COMPARISON OF VARIOUS CHIRAL STATIONARY PHASES FOR THE CHROMATOGRAPHIC SEPARATION OF CHIRAL PHARMACEUTICALS

Sherry E. Layton

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Approved by

Advisory Committee

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Chair

Accepted by

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Dean, Graduate School

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ABSTRACT

Many pharmaceuticals contain active ingredients that have more than one stereoisomer. An important concern is the recognition that these different stereoisomers do not necessarily have identical, or even desirable biological activity. Consequently, analytical methods for the analysis and separation of enantiomers are important in the proper development of a marketed pharmaceutical product.

In this research, direct HPLC methods for the chromatographic separation of oxyphene optical isomers have been developed and optimized using three types of chiral stationary phases. The research carried out a systematic study of the conditions for the separation of oxyphene optical isomers using synthetic polymer chiral stationary phase of cellulose tris (3, 5-dimethylphenylcarbamate) Chiralcel OD, ß-cyclodextrin chiral stationary phase, and α_1-acid glycoprotein chiral stationary phase. The methods using the ß-cyclodextrin and Chiralcel OD columns provide for the accurate determination of the optical purity (as low as 0.1%) of each enantiomer, in the presence of the other major enantiomer. The performance of these chiral stationary phases is also compared.

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DEDICATION

I would like to dedicate this thesis to my husband, Keith, whose constant support and encouragement has meant more to me than he will ever know.

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INTRODUCTION

Many of the most widely prescribed drugs today are chiral molecules. These drugs are often sold as racemic mixtures, a mixture of the two stereoisomers that are non-superimposable mirror images of each other. Pharmaceutical companies can manage the life of their drugs by patenting the racemic mixture as well as the individual isomers. They may also switch the isomer in a drug as a way to prolong the total life of the patent, which is known as a racemic switch. Frequently, the individual isomers of the racemic mixture differ in pharmacological or metabolic activity. These mirror images share the same physical properties such as melting point, boiling point, solubility in various solvents, etc., but differ in the direction in which they rotate plane polarized light. Chromatographic separation of the stereoisomers of a chiral molecule can be difficult because of their identical physical properties. Approximately two thirds of drugs currently on the market are chiral. If a drug is chiral, generally, one of the enantiomeric forms is pharmacologically active, and the other is often not effective or can be harmful (Bauer). There are various examples in which the stereoisomers of chiral drugs show differences in terms of their bioavailability, distribution, and metabolic behavior. The stereoisomers have a fundamental difference in their action and disposition in biological systems. As a result, there is an increasing demand for the separation and isolation of chiral pharmaceuticals.

Pharmaceutical companies that submit chiral pharmaceuticals for registration must adhere to strict guidelines, defined by the Food and Drug Administration (FDA). Before being approved by the FDA, all chiral forms of a drug must be rigorously tested for possible side effects as well as for chiral stability in biological systems (Rhodes). The

FDA demands full documentation of the separate pharmacological and pharmacokinetic (activity and toxicity) profiles of the individual isomers, as well as the racemic mixture (FDA, 1992). Therefore, it is necessary to have reliable analytical methods for the detection and quantitation of each individual isomer. The separation and isolation of the isomers of chiral pharmaceuticals is necessary to determine the enantiomeric purity (analytical chromatography) and to isolate the pure enantiomer (preparative chromatography). Separation of mixtures using preparative chromatography is carried out by overloading the mass and volume conditions in order to increase product throughput.

Pure enantiomers can be obtained either by asymmetric synthesis or by racemic resolution. Asymmetric synthesis is useful when very large quantities are required, however, the time needed to develop the synthesis can make it inappropriate for the small quantities necessary in the early phases of the drug discovery process. The racemic resolution method includes enzymatic resolution, the formation of diastereomers to be separated by crystallization or conventional chromatography and direct chromatographic separation of enantiomers using a chiral stationary phase (Kozma, 2002; Allenmark, 1991; Jones, 1976). Preparative chromatography separation on chiral stationary phases is considered today to be the most efficient and least time-consuming general route to obtain high optical purity of enantiomers, and has become an efficient tool in pharmaceutical research and early drug development (Miller, 1999).

High performance liquid chromatography (HPLC) is becoming more widely used as a technique for the direct separation of chiral compounds. An advantage of HPLC is that it can be used for many enantiomers that are non-volatile, polar, or ionic. It can also

be used for isolation of enantiomers (preparative chromatography). The separation of chiral compounds can also be achieved using gas chromatography (GC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE).

High efficiency and sensitivity are important advantages of the separation of volatile enantiomers by GC. GC is most useful for small, volatile molecules. The disadvantages of GC are that high column temperatures can cause racemization of the chiral stationary phase and the analyte. Derivatization of the analyte is needed to provide volatility or to improve chiral recognition, and preparative separations are generally infeasible.

SFC can usually be used for high molecular weight compounds that cannot be separated by GC or LC efficiently, or in a reasonable amount of time. The higher diffusivity and lower viscosity of supercritical fluids enable three to ten times faster analysis time with SFC in comparison to HPLC. Contrary to GC, SFC uses lower temperatures, therefore, racemization of the stationary phase or analyte is less likely to occur. The disadvantage of using SFC for the separation of chiral molecules is its limited polarity range, that is, it can only be used with compounds that dissolve in methanol or less polar solvents (Majors, 1997). Compounds that are ionic are not good candidates for SFC.

