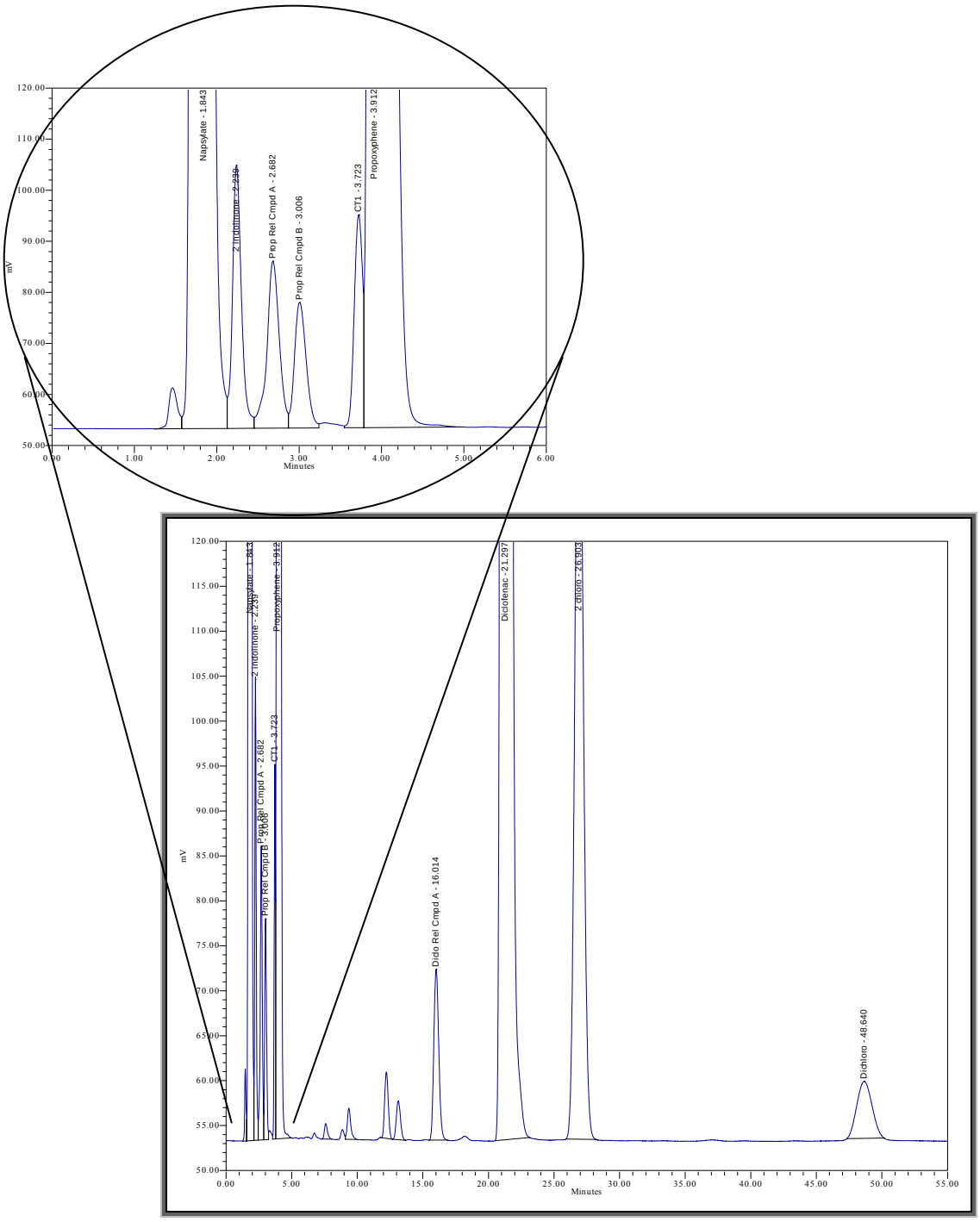


Figure 10. Chromatogram of mixture solution for isocratic method using 40:60, acetonitrile:phosphate buffer, pH 2.5, as the mobile phase

Table 5. Predicted retention times versus actual retention times for isocratic separation using a mobile phase buffer, pH 2.5 and acetonitrile in a volumetric ratio of 60:40

Component	**Measured Retention Time (minutes)**	**Predicted Retention Time (minutes)**
Napsylate	1.8	1.8
2-Indolinone	2.3	2.2
Propoxyphene Related Compound A	2.8	2.7
Propoxyphene Related Compound B	3.2	3.2
CT1	3.9	4.1
Propoxyphene	4.1	4.5
Diclofenac Related Compound A	16.4	18.8
Alcohol	25.8	24.3
Diclofenac	21.7	24.4
2 Chloro	28.1	29.3
Dichloro	47.4	46.6
Aldehyde	48.2	47.1

diclofenac and alcohol peaks, and the dichloro and aldehyde peaks coeluted and many peaks eluted too close to the solvent front. Thus, adequate resolution was not achieved. Because the isocratic method using acetonitrile and the aforementioned isocratic conditions was not possible for this system, a gradient method was developed.

There are many advantages of gradient methods such as improving separation, shortening the run time and improving the sensitivity. A gradient run was performed using an X-Terra MS 150 x 4.6 mm, 3.5 μm particle size column with mobile phase A composed of 50 mM potassium phosphate buffer, pH 2.5:acetonitrile in a volumetric ratio of 90:10 and mobile phase B composed of 50 mM potassium phosphate buffer, pH 2.5:acetonitrile in a volumetric ratio of 35:65. The flow rate was 1.0 mL/minute and 20 μL of a mixture solution and all independent solutions were injected and collected at 217 nm. A linear gradient with a slope of 1.2% per minute (5% to 95% mobile phase B in 40 minutes) was implemented. As noted in the chromatogram shown in Figure 11, the diclofenac and alcohol peaks coeluted. The gradient could not be further optimized to decrease the run time. There was no empty space in the beginning or the end of the run and all solutes were scattered during the run time.

Optimization of pH

Understanding the selectivity of pH on different solutes is beneficial when optimizing the analytical method. The pH effects were summarized by plotting, ln *k'* versus pH when the organic percentage was held constant at 35% (Figure 12). From this graph, the pH selectivity can be observed. It was demonstrated that the retention times of basic solutes increased as the pH is increased. The retention time of diclofenac, which is an acidic solute, decreased with the increasing pH. Each solute's sensitivity to pH varies

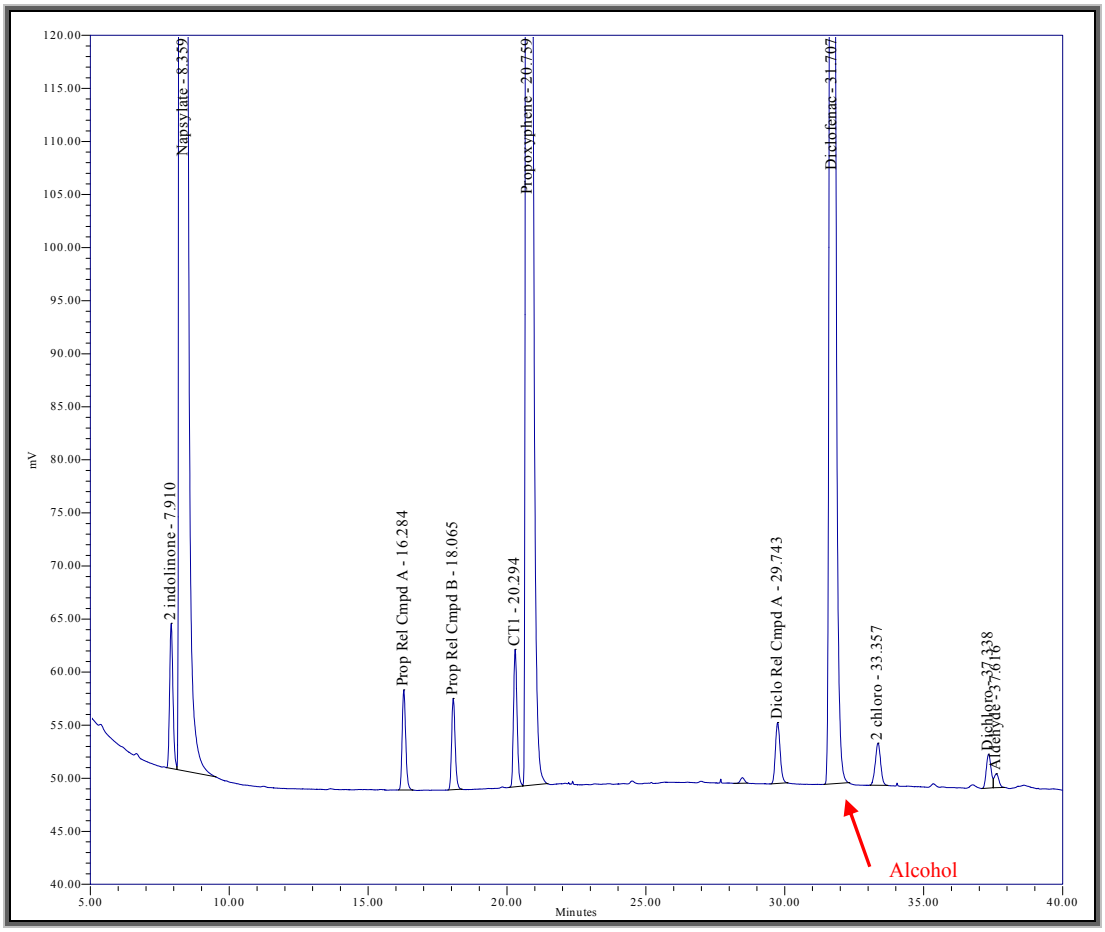
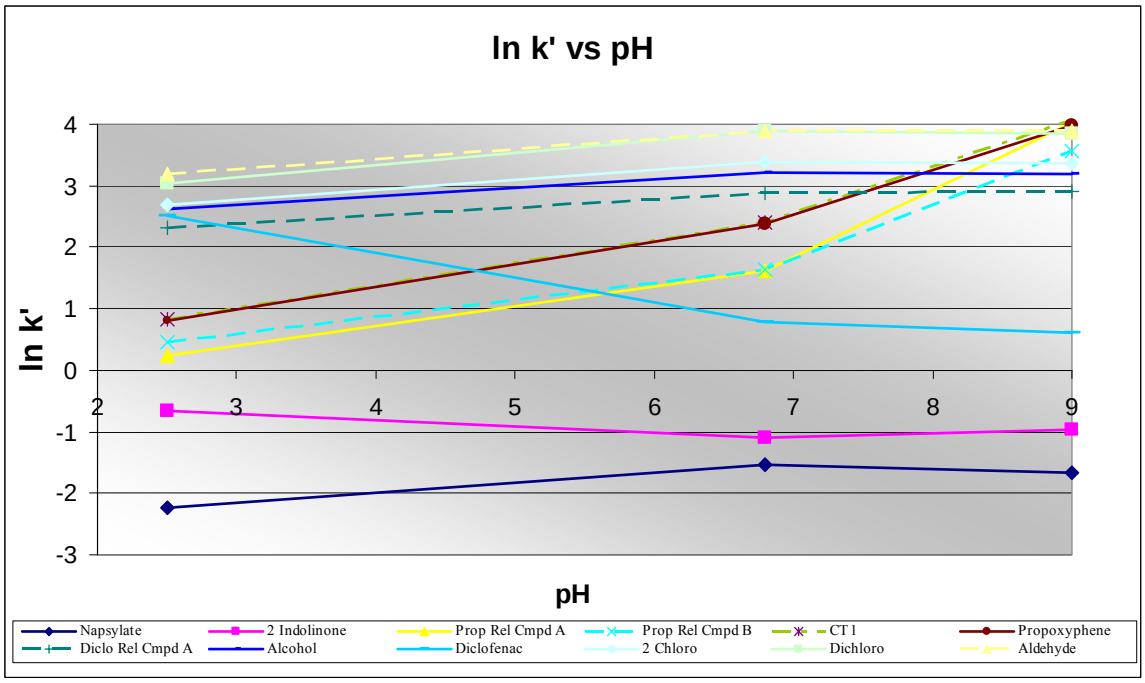


Figure 11. Chromatogram of mixture solution, 1.2% per minute gradient slope, acetonitrile as organic modifier in mobile phase, pH 2.5