In CE, enantiomers may be recognized stereoselectively only on the basis of their interaction with a chiral selector. Enantiomer separation relies on enantioselective non-covalent intermolecular interaction between the analyte and a chiral selector, which may be expressed as the effective mobility differences, and results in separation in CE. In direct enantioseparation by CE, a chiral selector, usually one of the cyclodextrins (α, ß, or

γ), or crown ethers is added to the running buffer where it interacts with the optical isomers to form an inclusion complex. This changes the electrophoretic mobilities of the isomers, which are otherwise equal. The separation can be optimized by adjusting the pH of the buffer, the temperature, and the applied voltage. There are several advantages of CE. One is that it is rapid and highly efficient, which results in higher resolution. Another is that one single CE can be subsequently filled with electrophoresis buffer containing different chiral selectors for the separation of various enantiomers. Also, because the volume of the CE system is small, very little chiral selector is required. Chiral selectors often are very expensive or are not commercially available. The major disadvantage of CE is that it cannot be used for the isolation of isomers on a preparative scale.

CE carried out in the presence of micelles is known as micellar electrokinetic chromatography (MEKC). The separation in MEKC results from the distribution of enantiomers between micelles and the aqueous mobile phase in the presence of electroosmotic flow. Micelles act as a pseudo-stationary phase. This technique involves the addition to the operating buffer of a chiral surfactant sometimes mixed with a conventional surfactant. When a sample is introduced into this system, the components are distributed between the aqueous phase and the hydrocarbon phase of the micelle's interior. The differential partitioning of the chiral molecule into the asymmetric chiral micelle results in separation (Majors, 1997). The mechanism of separation in LC and MEKC is the same, and depends upon differences in the distribution coefficients for analytes between the mobile aqueous phase and the hydrocarbon micelle phase. One advantage of this technique over HPLC is that it has much higher column efficiencies.

Also, changing the second phase in MEKC is simple. It only requires changing the micellar composition of the buffer. In HPLC, the second phase can only be changed by changing the type of column packing (Skoog, 1998).

Separation of chiral isomers can be carried out using HPLC through direct and indirect methods. Indirect methods are based on adding a chiral additive to the mobile phase. The optical isomers react with the chiral additive, and then the derivatives are separated on an achiral stationary phase. Direct methods separate the isomers on a chiral stationary phase. For enantiomers to be separated directly over a chiral stationary phase, they must form short-lived diastereomeric molecular complexes of non-identical stability by interacting rapidly and reversibly (Perrin, 1991). There are five different categories of HPLC chiral stationary phases which are commercially available: brush type, synthetic polymer, cyclodextrin bonded, ligand exchange, and protein. This research addresses the HPLC separation of oxyphene optical isomers using direct methods.

Brush Type Chiral Stationary Phase

Brush type chiral stationary phases are also known as Pirkle chiral stationary phases. These types of chiral stationary phases rely on multiple discreet molecular interactions to discriminate between enantiomers. Brush type chiral stationary phases have been prepared using low molecular weight selectors which have been designed to contain only those interaction sites which are essential for the differentiation of the enantiomers. Brush type columns have a wide range of physical compositions ranging from amino acids to substituted naphthyl rings. Separation on a brush type chiral stationary phase is based on at least three point attractive interactions. Brush type chiral stationary phases form complexes with the analyte through attractive interactions, such as

hydrogen bonding, pi-pi interactions, dipole-dipole interactions, and minimization of repulsive (steric) interactions (Pirkle, 1992). Aromatic rings are potential sites for pi-pi interactions. Acidic sites donate protons for potential intermolecular hydrogen bonding. The hydrogen involved often comes from an amide, carbamate, urea, amine, or alcohol. Basic sites provide non-bonding or pi electrons, and pi-pi interactions occur between aromatic rings within the analyte and those in the chiral stationary phase (Perrin, 1991). Basic sites such as sulfinyl or phosphinyl oxygens, hydroxyl or ether oxygens, or amino groups may also contribute to hydrogen bond formation. For most analytes, the sites of interaction must be located at or near the chiral center. The analyte must have the necessary three interaction sites for separation of the isomers on this type of chiral stationary phase. Brush type HPLC chiral stationary phases are most often used with non-polar mobile phases composed of hexane and a polar mobile phase modifier. Isopropyl alcohol, dichloromethane, or ethanol are typical choices for the polar mobile phase modifier. The structure, type, and concentration of the polar modifier can greatly affect the retention and stereoselectivity of the molecule. Non-polar mobile phases are used to maximize the polar attractive interactions between the analyte and the chiral stationary phase (Wainer, 1988). For analytical purposes, the retention of the analyte can be adjusted by varying the mobile phase composition in order to minimize the run time.

When the chiral stationary phase contains a pi-acid, amides containing a naphthyl group are commonly formed. When the chiral stationary phase contains a pi -base, carbamates are usually formed. Sometimes it is necessary to derivatize the analyte to increase the separation of enantiomers.

Synthetic Polymer Chiral Stationary Phase

The most widely used synthetic polymer based chiral stationary phases are polysaccharides. Polysaccharides can be coated on silica gel. The polysaccharide phase is comprised of derivatized cellulose or amylose coated on a silica support. Derivatization of the polysaccharide hydroxyl groups with various side chains give different helical supramolecular structures. Table 1 illustrates the various types and structures of the various Chiralcel stationary phases (Chiral Technologies, 2004). The curved groove of the helix is chiral, and can greatly favor the binding of one enantiomer over the other. The result is separation of the enantiomers. Interaction between analyte and synthetic polymer chiral stationary phases are based on both attractive interactions (H-bonding, pi-pi interaction, and/or dipole stacking) and inclusion complexes. Instead of a silica surface, inclusion complexes utilize cavities in which the analyte fits (Wainer, 1987). Synthetic polymer chiral stationary phases are most commonly used with non-polar mobile phases of alkanes (hexane or heptane) and a polar mobile phase modifier. An alcohol, such as ethanol or isopropyl alcohol, is generally the polar mobile phase modifier of choice. The structure of the alcohol plays an important role in the retention and resolution due to competition at or near the binding site.