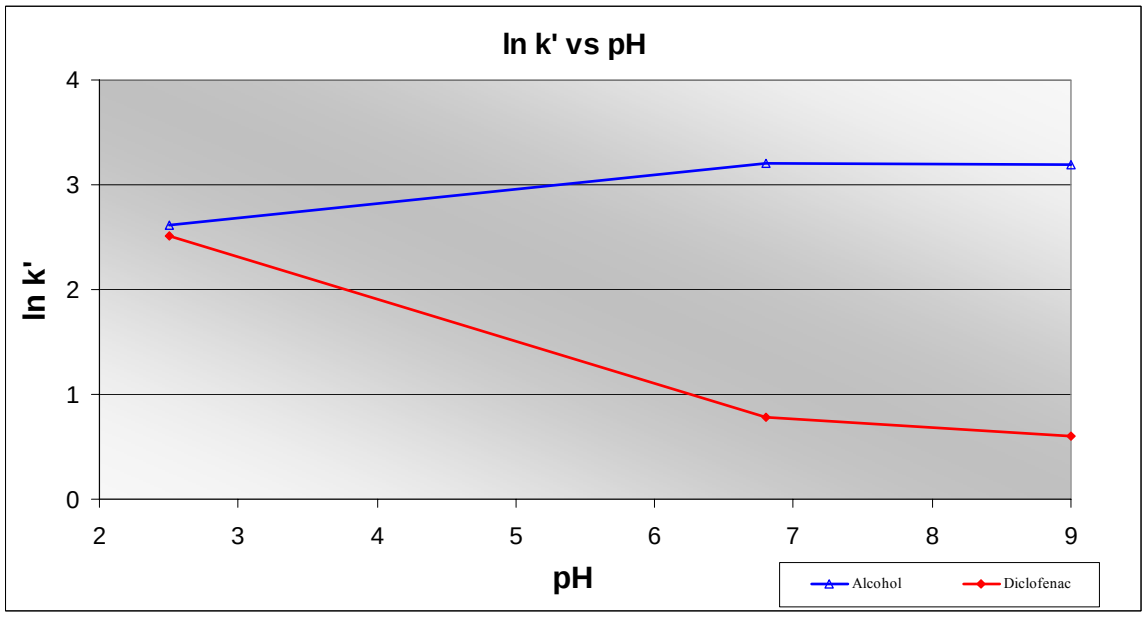


Figure 12. A plot of ln *k*' vs. pH when the percentage of organic in the mobile phase is held at 35%

which allowed the optimum pH to be selected. The selectivity of the diclofenac is different than other components. This was due to the pKa of diclofenac being lower than that of the other compounds. Diclofenac is a carboxylic acid and its pKa is about 4.0. If the pH is lower than 4.0, diclofenac is mainly non-ionic; if the pH is higher that 4.0, diclofenac is mainly ionized. Ionized forms are not retained on the non-polar reversed phase column as well as non-ionic forms. Therefore, decreased retention time of diclofenac by increased pH was expected.

By referring to the pH selectivity of diclofenac and alcohol in Figure 12, increasing the pH of the mobile phase increases the retention time of alcohol and decreases the retention time of diclofenac. The pH of the mobile phase was increased to 3.5 and the resulting chromatogram is shown in Figure 13. By making a simple adjustment of pH to the mobile phase, resolution of all solutes was obtained. A developed gradient method implements an X-Terra MS 150 x 4.6 mm, 3.5 μm particle size column with mobile phase A as 50 mM potassium phosphate buffer, pH 3.5:acetonitrile in a volumetric ratio of 90:10 and mobile phase B 50 mM potassium phosphate buffer, pH 3.5:acetonitrile in a volumetric ratio of 35:65 with a gradient slope of 1.2% per minute (5% to 95% mobile phase B in 40 minutes). The flow rate was 1.0 mL per minute with an injection volume of 20 μL. The wavelength was set at 217 nm for the first 24 minutes then switched to 254 nm at 25 minutes. The wavelength was changed after 25 minutes to 254 nm to decrease the absorption of gradient peaks and to optimize the absorption of diclofenac and diclofenac related substances.

Isoelutropic Binary Method Development

A gradient method for assay and impurities was developed in which all solutes

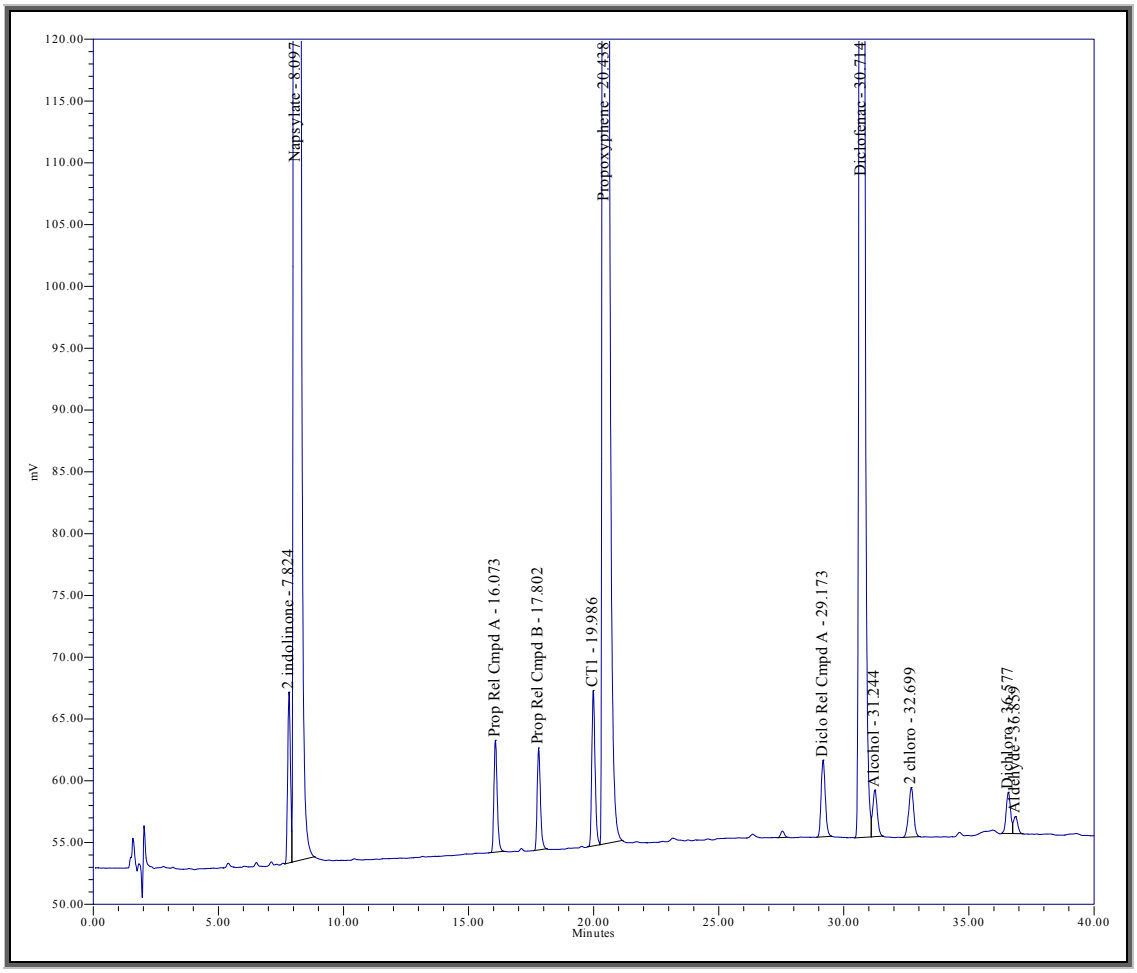


Figure 13. Chromatogram of mixture solution, 1.2% per minute gradient slope, acetonitrile as organic modifier, pH 3.5

were adequately separated. However, an isocratic method was preferred because it has better reproducibility, less complications and clean chromatography which is free from gradient artifacts. By changing the organic solvent to methanol, the selectivities of the peaks were affected and provide different separation. It is known that methanol will cause a greater back pressure on the system due to the higher viscosity of methanol/buffer compared to acetonitrile/buffer. Therefore, the column was changed to 5 μm particle size which would decrease the pressure by about one half compared with the 3.5 μm particle size column. Theoretically, the particle size should not affect the retention time of solute. However, practically, it is difficult to reproduce the exact properties of the packing material. Therefore, the previous experiment performed to predict the retention times may not be accurate. In order to accurately predict the retention time behavior on the 5 μm column, the Schoenmakers' two gradient approach was applied using acetonitrile.

Two gradient runs, each with different gradient slopes were performed. The experiments employed the X-Terra MS 150 x 4.6 mm, 5 μm particle size column. Because the particle size was increased to 5 μm, it was possible to increase the flow rate to 2.0 mL/min and obtain an acceptable back pressure. Mobile phase A was buffer, pH 3.5 and acetonitrile in a volumetric ratio of 90:10 and mobile phase B was buffer, pH 3.5 and acetonitrile in a volumetric ratio of 35:65. The first gradient slope was 2.5% per minute or 5% to 95% mobile phase B in 20 minutes and the second gradient slope was 1.2% per minute or 5% to 95% mobile phase B in 40 minutes. After the completion of the gradient, the mobile phase remained at the final composition for at least 10 minutes to ensure all solutes elute during the run time. All solutes eluted before the completion of the gradient. The retention time of each solute was measured for each gradient run. The

slope, s and capacity factor in pure weak solvent ($k_{j,w}$) were calculated by SAS (Table 6) and a plot of ln k'_j versus the fraction of organic (φ) was established (Figure 14).

From the plot, 34% acetonitrile was selected as the optimum amount of acetonitrile in the mobile phase. An isocratic run was performed using an X-Terra RP C18 150 x 4.6 mm, 5 μm particle size with a mobile phase composition 50 mM potassium phosphate buffer, pH 3.5 and acetonitrile in a volumetric ratio of 66:34 with a flow rate of 2.0 mL/minute. The column temperature was at ambient laboratory conditions and 20 μL was injected. The resulting chromatogram is shown in Figure 15. As expected by the predictions from the plot in Figure 14 and as observed from studies using the 3.5 μm particle size column, diclofenac coelutes with the alcohol and 2-chloro impurities. Because separation could not be achieved with acetonitrile, the organic modifier was changed to methanol. The isoelutropic solution based on Herman's cubic equation (Equation 18) was used to determine an amount of methanol equivalent to 34% acetonitrile. It was determined that 34% acetonitrile is equivalent to 44% methanol. An isocratic run using 44% methanol as the organic modifier was performed using the X-Terra RP C18 150 x 4.6 mm, 5 μm particle size with a mobile phase composition 50 mM potassium phosphate buffer, pH 3.5 and methanol in a volumetric ratio of 56:44 with a flow rate of 2.0 mL/minute. The column temperature was at ambient laboratory conditions and 20 μL was injected. The resulting chromatogram is shown in Figure 16.

A comparison of the two chromatograms is shown in Figure 17. The run time of the 44% methanol run was significantly longer than the 34% acetonitrile run that was

Table 6. Calculated characteristic values of s and $k'_{j,w}$ at pH 2.5 using SAS, based on Schoenmakers' equations

Components	**s**	$\boldsymbol{k'_{j,w}}$
Napsylate	-15.462	3.712
2-Indolinone	-10.298	2.867
Propoxyphene Related Compound A	-15.257	5.473
Propoxyphene Related Compound B	-16.392	6.081
CT1	-18.160	7.043
Propoxyphene	-17.592	6.941
Diclofenac Related Compound A	-12.150	6.914
Alcohol	-12.235	7.493
Diclofenac	-12.811	7.691
2 Chloro	-12.161	7.471
Dichloro	-11.844	8.002
Aldehyde	-11.978	8.000

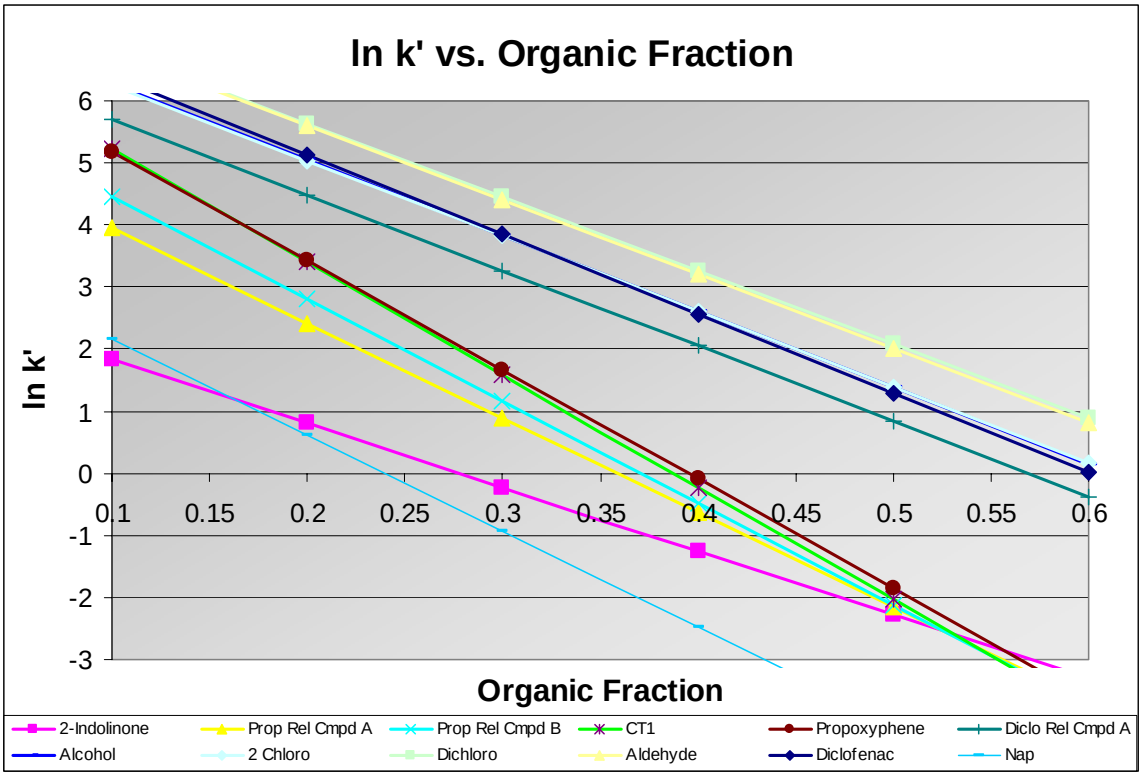


Figure 14. A plot of ln *k*' vs. organic fraction, pH 3.5, 5 µm particle size column

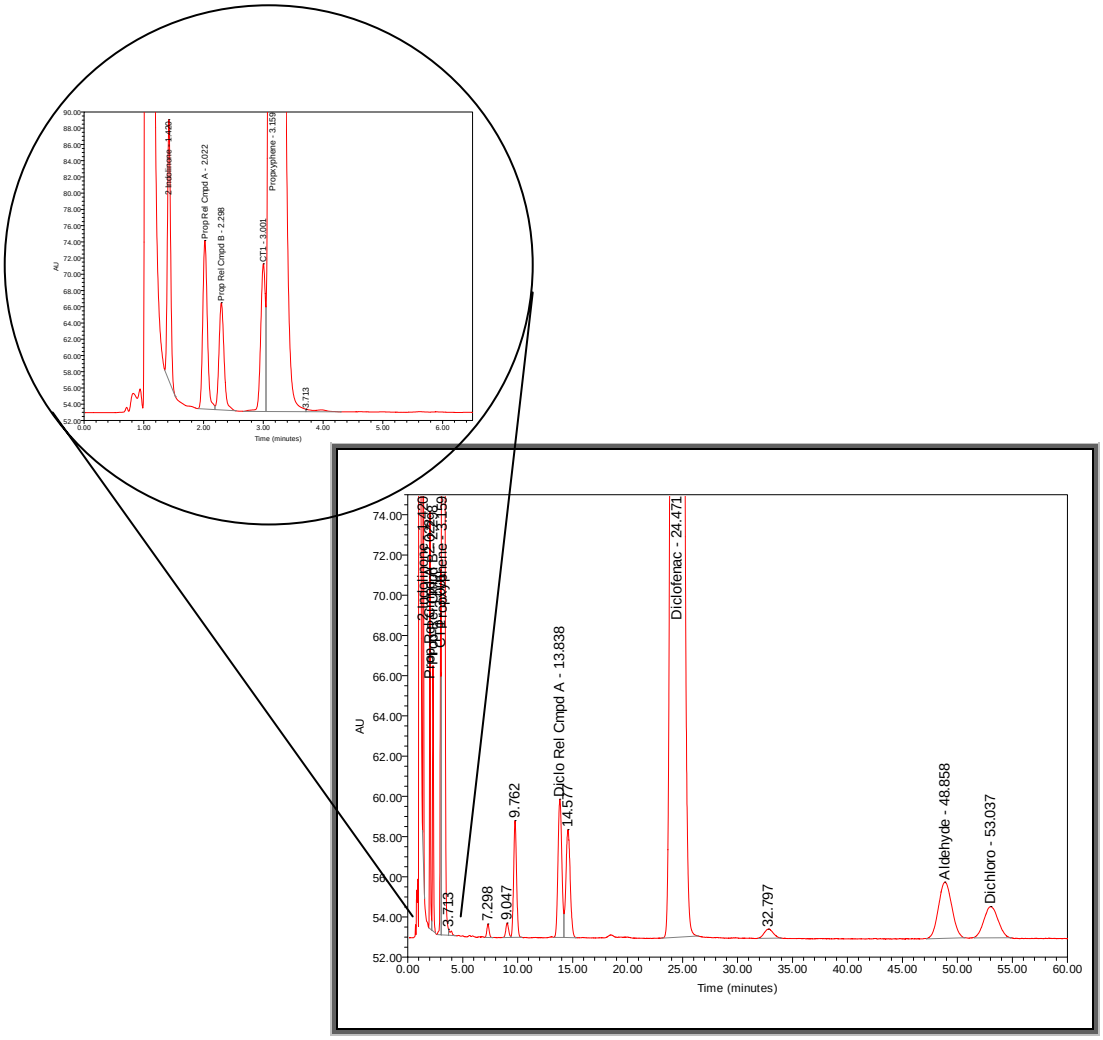


Figure 15. Chromatogram of a mixture solution from an isocratic run using 34% acetonitrile, pH 3.5

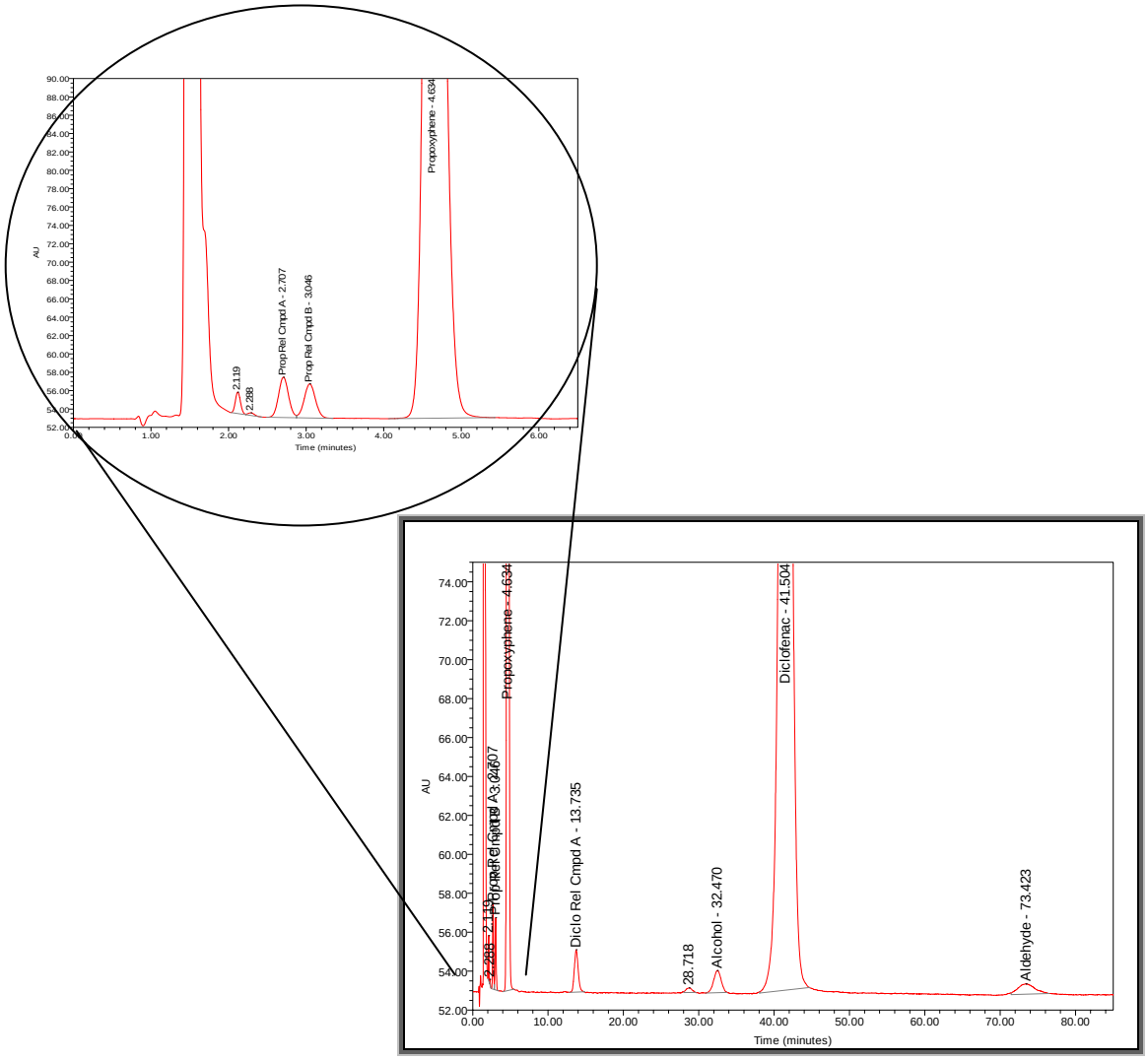


Figure 16. Chromatogram of a mixture solution, isocratic method using 44% methanol in the mobile phase, pH 3.5

predicted by Herman's isoelutropic expression. Therefore, the strength of methanol equivalent to 34% acetonitrile was determined by Schoenmakers' isoelutropic solution (Equation 16). According to Schoenmakers' expression, 34% acetonitrile is equivalent to 47% methanol. An isocratic run using 47% methanol as the organic modifier was performed using the X-Terra RP C18 150 x 4.6 mm, 5 μm particle size with a mobile phase composition 50 mM potassium phosphate buffer, pH 3.5 and methanol in a volumetric ratio of 53:47 with a flow rate of 2.0 mL/minute. The column temperature was at ambient laboratory conditions and 20 μL was injected. The resulting chromatogram is shown in Figure18. The similar run times of the 34% acetonitrile isocratic run and the 47% methanol isocratic run can be seen in Figure 19. Schoenmakers' prediction more accurately estimated the isoelutropic equivalence compared to Herman's. The separation of alcohol and diclofenac was achieved from the different selectivity of methanol. However, CT1 and propoxyphene coelute when using methanol as the organic modifier. Because an isocratic separation was not achievable using acetonitrile or methanol, a ternary separation was evaluated.

Applying Mixtures of Organic Solvents

According to the mixture design, for a ternary system, at least three experiments must be performed to determine the composition of mobile phase that would provide an optimum separation of all peaks (Figure 20). The binary systems were performed previously for acetonitrile and methanol. Using the mixture design, a study was performed using a ternary system (mixture of acetonitrile, methanol and phosphate buffer) at a point between the optimum binary conditions of methanol and acetonitrile. An isocratic run was performed using the X-Terra RP C18 150 x 4.6 mm, 5 μm particle

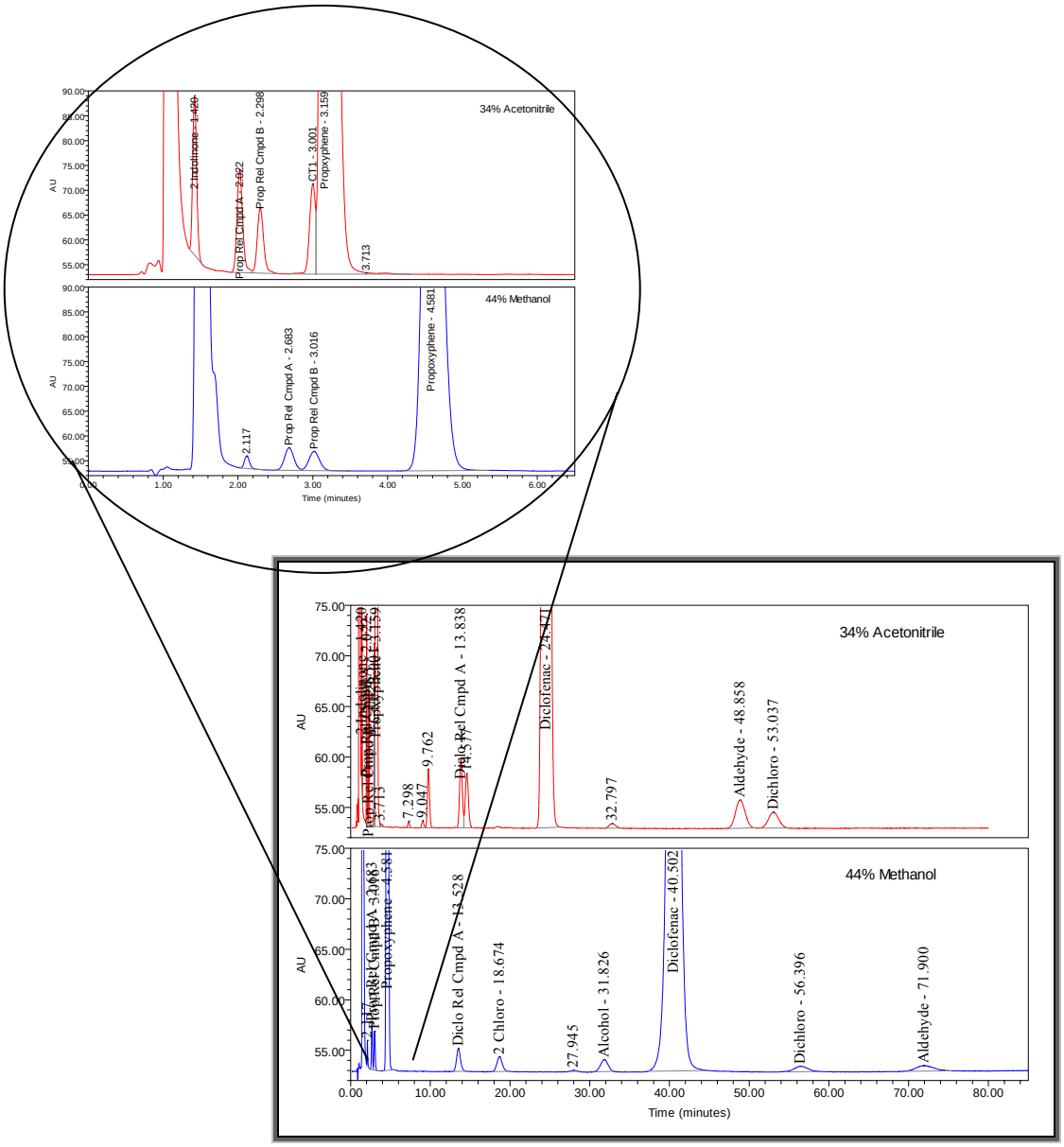


Figure 17. Chromatogram overlay of mixture solution for isocratic runs using 34% acetonitrile and 44% methanol