Reverse phase versions of polysaccharide columns (Chiralpak AD-RH, AS-RH, Chiracel OD-RH and OJ-RH) are also available and can be used for samples which do not dissolve in an alkane/alcohol solvent mixture, or require aqueous mobile phase (for example, biological samples).

Recently, a new generation of polysaccharide derivative columns was developed by immobilizing it on silica gel support instead of coating it on silica. The

Table 1. Polysaccharide phases of derivatized cellulose or amylose coated on silica support

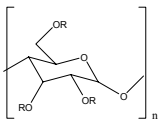
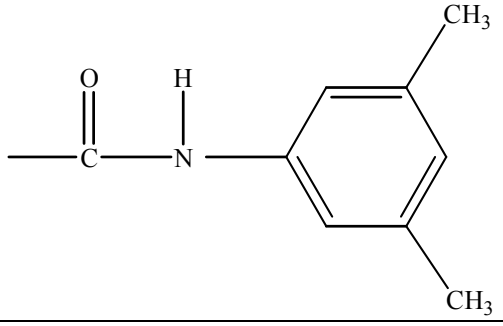
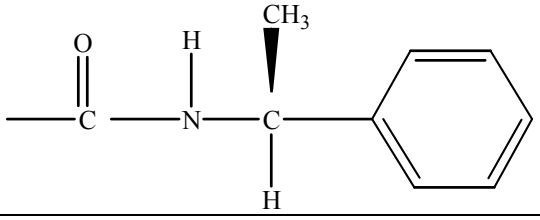
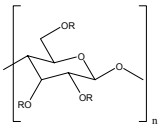
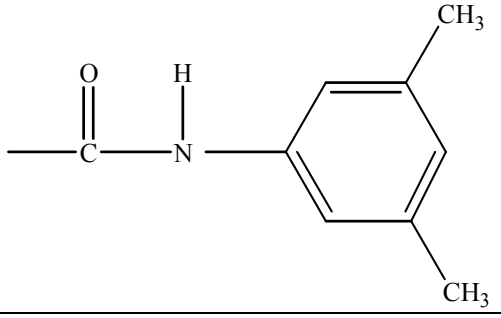
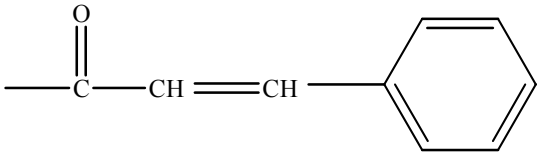
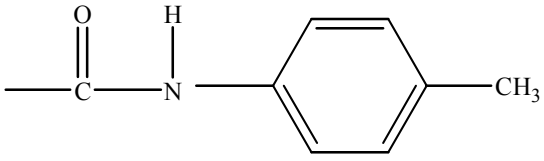
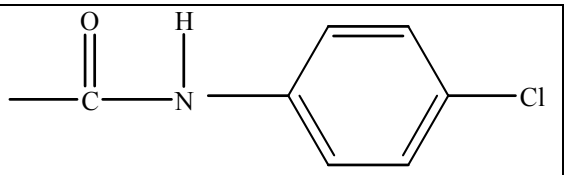
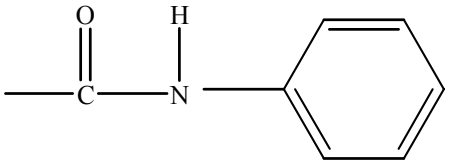
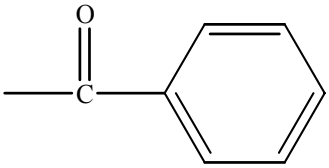
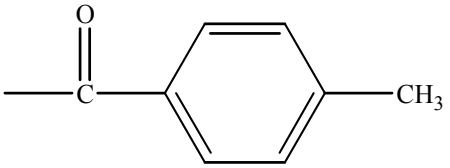
	Side Group (R)	
Series	Name	Structure
Derivatized Amylose (amylose derivative coated on silica gel) 	AD 3,5-dimethylphenyl carbamate	
	AS (S)- α-methylbenzyl carbamate	
Derivatized Cellulose (cellulose derivative coated on silical gel) 	OD 3,5-dimethylphenyl carbamate	
	OK cinnamate	
	OG 4-methylphenyl carbamate	

Table 1. cont.

Derivatized Cellulose	OF 4-chlorophenyl carbamate	
	OC phenyl carbamate	
	OB benzoate	
	OJ 4-methylbenzoate	

immobilization allows free choice of any miscible solvent. Using either non-polar or aqueous mobile phase greatly expands the application domain of the polysaccharide derivative chiral stationary phases. Other series of synthetic polymer columns consist of polyacrylamide based and N, N'-diallyltartardiamide (L-DATD) based polymers.