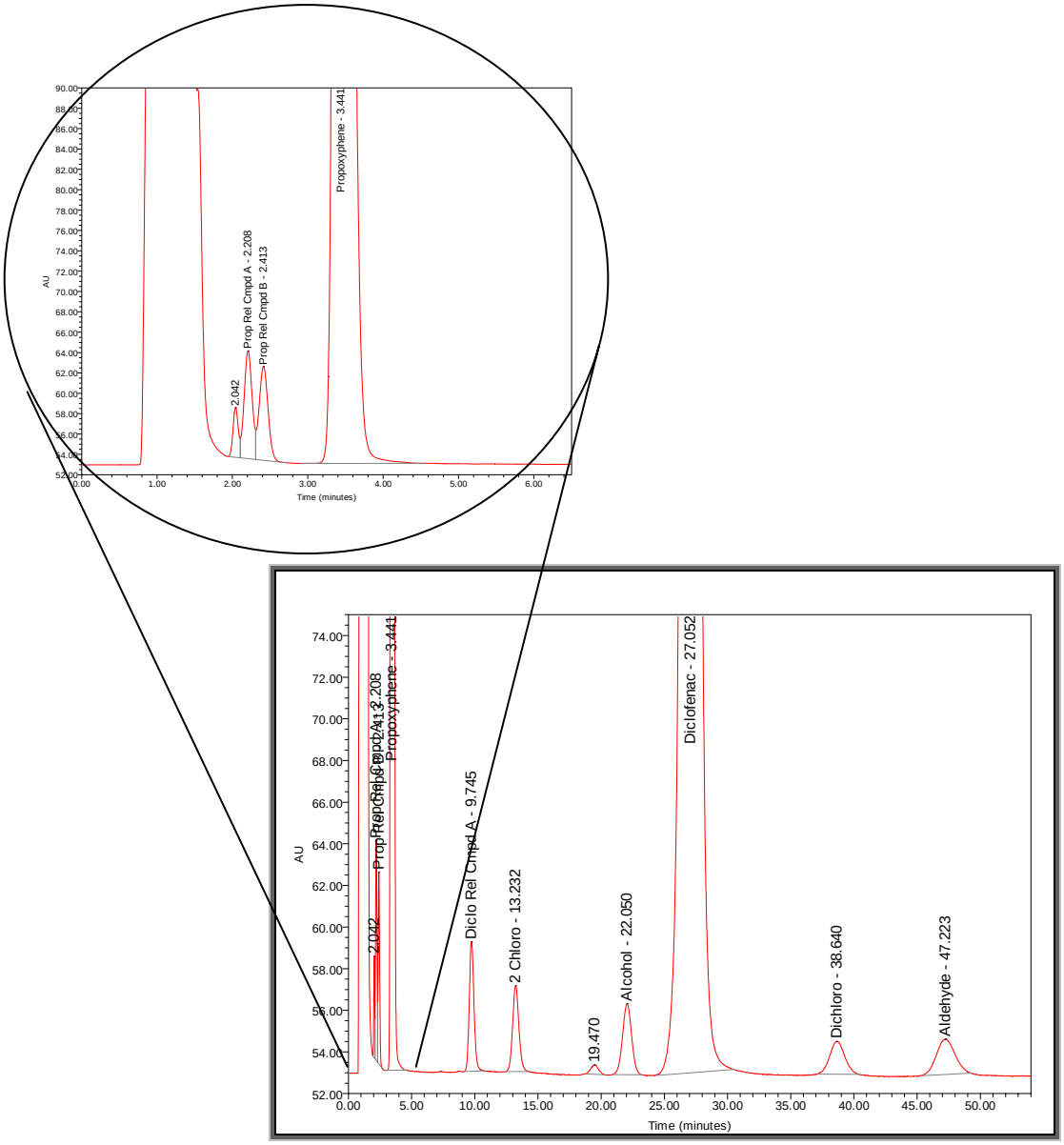


Figure 18. Chromatogram of a mixture solution, 47% methanol

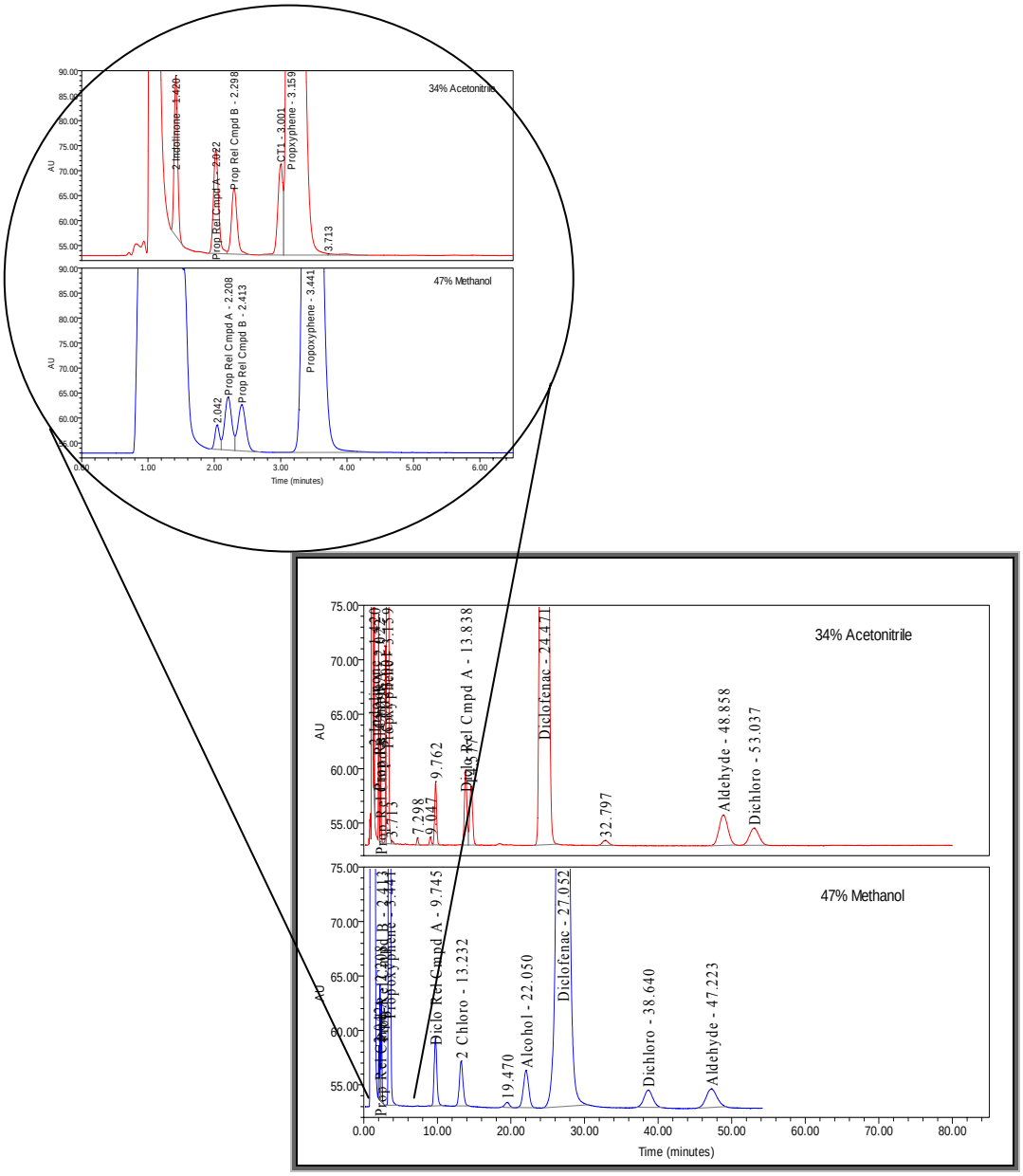


Figure 19. Chromatogram overlay of mixture solution at 34% acetonitrile and 47% methanol.

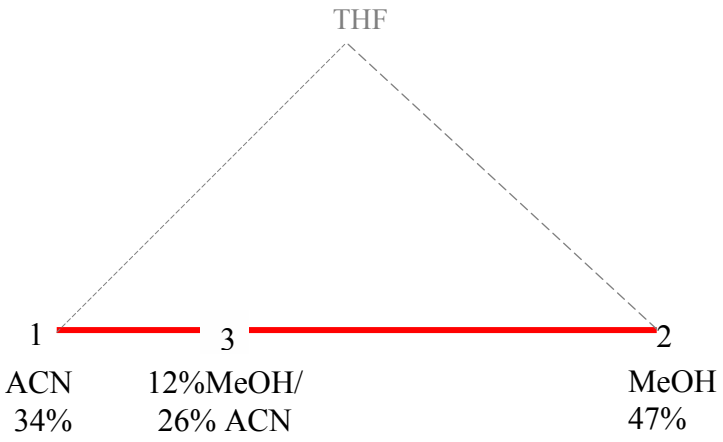


Figure 20. Mixture design model for ternary mobile phase

size with a mobile phase composition 50 mM potassium phosphate buffer, pH 3.5, methanol and acetonitrile in a volumetric ratio of 62:12:26 with a flow rate of 2.0 mL/minute. The column temperature was at ambient laboratory conditions and 20 µL injections of samples were performed. The resulting chromatogram is shown in Figure 21.

The retention time of each peak was measured and recorded for each mobile phase, binary with acetonitrile, binary with methanol and ternary with acetonitrile and methanol. The natural logarithms of the capacity factor for each solute (ln k'_j) were calculated at each different composition of mobile phase. Using the ln k'_j, the three constants in equation 26, a_1, a_2, $a_{1,2}$ were calculated.

$$\ln k' = a_1 x_1 + a_2 x_2 + a_{1,2} x_1 x_2 \tag{26}$$

After inserting these constants into Equation 26, the optimum mobile phase was calculated by iterative calculation at different compositions of acetonitrile (x_1) and methanol (x_2) to determine which composition would give the greatest difference in retention time for critical pairs.

Using the same approach, the resolutions (R) at different compositions of acetonitrile (x_1) and methanol (x_2) were also determined. The resolutions between all successively eluting peaks were measured and recorded for each mobile phase, binary with acetonitrile, binary with methanol and ternary with acetonitrile and methanol. Using the resolutions, the three constants in equation 27, a'_1, a'_2, $a'_{1,2}$ were calculated.

$$R = a'_1 x_1 + a'_2 x_2 + a'_{1,2} x_1 x_2 \tag{27}$$

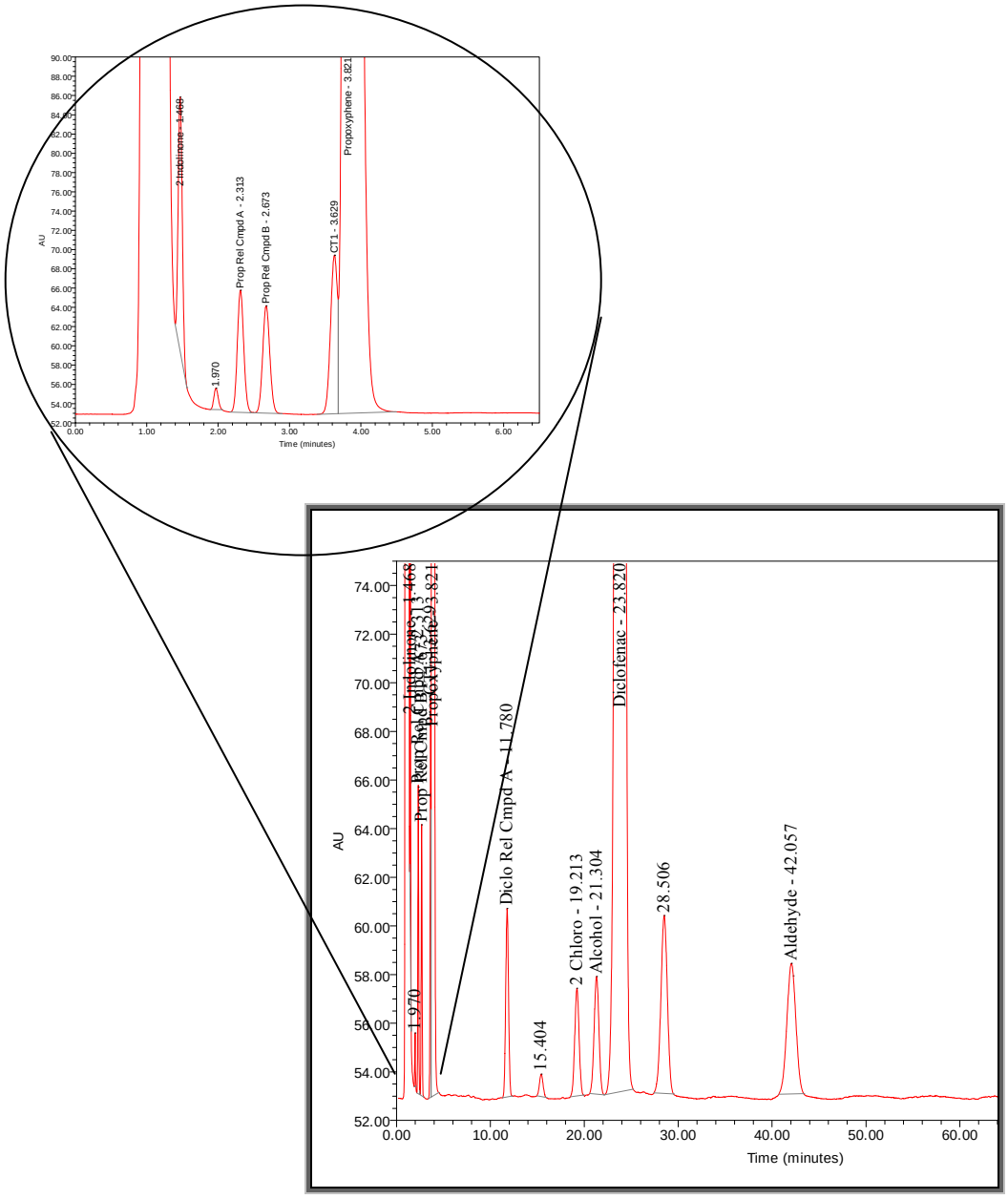


Figure 21. Chromatogram of mixture solution in ternary system (12:26:62, methanol:acetonitrile:phosphate buffer, pH 3.5)

After inserting these constants into Equation 27, the optimum mobile phase was calculated by iterative calculation at different compositions of acetonitrile (x_1) and methanol (x_2) to determine which composition gives the greatest resolution of critical pairs.

The optimum ternary composition of mobile phase using ln *k'* or resolution was predicted to be methanol, acetonitrile and phosphate buffer in a volumetric ratio of 7:29:64. An isocratic run was performed using the X-Terra RP C18 150 x 4.6 mm, 5 µm particle size with a mobile phase composed of methanol, acetonitrile and phosphate buffer, pH 3.5 in a volumetric ratio of 7:29:64 with a flow rate of 2.0 mL/minute. The column temperature was at ambient laboratory conditions and 20 µL injections of samples were performed. The resulting chromatogram is shown in Figure 22.

Table 7 and 8 compare the calculated resolutions/retention times with actual resolutions and retention times based on the polynomial expression utilized in Equation 26. As observed in Figure 22, all process and degradation impurities have adequate resolution for quantitation. This systematic and mathematical approach demonstrated an acceptable analytical method for the assay of propoxyphene, diclofenac and all related process and degradation impurities.

Column Temperature Optimization

Although an isocratic ternary method was developed which separated all in-process and degradation products, and since the 5 µm column behaved differently than the 3.5 µm column, it was desired to simplify the method to one solvent for finished product testing. In finished product testing, it is not necessary to quantitate in-process impurities. Therefore, separation is not needed for dichloro and 2-chloro. An isocratic

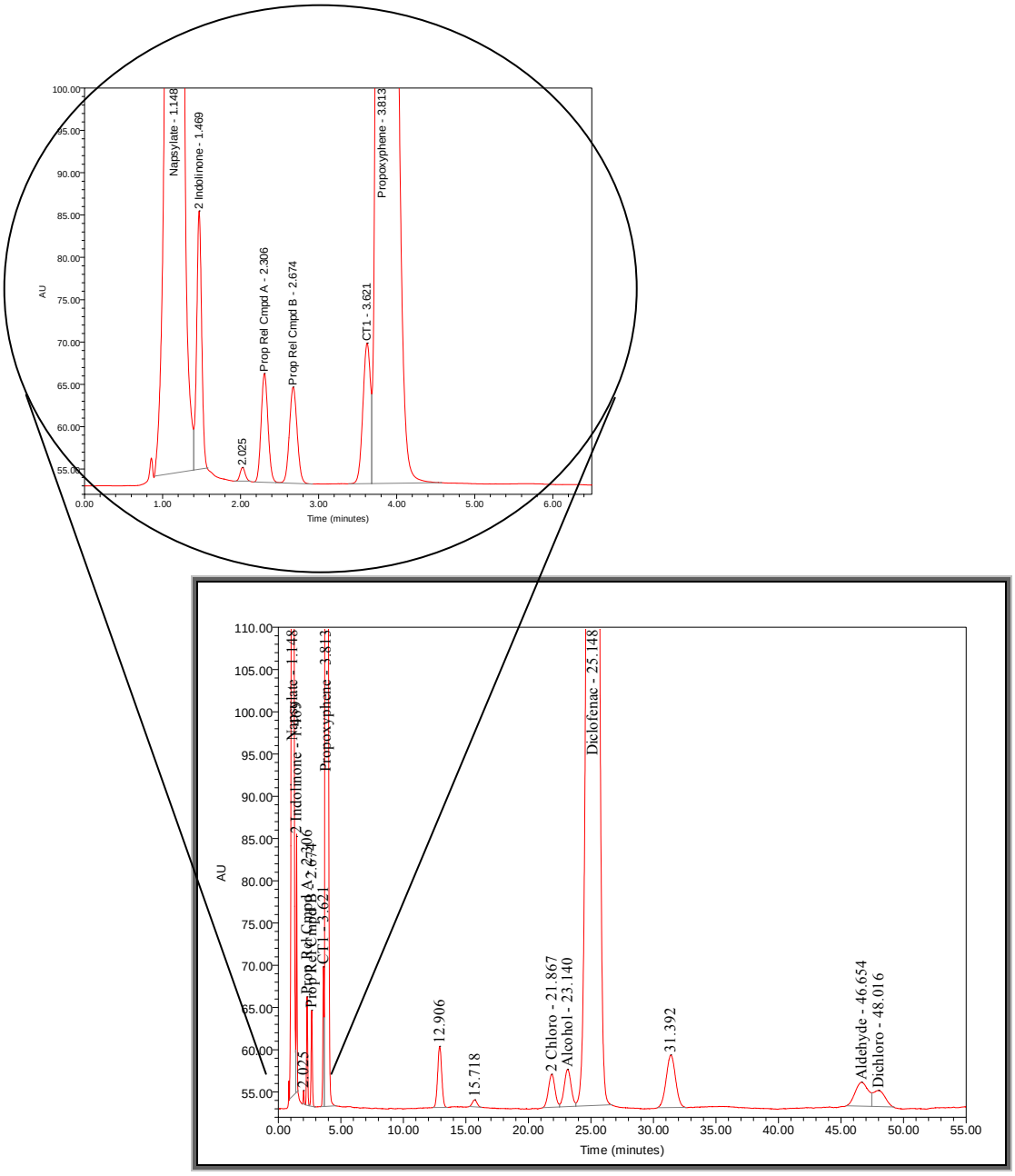


Figure 22. Chromatogram of mixture solution for optimal isocratic ternary system

Table 7. Resolution measurements for binary systems with acetonitrile and methanol and ternary system of acetonitrile and methanol

Successive Components	**Calculated Resolution**	**Measured Resolution**
Napsylate/2-Indolinone	2.1	1.7
2-Indolinone/Prop Rel Cmpd A	5.5	5.8
Prop Rel Cmpd A/Prop Rel Cmpd B	2.0	2.1
Prop Rel Cmpd B/CT1	4.4	4.7
CT1/ Propoxyphene	0.7	0.7
Propoxyphene/Diclo Rel Cmpd A	19.7	19.7
Diclo Rel Cmpd A/2 Chloro	10.9	11.3
2 Chloro/Alcohol	1.4	1.3
Alcohol/Diclofenac	1.5	1.8
Diclofenac/Unknown Impurity	4.6	4.8
Unknown Impurity/Aldehyde	8.5	8.2
Aldehyde/Dichloro	1.0	1.0

Table 8. Retention time measurements for binary systems with acetonitrile and methanol and ternary system of acetonitrile and methanol

Component	**Calculated Retention Time (minutes)**	**Measured Retention Time (minutes)**
2-Indolinone	1.5	1.5
Propoxyphene Related Compound A	2.2	2.3
Propoxyphene Related Compound B	2.5	2.7
CT1	3.4	3.6
Propoxyphene	3.6	3.8
Diclofenac Related Compound A	12.6	12.9
2 Chloro	21.0	21.9
Alcohol	22.5	23.1
Diclofenac	24.2	25.1
Unknown Impurity	30.2	31.4
Aldehyde	44.5	46.7
Dichloro	46.0	48.0

run was performed using the X-Terra RP C18 150 x 4.6 mm, 5 μm particle size with a mobile phase composed of 50 mM potassium phosphate buffer, pH 3.5 and acetonitrile in a volumetric ratio of 66:34 with a flow rate of 2.0 mL/minute. The column temperature was at ambient laboratory conditions and 20 μL injections of samples were performed. The resulting chromatogram is shown in Figure 15. The measured retention times were similar to the retention times predicted from the plot in Figure 14 (Table 9).

Because diclofenac and alcohol still coelute, the temperature of the column was evaluated. The column temperature often aids in the separation of components because of different heat of enthalpy values (ΔH) for each solute. The temperature effects were studied by injecting solutes using different column temperatures while all other variables were held constant. An isocratic run was performed using the X-Terra RP C18 150 x 4.6 mm, 5 μm particle size with a mobile phase composed of 50 mM potassium phosphate buffer, pH 3.5 and acetonitrile in a volumetric ratio of 66:34 with a flow rate of 2.0 mL/minute and 20 μL injection volume. The resulting chromatograms are shown in Figures 23 and 24.

The separation of the alcohol and diclofenac, and the CT1 and propoxyphene improved as the temperature increased. By measuring the retention times of each solute at each temperature, the plot of ln *k'* vs. 1/T is observed in Figure 25. Using the data at different temperatures, ΔH's in equilibrium between the mobile phase and the stationary phase, were calculated using the following equation (Table 10).

$$\ln k = \frac{-\Delta H}{RT} + \text{Constant} \tag{28}$$

Table 9. Predicted retention times versus actual retention times for isocratic separation using a 5 µm particle size column a mobile phase buffer, pH 3.5 and acetonitrile in a volumetric ratio of 66:34

Component	**Measured Retention Time (minutes)**	**Predicted Retention Time (minutes)**
2-Indolinone	1.4	1.3
Propoxyphene Related Compound A	2.0	2.0
Propoxyphene Related Compound B	2.3	2.3
CT1	3.1	2.9
Propoxyphene	3.2	3.1
Diclofenac Related Compound A	14.1	14.6
Alcohol	24.8	24.7
Diclofenac	25.2	24.7
Aldehyde	50.2	44.0