Cyclodextrin Bonded Chiral Stationary Phase

The ability of cyclodextrins to form inclusion complexes was recognized by Freundenberg, et al. (Freundeberg, 1948). The cyclodextrin bonded chiral stationary phase is prepared by bonding various cyclodextrins to silica support. Cyclodextrins are chiral molecules composed of six or more glucose residues chair conformations linked in a ring. Cyclodextrins are referred to by the number of glucose residues they contain: α-cyclodextrin contains six, ß-cyclodextrin contains seven, and γ-cyclodextrin contains eight. The structure of ß-cyclodextrin is shown in Figure 1 (Menges, 1991). Cyclodextrins with fewer than six glucose residues have not been found, and cyclodextrins with more than eight glucose residues have been identified (Menges, 1991). For cyclodextrin bonded chiral stationary phases the mechanism of interaction consists of inclusion complexes. All or part of the analyte enters into chiral cavities within the chiral stationary phase to form inclusion complexes (Wainer, 1988). Complexation involves interaction of a nonpolar portion of the analyte with the nonpolar cyclodextrin cavity. The size, shape, and polarity of the analyte are the most critical factors influencing the inclusion complex. If the analyte is too large to fit into the hydrophobic cavity, then inclusion will not occur (Menges, 1991). In general, the better the molecular fit of the analyte into the cavity, the greater the potential for chiral recognition. This fit into the

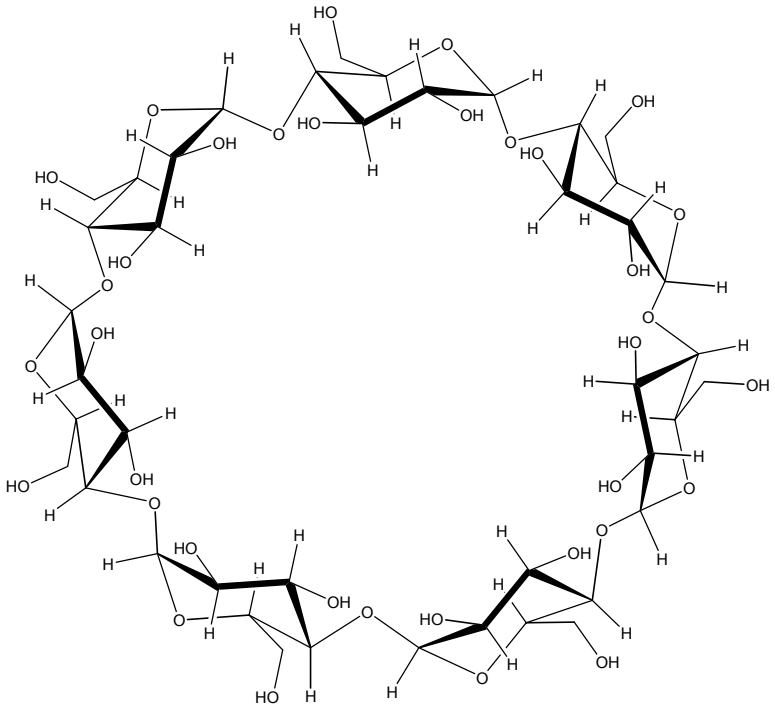


Figure 1. Structure of β-cyclodextrin

cavity is most often achieved when the analyte contains at least one or more rings. Most of the time, an aromatic ring is necessary for the inclusion complex to occur.

Ligand Exchange Chiral Stationary Phase

In ligand exchange chiral stationary phases, an amino acid such as L-proline is bonded to silica gel support. The separation is based on the formation of an enantioselective ternary complex between amino acid (fixed ligand), a transition metal ion in mobile phase (usually copper ion), and the solute (mobile ligand). The difference in stability between complexes with R and S form of solute (chiral analyte) results in separation of the enantiomers. Factors affecting the selectivity and efficiency of the separation include the pH, the ionic strength of the mobile phase, and the temperature. For the separation to be successful, the solute must have two polar functional groups with the correct spacing, which can simultaneously act as ligands for the copper ion. For this reason, ligand exchange chiral stationary phases are very good at separating underivatized amino acids and carboxylic acids. However, there are a limited number of classes of analytes that can be separated on this type of chiral stationary phase.

Protein Chiral Stationary Phase

The protein chiral stationary phase is a protein immobilized on silica support. Proteins are complex, high molecular weight polymers composed of amino acids, most of which are chiral. Some proteins such as α_1 acid glycoprotein (AGP), bovine serum albumin (BSA), human serum albumin (HSA), or ovomucoid (OVM) can reversibly bind with small molecules and these binding interactions sometimes can be stereospecific (Hermansson, 1989; Allenmark, 1986; Domencici, 1990; Miwa, 1987). Such specific

stereoselective interactions of these proteins with solutes have been used to develop protein-base chiral stationary phases and these chiral stationary phases have an extremely wide range of application. Proteins that tolerate organic solvents as well as high temperatures and function properly over a wide pH range are particularly useful in preparing a chiral stationary phase (Narayanan, 1992). Because of their complex structures, the mechanism of chiral recognition by proteins is largely unknown. The solute is retained on this type of chiral stationary phase mainly by combinations of ionic bonding, hydrophobic interaction, hydrogen bonding, and charge transfer interaction. Retention and separation of isomers can be manipulated by varying the temperature, pH, ionic strength, organic modifier concentration, and flow rate. Protein chiral stationary phases most often use aqueous mobile phases that contain phosphate buffer. For enantioselective separation a three point interaction is necessary. The analyte needs to have at least one binding group and a ring structure near the chiral center in order to be resolved on protein chiral stationary phases. The distance between the binding groups and the chiral center is also important in determining the degree of enantioselectivity. Hydrogen bonding groups are best if they are near the chiral center. Resolution is also influenced by substitution of basic nitrogen atoms (Narayanan, 1992). The wide variety of chiral analytes that can be separated on the protein chiral stationary phases, and the use of aqueous mobile phases make them a good analytical tool for the separation of chiral pharmaceuticals. Loading capacity of protein-based chiral stationary phases is small compared to the other chiral stationary phases, and as a result, these chiral stationary phases cannot be used for scale-up preparative application for isolation of chiral compounds.