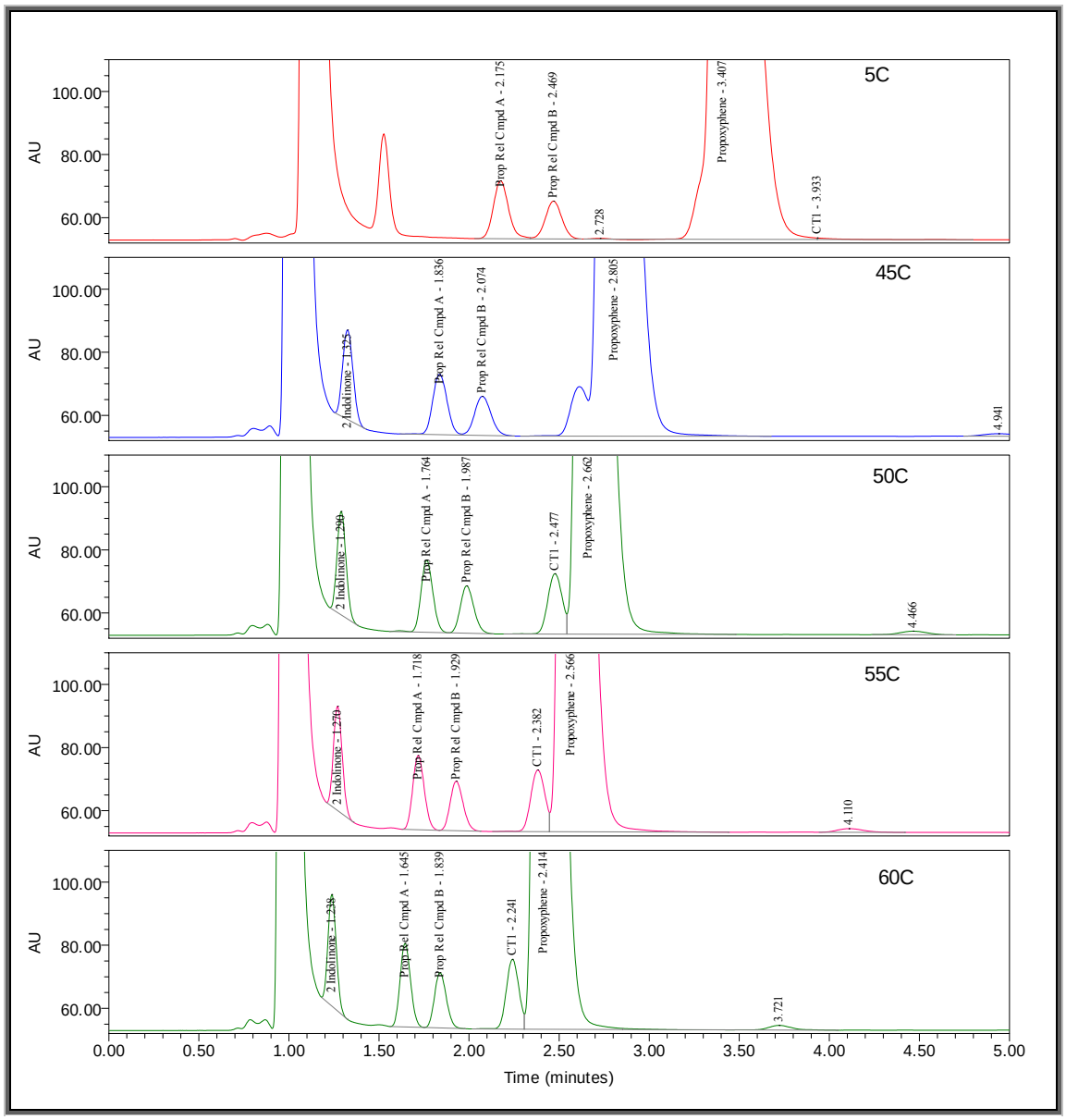


Figure 23. Chromatogram of mixture solution from an isocratic run having a mobile phase of 34:66 acetonitrile:buffer, pH 3.5 at various column temperatures from 0-5 minutes

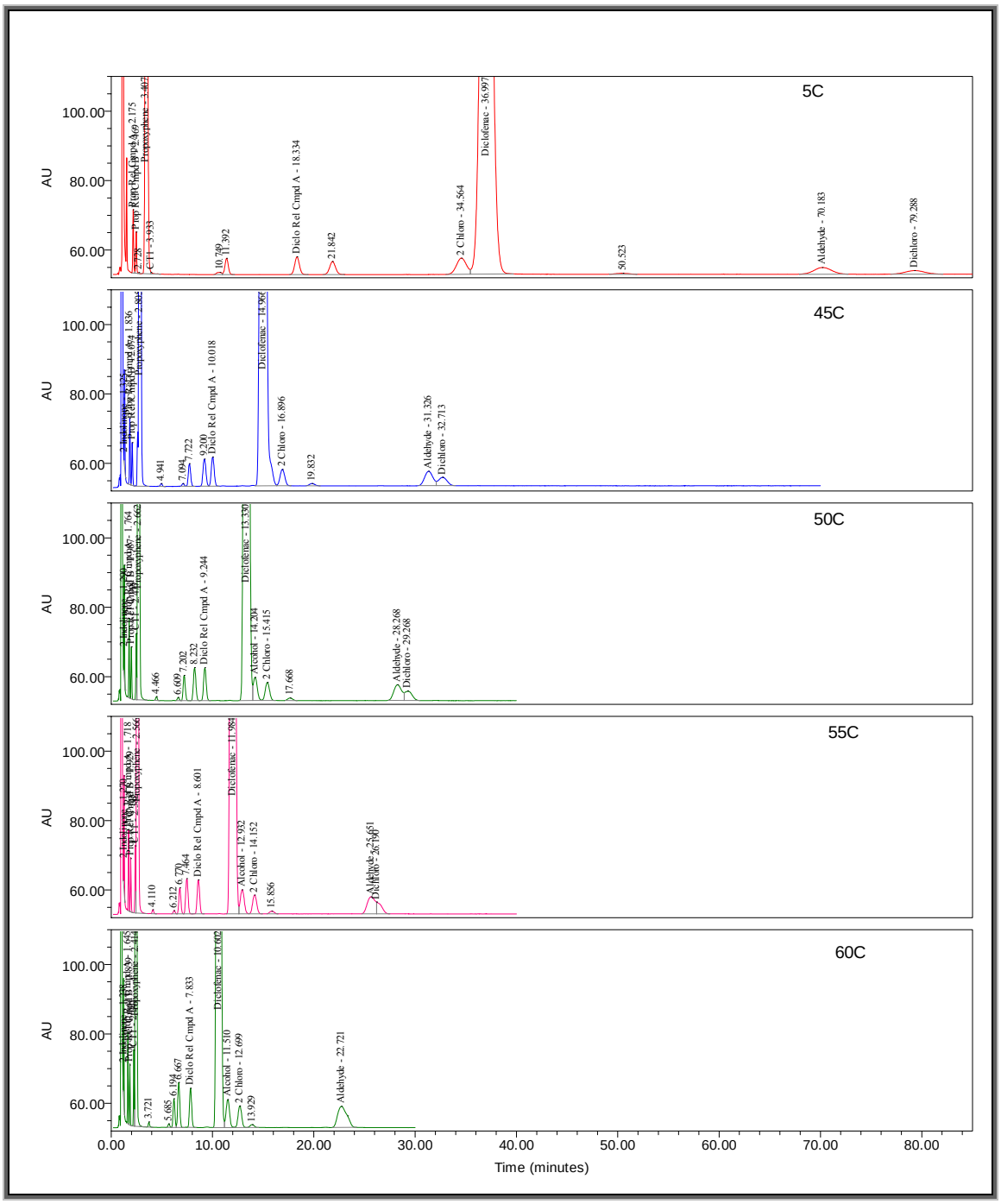


Figure 24. Chromatogram of mixture solution from an isocratic run having a mobile phase of 34:66 acetonitrile:buffer, pH 3.5 at various column temperatures from 0-85 minutes

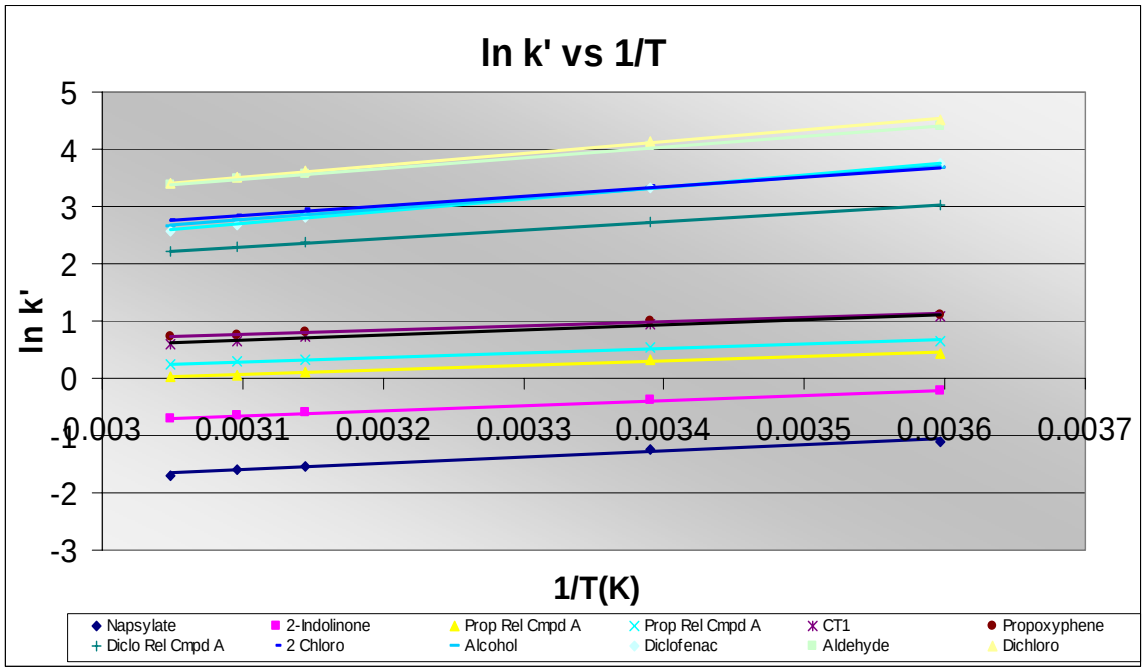


Figure 25. Plot of ln *k*' vs. the inverse of the temperature where the ΔH = -slope × R

Table 10. Calculated ΔH in equilibrium between the mobile phase and stationary phase

Components	**ΔH (kcal/mol)**
Napsylate	-2.127
2-Indolinone	-1.771
Propoxyphene Related Compound A	-1.564
Propoxyphene Related Compound B	-1.505
CT1	-1.787
Propoxyphene	-1.449
Diclofenac Related Compound A	-2.943
Alcohol	-3.742
Diclofenac	-4.259
2 Chloro	-3.335
Dichloro	-4.048
Aldehyde	-3.726

The negative slope multiplied by the molar gas constant gives the ΔH. If the ΔH's are similar, the components are expected to behave similarly as temperature varies. A greater difference in ΔH will result in a greater improvement in resolution when the column temperature is increased.

Since the best resolution was observed at 60°C, Schoenmakers' two gradient system was implemented to determine the optimum mobile phase at the optimum temperature of 60°C. The experiments utilized the X-Terra MS 150 x 4.6 mm, 5 μm particle size column maintained at 60°C. Mobile phase A was buffer, pH 3.5 and acetonitrile in a volumetric ratio of 90:10 and mobile phase B was buffer, pH 3.5 and acetonitrile in a volumetric ratio of 35:65 with a flow rate of 2.0 mL/minute. The first gradient slope was 2.5% per minute or 5% to 95% mobile phase B in 20 minutes and the second gradient slope was 1.2% per minute or 5% to 95% mobile phase B in 40 minutes. After the completion of the gradient, the mobile phase remained at the final composition for at least 10 minutes to ensure all solutes elute during the run time. All solutes eluted before the completion of the gradient. The retention time of each solute was measured for each gradient run. The slope, s, and capacity factor in pure weak solvent ($k_{j,w}$) for each solute was calculated by SAS (Table 11) and a plot of ln k'_j versus the fraction of organic (φ) was established (Figure 26).