EXPERIMENTAL

Equipment

The high performance liquid chromatography (HPLC) equipment used for the synthetic polymer and cyclodextrin bonded chiral stationary phases was a Micromeritics 728 autosampler, Hitachi L-7100 pump, Applied Biosystems 785A absorbance detector, and Eppendorf CH-30 column heater. The HPLC equipment used for the brush type and and protein chiral stationary phases was a Hewlett-Packard 1100 equipped with a gradient pump, autosampler, and temperature controlled column compartment. The detector used for the brush type and protein chiral stationary phases was an Applied Biosystems 785A absorbance detector. The following columns were used: Pirkle Covalent L-Phenylglycine, 250 x 4.6 mm, 5 µm particle size column supplied by Rexchrom Regis (brush type), Chiralcel OD-R, 250 x 4.6mm, 10 µm particle size column supplied by Daicel Chemical Industries, Ltd. (synthetic polymer), Chiral CD-Ph, 250 x 4.6 mm, 5 µm particle size column supplied by Shiseido (cyclodextrin bonded), Chiral AGP, 100 x 4.6 mm, 5 µm particle size column supplied by ChromTech (protein). All columns were selected because they were readily available. A ligand exchange chiral stationary phase is not suitable for separation of the isomers of this class of analyte. Therefore, it was not used for any experiments in this research. Various phosphate and acetate buffer concentrations were used for the aqueous portion of the mobile phases, where applicable. Hexanes (Mallinckrodt, ACS grade), 1, 2-dichloroethane (Aldrich, anhydrous), ethanol (Aaper, 200 proof), methanol (Fisher, HPLC grade), acetonitrile (Fisher, HPLC grade), and isopropyl alcohol (Burdick and Jackson, HPLC grade) were

the organic solvents used. All sample injections were analyzed at a detection wavelength of 260 nm.

Computer Software and Program

Waters Millennium version 4.0 was used as the data acquisition program to collect all chromatographic data and to measure resolution values, tailing factors, and retention times.

Chemical Information

The following list shows the names of the actives and derivatizing agents that were used in this research.

List of Components

	Component Type
(+)-(2S, 3R)-4-Dimethylamino-3-methyl-1,2-diphenyl-2-butanol ((+)-oxyphene, Fluka, > 97%)	Active
(-)-(2R, 3S)- 4-Dimethylamino-1,2-diphenyl-3-methyl-2-butanol ((-)-oxyphene, Aldrich, 99%)	Active
1-Naphthylisocyanate (Aldrich, 98%)	Derivatizing agent
n-Butyllithium (Aldrich, 2.5 M solution in hexanes)	Reagent

The structure of α-(+)-oxyphene is shown in Figure 2 (Fadnavis, 2001).

Procedure

The goals of this research were to separate and optimize the oxyphene optical isomers on the various chiral stationary phases, and to compare the performance of the various chiral stationary phases for analytical and preparative purposes. A method for the separation of oxyphene optical isomers on a protein and cyclodextrin based columns is

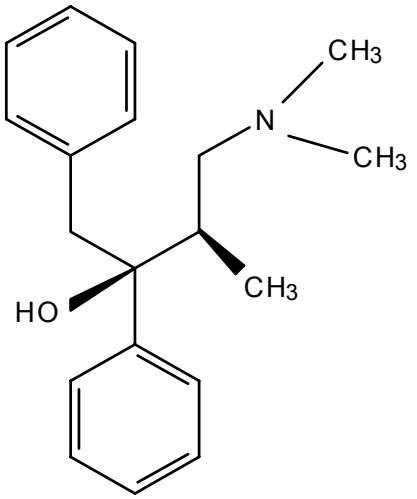


Figure 2. Structure of α-(+)-oxyphene

not reported in the literature. Therefore, new analytical methods were developed and optimized for the separation on these types of chiral stationary phases. A method for the separation of oxyphene isomers on a polysaccharide type chiral column (Chiralcel OD) was found in the literature. Due to the unavailability of the Chiralcel OD column, a Chiralcel OD-R was used. Both Chiralcel OD and Chiralcel OD-R are made with cellulose tris (3, 5-dimethylphenylcarbamate) coated on 10 μm silica gel; the Chiralcel OD-R can be used as Chiralcel OD after it is washed with isopropyl alcohol. This method was optimized for analytical use. An available brush type column (L-phenylglycine) was also tried for the separation of oxyphene isomers.

RESULTS AND DISCUSSION

Cyclodextrin Bonded Chiral Stationary Phase

Background

Commercially available ß-cyclodextrin bonded stationary phases have been successfully used for the separation of a variety of enantiomers by HPLC (Armstrong, 1985; Armstrong, 1986; Thuaud, 1994; Hinz, 1985; Moller, 1994). The chiral recognition mechanism of cyclodextrins (CDs) usually results from the formation of inclusion complexes between the hydrophobic moiety of the analyte and the relatively non-polar interior of the CD cavity (Bressolle, 1996). Most enantioseparations are usually obtained in the reversed phase mode based on hydrophobic interactions and hydrogen bonding (Han, 1989). Nevertheless, the exact mechanism of chiral recognition remains unknown.

Method Development

A Chiral CD-Ph, 250 mm x 4.6 mm, 5 µm column was used for all method development and optimization experiments involving a cyclodextrin bonded chiral stationary phase. A solution representing the racemic mixture, 50:50 of each oxyphene optical isomer, was used for the method development. In order to evaluate and compare the chromatographic separation, the enantioselectivity, resolution, and tailing factors were calculated and compared. The enantioselectivity, α, was calculated using Equation 1;

$$\alpha = \frac{tr_2 - t_0}{tr_1 - t_0} \tag{1}$$

where t_{r1} and t_{r2} are the retention times of the less and more retained isomers, respectively, and t_0 is the column hold-up time. The column hold-up time, the time it takes to elute a non-retained component, is determined from Equation 2;

$$t_0 = \frac{V_0}{F_v} \tag{2}$$

where t_0 is the column hold-up time (minutes), V_0 is the column hold-up volume (mL), and F_v is the mobile phase flow rate. The volume of the mobile phase required to elute a non-retained component is the column hold-up volume. The column hold-up time is inversely proportional to flow rate. It was determined from the solvent front to be 2.1 minutes at a 1 mL/min flow rate, 4.1 minutes at a 0.5 mL/min flow rate, and 1.1 minutes at a 2 mL/min flow rate. Resolution was calculated using Equation 3;

$$Rs = \frac{2(t_{r2} - t_{r1})}{(w_{b1} + w_{b2})} \tag{3}$$

where w_{b1} and w_{b2} are the corresponding widths of peaks 1 and 2 at the base of the peaks. Tailing was calculated using Equation 4;

$$T = \frac{W_{0.05}}{2f} \tag{4}$$

where $W_{0.05}$ is the width of the peak at 5% of the peak height, and f is the distance from the peak maximum to the leading edge of the peak at 5% of peak height. In general, higher selectivity, higher resolution, and less tailing results in better separation.

The starting chromatographic parameters for the method development were a typical mobile phase for a reverse phase HPLC system consisting of 0.05 M, pH 4 ammonium acetate buffer and acetonitrile in a volumetric ratio of 50:50, flow rate of 1.0 mL/min, 75 µL injection volume, and detection wavelength of 260 nm. The first sample solution evaluated was a mixture of the isomers at a concentration of 0.6 mg/mL for each isomer. Using the starting chromatographic parameters, the isomers were separated with a resolution factor of 1.0. Tailing factors for (+)-oxyphene and (-)-oxyphene were 5.2 and 4.8, respectively. A chromatogram of sample representing the racemic mixture using the starting chromatographic parameters is shown in Figure 3. The chromatogram shows acceptable resolution of the two isomers, however, the tailing is high and unacceptable. Therefore, the tailing must be reduced.

Influence of Buffer Type

It is known that the untreated silica of the reversed phase stationary phase has strong interactions with the solute, resulting in large tailing. Triethylamine (TEA) is commonly used to cover the untreated silica and reduce peak tailing. Therefore, a

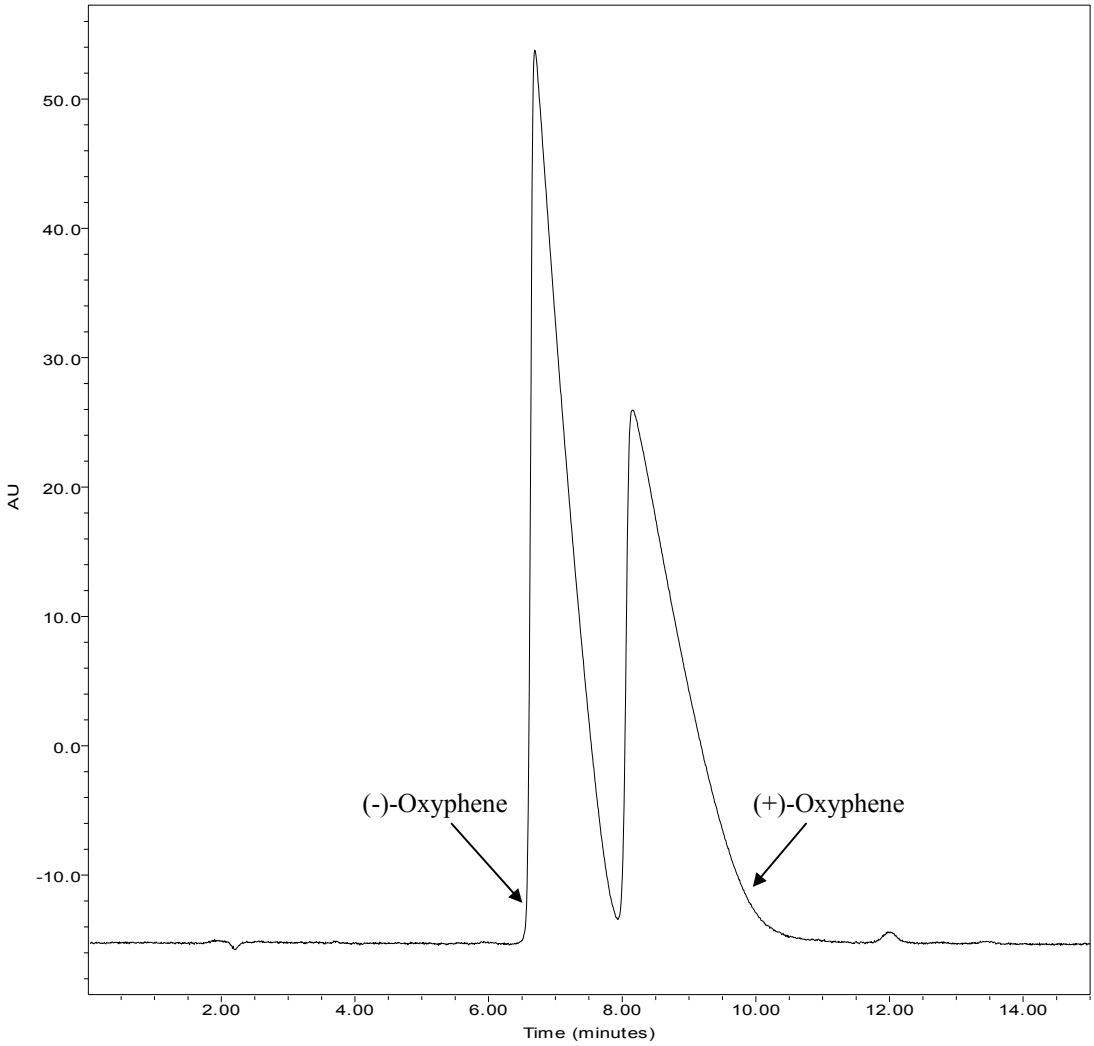


Figure 3. Chromatogram of the racemic mixture using the starting chromatographic conditions