The plot in Figure 26 demonstrated that 35% acetonitrile with a column temperature of 60°C would provide adequate resolution of all degradation products. Therefore an isocratic run was performed using the X-Terra RP C18 150 x 4.6 mm, 5 μm particle size with a mobile phase composed of 50 mM potassium phosphate buffer, pH

Table 11. Calculated characteristic values of s and $k'_{j,w}$ at pH 3.5 and column temperature of 60°C using SAS, based on Schoenmakers' equations

Components	**s**	**$k'_{j,w}$**
Napsylate	-19.371	3.333
2-Indolinone	-14.876	2.789
Propoxyphene Related Compound A	-15.917	4.828
Propoxyphene Related Compound B	-16.911	5.421
CT1	-16.961	5.897
Propoxyphene	-17.079	6.074
Diclofenac Related Compound A	-12.121	6.122
Alcohol	-11.810	6.475
Diclofenac	-12.030	6.640
2 Chloro	-12.858	6.725
Dichloro	-11.384	7.048
Aldehyde	-11.524	7.083

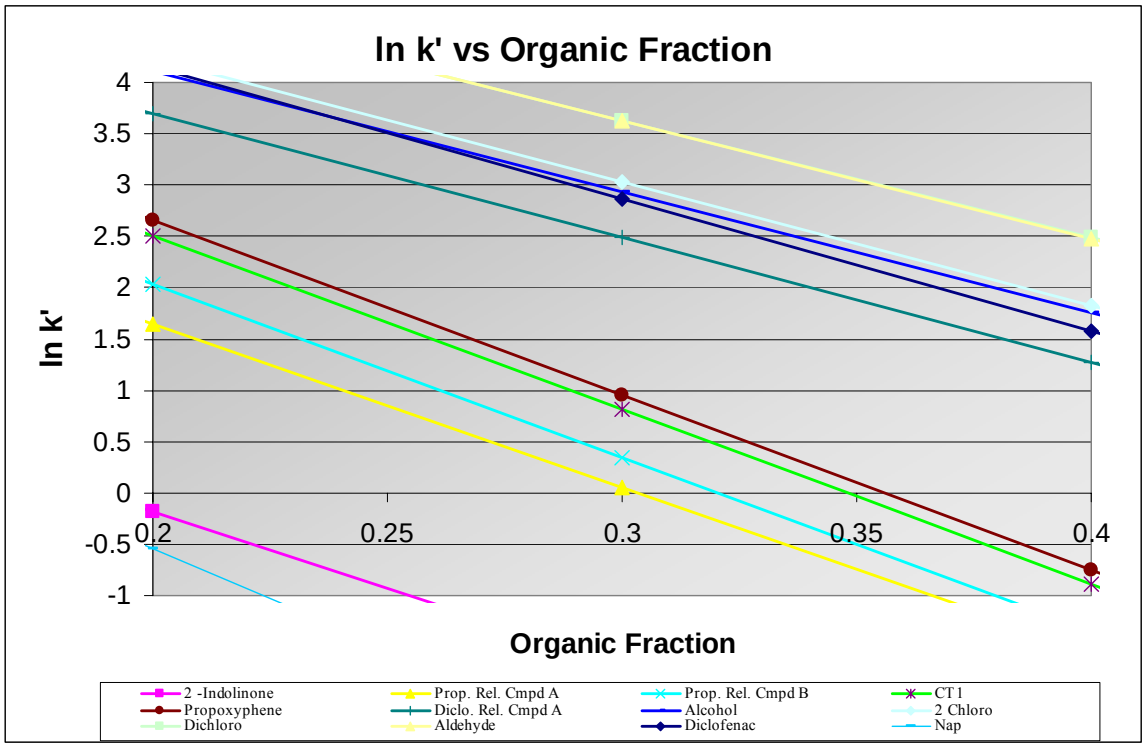


Figure 26. A plot of ln *k*' vs. organic fraction, pH 3.5, 5 µm particle size column at 60°C

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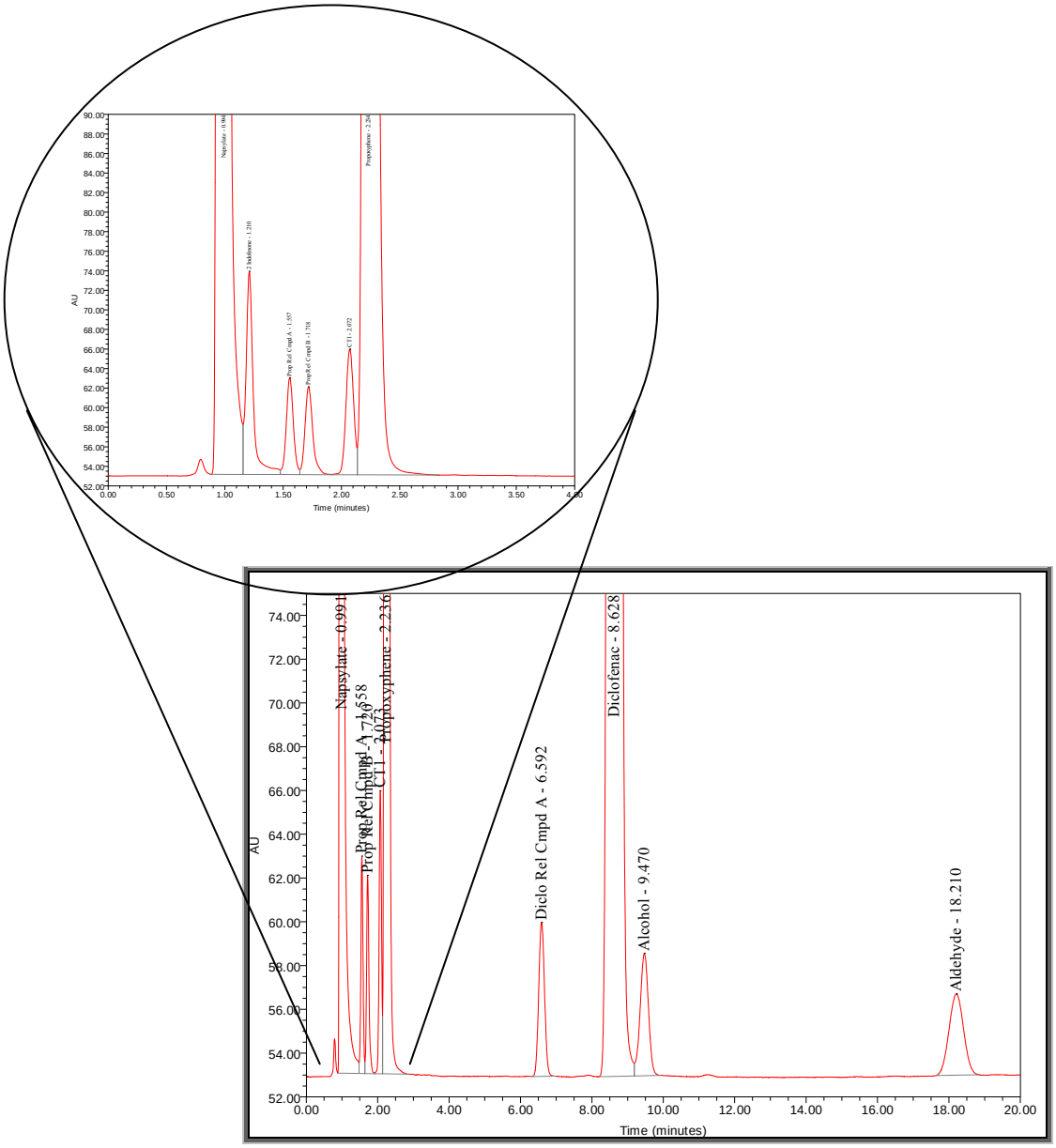

Figure 27. Chromatogram of mixture solution with 5 µm particle size column at 60°C with mobile phase of buffer, pH 3.5:acetonitrile 65:35.

Table 12. Predicted retention times vs. actual retention times for isocratic separation using a 5 µm particle size column at 60°C, a mobile phase of buffer, pH 3.5 and acetonitrile in a volumetric ratio of 65:35

Component	**Measured Retention Time (minutes)**	**Predicted Retention Time (minutes)**
2-Indolinone	1.2	0.8
Propoxyphene Related Compound A	1.5	1.3
Propoxyphene Related Compound B	1.7	1.3
CT1	2.0	1.5
Propoxyphene	2.2	1.7
Diclofenac Related Compound A	6.4	6.0
Diclofenac	8.3	8.1
Alcohol	9.2	9.0
Aldehyde	17.5	17.4

3.5 and acetonitrile in a volumetric ratio of 65:35 with a flow rate of 2.0 mL/minute and a 20 µL injection volume. The resulting chromatogram is shown in Figure 27. The predicted retention times correlated well with the measured retention times (Table 12). All peaks of interest were adequately separated.

Dissolution Method Development

Dissolution profiles are the often performed to demonstrate how a product may behave in man as well as how it may behave over time. When performing dissolution profiles with 6 to 12 tablet dissolutions, the number of samples to be tested is significant. Therefore, it is important to develop a method with a short run time to allow the total testing time to be efficient. A dissolution method was developed based on the plot of ln *k'* versus. organic fraction (Figure 8). From the plot, the percentage of acetonitrile in the mobile phase can be estimated. At 45% acetonitrile, the plot predicted propoxyphene to elute just after the solvent front at 2.9 minutes and diclofenac should elute at 14.6 minutes. As observed in Figure 12, if the pH of the mobile phase is increased, diclofenac would have a shorter retention time and propoxyphene would have a longer retention time which would result in a shorter run time and better separation from the solvent front. Therefore the pH of the mobile phase was increased to 3.5. The following instrument parameters were selected for the dissolution method.

HPLC Parameters:

Column: X-Terra MS 150 x 4.6 mm, 3.5 µm particle size
Mobile Phase A: 55:45, 50 mM potassium phosphate buffer, pH 3.5:acetonitrile
Flow Rate: 1.0 mL/minute
Wavelength: 217 nm
Injection Volume: 10 µL
Temperature: Ambient

Dissolution Parameters:
Apparatus: USP II (Paddles)
Paddle Speed: 50 RPM, 250 RPM at infinity
Medium: 900 mL 50 mM K_2HPO_4, pH 6.8
Temperature: 37.0 ± 0.5°C
Pull Volume: 10 mL
Pull Times: 10, 20, 30, and 45 (infinity) minutes
Filter: 0.45 μm

Paddles were selected since the final dosage product will be in a soft gelatin capsule. Typically baskets are used for capsules at 100 rotations per minute and paddles are used for tablets and soft gelatin capsules at 50 rotation per minute. The dissolution medium is often dilute 0.1 N hydrochloric acid to mimic the aqueous environment of the stomach. However, diclofenac is not soluble in an acidic environment and therefore, the approximate pH of the intestine, pH 6.8, was used. The temperature of the medium was set at the temperature of the body, 37°C. A profile was implemented to demonstrate the release rate of each active once it comes into contact with the intestines. Immediate release is desirable for a fast onset. Finally, a nylon filter was selected as a suitable filter for an aqueous solution. For the HPLC parameters, the injection volume was decreased from the assay method which would provide minimal detection of impurities for less interference but still have an adequate area response for a robust method. Figure 28 is an overlay of a standard solution and sample solution. Propoxyphene was well resolved from the solvent front and diclofenac eluted in a reasonable time to give an efficient run time. A dissolution test was performed for a research and development prototype of the soft gelatin capsule fill solution. The results are in Table 13. As anticipated, after 10 minutes in the intestines, the active ingredients are almost completely dissolved.

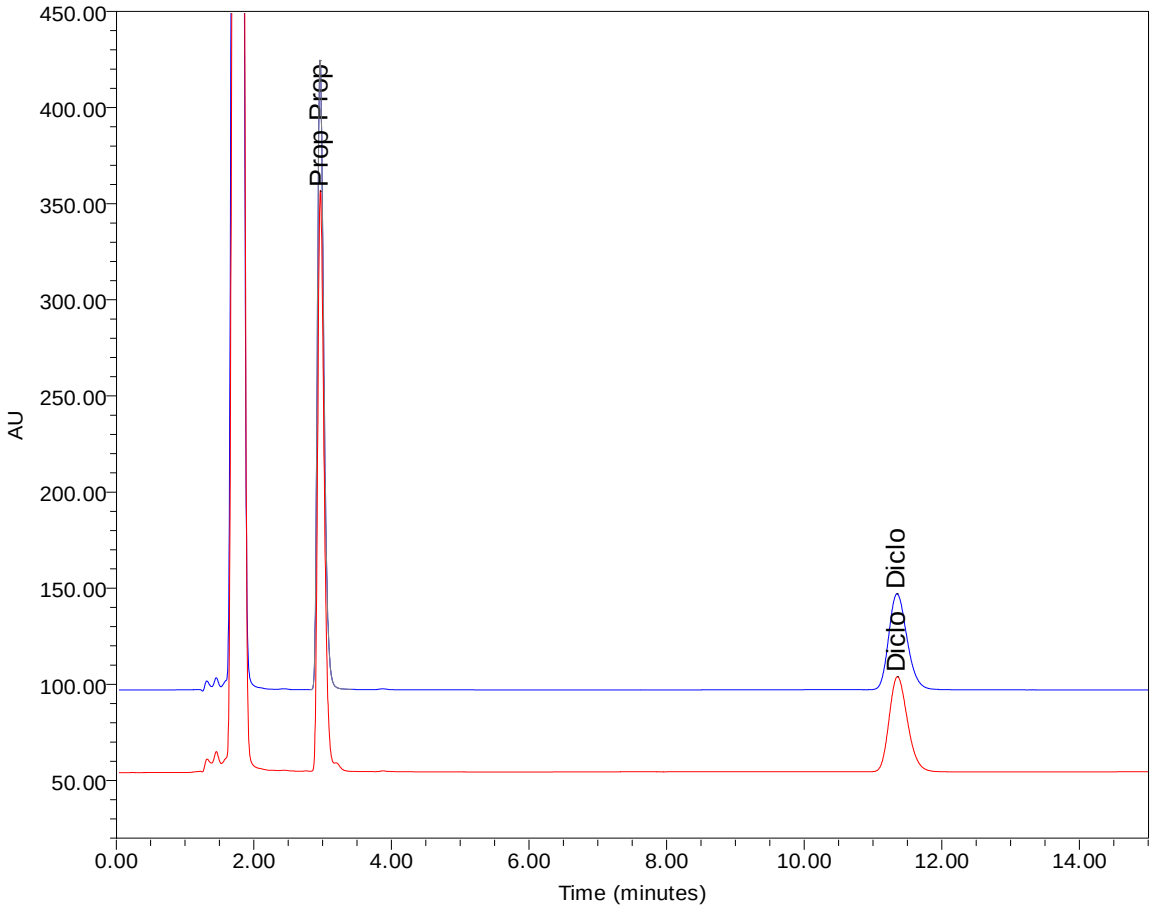

Figure 28. Chromatogram overlay of a standard and sample solution using a mobile phase of 45:55, acetonitrile:phosphate buffer, pH 3.5.