mixture of triethylamine and acetic acid was used as the buffer, instead of ammonium acetate. The sample concentration was increased to 3 mg/mL for each isomer, and the injection volume was reduced to 15 µL. This column load (0.045 mg) is the same as what was used during the method development with the original chromatographic parameters. A chromatogram of the racemic mixture using mobile phase of 1% (by volume) triethylamine adjusted to pH 4 with acetic acid and acetonitrile in the volumetric ratio 50:50 is shown in Figure 4. All other chromatographic conditions were the same as the starting chromatographic parameters. When this mobile phase was used, the resolution was 1.6 and the tailing factors were 2.5 for (+)-oxyphene, and 2.1 for (-)-oxyphene as shown in Table 2. With this buffer, the tailing decreased and the resolution increased. Therefore, triethylamine adjusted to pH 4 with acetic acid was selected instead of acetate buffer.

Influence of Mobile Phase pH

The influence of the pH of the mobile phase consisting of a mixture of 1% triethylamine and acetonitrile in the volumetric ratio 50:50 was evaluated in the range of pH 3 – 5, adjusted using acetic acid, as shown in Figure 5 and Table 3. The increase in pH caused the retention time of both enantiomers to increase. Changing the mobile phase pH had very little influence on enantioselectivity and resolution. Since acetate has better buffer capacity at pH 4 compared to pH 3, pH 4 was selected as the pH of the buffer. A pH lower than 3 was not evaluated because silica based stationary phases are not stable at low pH.

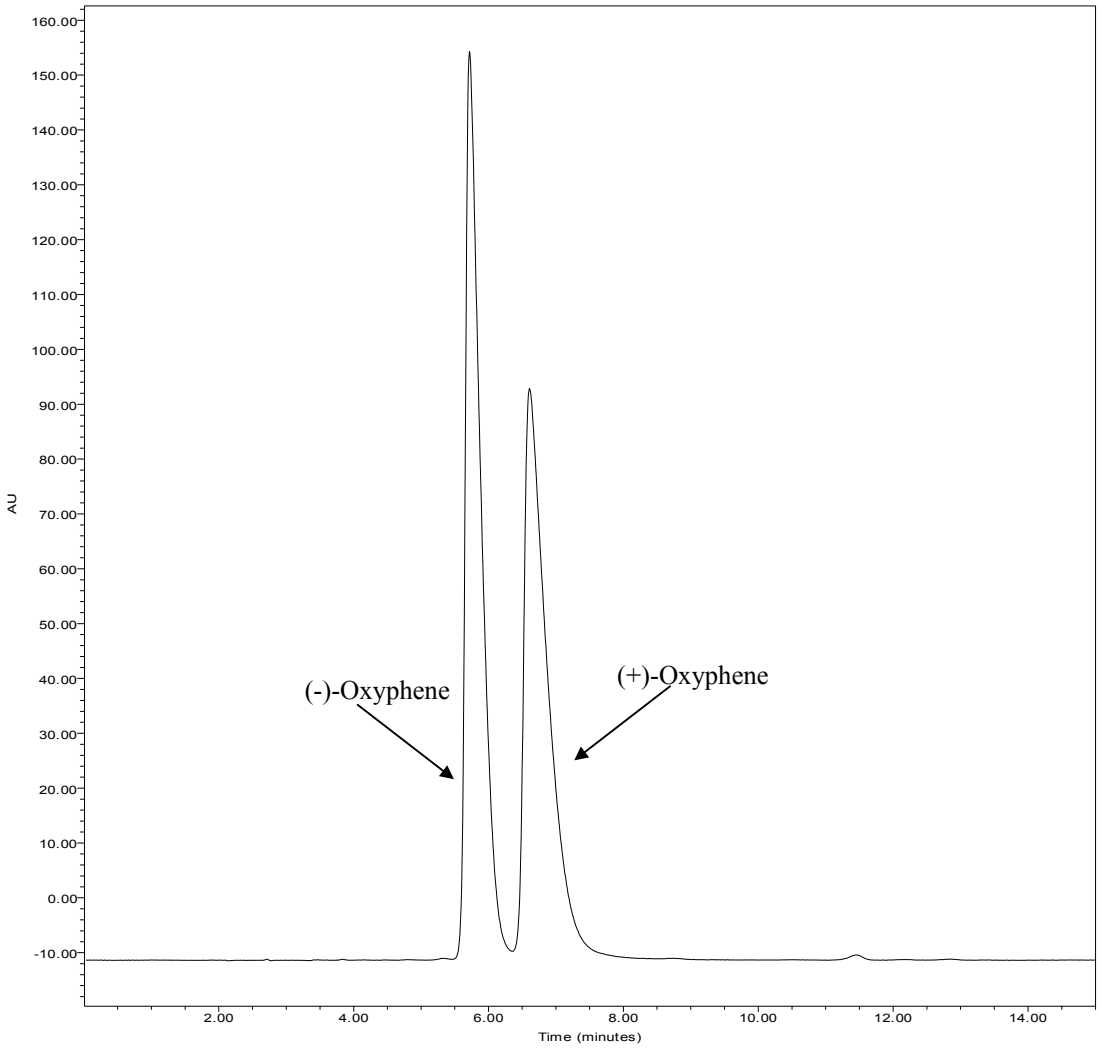


Figure 4. Chromatogram of the racemic mixture using mobile phase of 1% triethylamine adjusted to pH 4 with acetic acid and acetonitrile, 50:50