Table 13. Dissolution results of a research prototype

	% Diclofenac Dissolved			
Vessel	**10 minutes**	**20 minutes**	**30 minutes**	**45 minutes(infinity)**
1	104	104	105	105
2	106	107	106	107
3	106	106	107	107
Mean (3)	105	106	106	106
%RSD	0.9	1.5	0.9	1.1

	% Propoxyphene Dissolved			
Vessel	**10 minutes**	**20 minutes**	**30 minutes**	**45 minutes(infinity)**
1	93	94	93	95
2	95	96	96	95
3	94	94	95	96
Mean (3)	94	95	95	95
%RSD	1.5	1.2	1.6	0.8

CONCLUSION

Cost and time are major factors in the development of a pharmaceutical product. Each day saved in development may reflect in thousands of dollars in revenue for a company. Hence, it is of extreme importance to utilize an efficient and productive development plan for analytical to support the integrity of the drug product. Development and optimization of a reliable and robust method for a mixture of solutes is challenging, time consuming and labor intensive. The development time of optimal analytical methods using a complex multi-component system for diclofenac, propoxyphene, and their corresponding impurities was reduced using a mathematical approach and exploring the characteristics and behaviors of the solutes. In addition, the cost of testing the commercial product will be less as a result of one efficient analytical assay method for label claim, impurities and backend dissolution as opposed to multiple analytical assay methods. This in turn may be reflected in a lower cost of the product to the consumer.

A gradient method was developed which employed a 3.5 μm particle size column. This method was further optimized by exploring the pH effects on each solute's behavior. Understanding how each solute behaves for each variable is important when optimizing the method as demonstrated with the pH. The gradient method had adequate resolution between all components. However, an isocratic method was desired for improving the chromatogram baseline, reproducibility and simplicity.

Using the plot of the capacity factor of each solute versus the organic fraction, it was shown the mixture of solutes could not be separated using a binary solvent system (buffer and acetonitrile) when the column was maintained at room temperature. Because

acetonitrile could not separate all components, the isoelutropic binary expression was used to calculate the equivalent buffer-methanol composition. The equivalent strength of buffer-methanol showed different selectivity. However, as in the case with acetonitrile, the buffer and methanol binary system did not give successful separation of all solutes. Because methanol and acetonitrile had different selectivity, the mixture design theory was implemented to develop a ternary separation. By performing only three experiments, the optimum mixture composition of the mobile phase was selected. This ternary system resulted in adequate separation of all solutes.

Although an excellent ternary isocratic method was developed, an attempt was made to develop an isocratic binary method by exploring the column temperature. The characteristics of solutes varied differently with temperature. It was shown that at 60°C, an isocratic binary system having a mobile phase composition of 35% acetonitrile and 65% buffer could separate all actives and impurities necessary for analytical testing of the drug product.

A dissolution method was also developed using the data collected for the development of the assay methods. Using the plot of the capacity factor of each solute versus the organic fraction, the optimum mobile composition was determined and a dissolution method was developed with a short run time and good separation.

This research strongly demonstrates the benefits of using systematic and mathematical approaches when developing analytical methods for a complex mixture. Unlike the trial-and-error approach, which has a blinding effect with variable changes, knowing the behaviors of all the solutes allows logical directions for the development of

optimal analytical methods. In turn, this approach can strongly impact the final development and provide a final product at a reduced cost to the consumer.

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APPENDIX A

Derivation of a Mathematical Equation for the Prediction of Retention Times for Solutes in Gradient Elution Chromatography

In general, a gradient program can be described as a function of time.

$$x = \varphi(t) \tag{1}$$

This gradient program will need a certain time, delay time (τ), to reach the top of the column and additional time, z/u, to reach the point z in the column (u is the linear velocity of the mobile phase).

$$u = \frac{L}{t_0} \tag{2}$$

z is varied from 0 to L, where L is the length of the column. Therefore,

$$\varphi(z,t) = f(t - \frac{z}{u} - \tau) \tag{3}$$

After introducing the inverse function of φ, $f^{-1}(\varphi)$, Equation 3 can be written as shown in Equation 4 and Equation 5 (Schoenmakers, 1978 and 1991).

$$t = \frac{z}{u} + \tau + f^{-1}(\varphi) \tag{4}$$

$$dt = \frac{dz}{u} + df^{-1}(\varphi) \tag{5}$$

The migration velocity of the solute is given by Equation 6

$$\frac{dz}{dt} = \frac{u}{1 + k'_{\varphi}} \tag{6}$$

where $k'(\varphi)$ is the capacity factor at composition φ. By eliminating *dt* from Equation 5 and Equation 6, Equation 7 can be obtained.

$$\frac{df^{-1}(\varphi)}{k'_{\varphi}} = \frac{dz}{u} \tag{7}$$

From $t = 0$ to $t = \tau + \frac{z}{u}$, the gradient program is not reached to point *z*. Therefore, during this period, φ remains at the initial value (φ_i) and the capacity factor remains constant at $k'_{\varphi i}$. For solutes which elute before the gradient program reaches the final composition at the end of the column, the left side of the integral must be split into two parts, one from $t = 0$ to $t = \tau + \frac{z}{u}$ and another from $t = \tau + \frac{z}{u}$ to the column outlet ($z = L$ and $t = t_r$).

From Equation 4, when $t = 0$, $f^{-1}(\varphi) = -\tau$ and when $t = \tau + \frac{z}{u}$, $f^{-1}(\varphi) = 0$. Therefore, integrating Equation 7gives the following solution shown in Equation 8 which can be written as Equation 9.

$$\frac{1}{k'_{\varphi_i}} \int_{-\tau}^{0} df^{-1}(\varphi) + \int_{\varphi \, at \, \tau + \frac{z}{u}}^{\varphi \, at \, t_r} \frac{df^{-1}(\varphi)}{k'_{\varphi}} = \int_{0}^{L} \frac{dz}{u} = \frac{z}{u} = t_0 \tag{8}$$

$$\int_{\varphi \, at \, \tau + \frac{z}{u}}^{\varphi \, at \, t_r} \frac{df^{-1}(\varphi)}{k'_{\varphi}} = t_0 - \frac{\tau}{k'_{\varphi_i}} \tag{9}$$

Equation 9 is valid for any gradient program. For a linear gradient can be expressed as Equation 10.

$$\varphi(z,t) = \varphi_i + b\left(t - \tau - \frac{z}{u}\right) \tag{10}$$

Substituting $t - \tau - \frac{z}{u}$ with $f^{-1}(\varphi)$ according to Equation 4, Equation 10 can be written as Equation 11 or Equation 12.

$$\varphi = \varphi_i + bf^{-1}(\varphi) \tag{11}$$

or

$$f^{-1}(\varphi) = \frac{\varphi - \varphi_i}{b} \tag{12}$$

By differentiating Equation 12, Equation 13 is given.

$$df^{-1}(\varphi) = \frac{d\varphi}{b} \tag{13}$$

Also, according to Equation 10 at $t = \tau + \frac{z}{u}$, $\varphi = \varphi_i$ and at the end of the column ($t = t_r$, $z = L$), φ is equal to the expression shown in Equation 14.

$$\varphi = \varphi_i + b\left(t_r - \tau - \frac{L}{u}\right) = \varphi_i + b\left(t_r - \tau - t_0\right) \tag{14}$$

Substituting these values in Equation 9 gives the following solution shown in Equation 15, which is valid for linear gradient programs.

$$\frac{1}{b} \int_{\varphi_i}^{\varphi_i + b(t_r - \tau - t_0)} \frac{d\varphi}{k'_\varphi} = t_0 - \frac{\tau}{k'_{\varphi_i}} \tag{15}$$

k'_φ is given by Equation 16 where at φ_i it can be expressed as Equation 17 (Snyder, 1980; Jandera, 1985).

$$k'_{\varphi} = k'_{w} \times e^{s\varphi} \tag{16}$$

$$k'_{\varphi_i} = k'_{w} \times e^{s\varphi_i} \tag{17}$$

By eliminating $k'_{w,}$ between Equation 16 and Equation 17 gives a solution shown in Equation 18.

$$k'_{\varphi} = k'_{\varphi_i} \times e^{s(\varphi-\varphi_i)} \tag{18}$$

Substituting Equation 18 into Equation 15 gives Equation 19

$$\frac{1}{b \times k'_{\varphi_i}} \int_{\varphi_i}^{\varphi_i + b(t_r - \tau - t_0)} e^{-s(\varphi-\varphi_i)}\, d\varphi = t_0 - \frac{\tau}{k'_{\varphi_i}} \tag{19}$$

After integration and substitution, Equation 20 can be obtained (Schoenmakers, 1978 and 1991), which is valid for solutes that elute before the gradient program reaches the final composition at the column outlet.

$$t_{r_j} = -\frac{1}{s_j \times b} \times \ln\left\{1 - \left[s_j \times b \left(t_0 - \frac{\tau}{k'_{j_{\varphi i}}} \right) \times k'_{j_{\varphi i}} \right]\right\} + \tau + t_0 \tag{20}$$

In deriving Equation 19, it was assumed that solutes are eluted from the column before the gradient composition reaches the final composition (at the column outlet). For solutes that elute from the column after the gradient program reaches to the final composition at the column outlet, Equation 19 and 20 are no longer valid and must be modified. The left side of the integral in Equation 19, must be split into two parts, one running from an initial composition ($\varphi = \varphi_i$ to $\varphi = \varphi_f$) with a variable k'_{φ} the other from where φ reaches φ_f to the column outlet ($z=L$ and $t = t_r$) with constant k'_{φ_f}. At the time when

φ becomes φ_f, the value of $f^{-1}(\varphi)$ is given by Equation 12 when $\varphi = \varphi_f$ and $f^{-1}(\varphi) = \frac{\varphi_f - \varphi_i}{b}$. At the column outlet, the value of $f^{-1}(\varphi)$ is determined from Equation 4.

$$f^{-1}(\varphi) = t_r - \frac{L}{u} + - \tau \ = t_r - t_0 - \tau \tag{21}$$

Therefore, Equation 19 must be written as Equation 22.

$$\frac{1}{b \times k'_{\varphi_i}} \int_{\varphi_i}^{\varphi_f} e^{-s(\varphi - \varphi_i)} \, d\varphi + \frac{1}{k'_{\varphi_f}} \int_{\frac{\varphi_f - \varphi_i}{b}}^{t_r - t_0 - \tau} df^{-1}(\varphi) = t_0 - \frac{\tau}{k'_{\varphi_i}} \tag{22}$$

By integrating Equation 22 and defining $\Delta\varphi = \varphi_f - \varphi_i$, Equation 23 is observed

$$\frac{1}{b \times s \times k'_{\varphi_i}} \left[e^{-s(\Delta\varphi)} - 1 \right] - \frac{\Delta\varphi}{b \times k'_{\varphi_f}} + \frac{1}{k'_{\varphi_f}} \left(t_r - t_0 - \tau \right) = t_0 - \frac{\tau}{k'_{\varphi_i}} \tag{23}$$

When solving for t_r from Equation 23, Equation 24 is obtained which gives the retention time of any solute which elutes after the gradient program reaches the final composition.

$$t_r = t_0 + \tau + k'_{\varphi_f} \left(t_0 - \frac{\tau}{k'_{\varphi_i}} \right) + \frac{\Delta\varphi}{b} + \frac{k'_{\varphi_f}}{b \times s \times k'_{\varphi_i}} \left[e^{-s(\Delta\varphi)} - 1 \right] \tag{24}$$

Equation 18 was rewritten for the final composition of the gradient and the solution was rearranged and given in Equation 25

$$\frac{k'_{\varphi_i}}{k'_{\varphi_f}} = e^{-s(\Delta\varphi)} \tag{25}$$

By substituting Equation 25 into Equation 24, the final solution is given in Equation 26.

$$t_r = t_0 + \tau + k'_{\varphi_f} \left(t_0 - \frac{\tau}{k'_{\varphi_i}} \right) + \frac{\Delta\varphi}{b} + \frac{1}{b \times s} \left(\frac{k'_{\varphi_f} - k'_{\varphi_i}}{k'_{\varphi_i}} \right) \tag{26}$$

Equation 26 is the rederived equation that was found to be different than that derived by Schoenmakers (1978 and 1991).