Table 2. Influence of buffer type on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a ß-cyclodextrin column

Buffer	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
0.05 M ammonium acetate	1.31	1.0	5.2	4.8
1% TEA adjusted to pH 4 with acetic acid	1.24	1.6	2.5	2.1

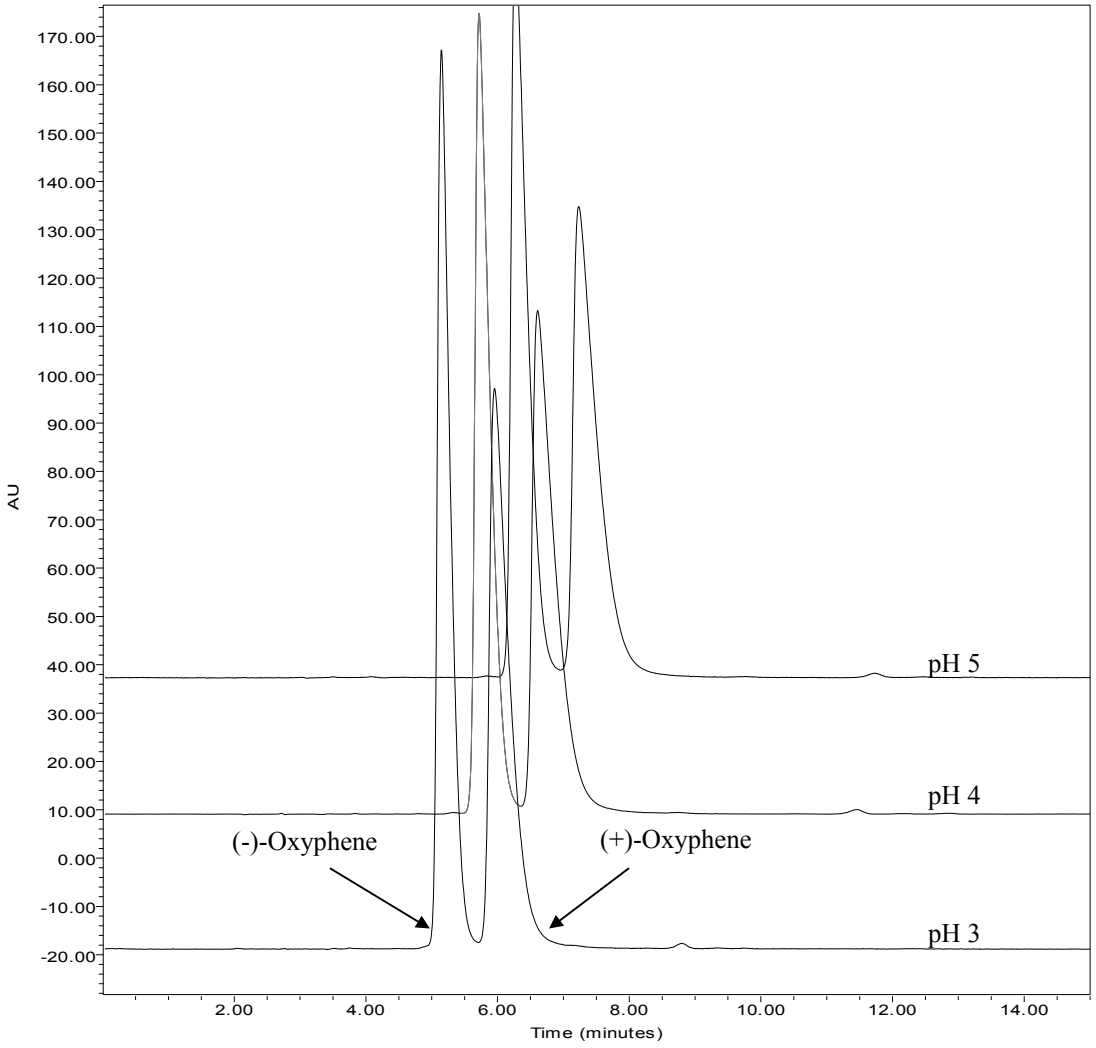


Figure 5. Chromatograms of the racemic mixture showing the influence of mobile phase buffer pH

Table 3. Influence of the mobile phase pH on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a ß-cyclodextrin column

Buffer pH (1% TEA)	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
3	1.26	1.7	2.4	1.9
4	1.24	1.6	2.5	2.1
5	1.23	1.6	2.6	2.2

Influence of Triethylamine Concentration

The effect of triethylamine concentration on the separation was studied by varying the concentration of triethylamine in the mobile phase. Figure 6 illustrates the chromatographic effect of the various buffer concentrations. Results of the selectivity, resolution, and tailing factors for the various buffer concentrations are show in Table 4. Tailing is very high and resolution is lower with 0.1% triethylamine. By increasing the triethylamine concentration, resolution is improved and tailing is reduced. The effect is less pronounced from 1% to 2% triethylamine. 2% triethylamine adjusted to pH 4 with acetic acid was selected as the optimal buffer concentration.

Influence of Organic Modifier Composition

The organic modifier composition of the mobile phase was also optimized for the separation of (+) and (-)-oxyphene. The initial mobile phase had an organic modifier content of 50% acetonitrile. Mobile phase organic compositions of 40%, 60%, and 70% acetonitrile were evaluated. The influence of the mobile phase organic modifier composition on retention, enantioselectivity, resolution, and tailing is shown in Table 5. The chromatographic effect of organic modifier composition is shown in Figure 7. As expected in a reverse phase system, a decrease in acetonitrile concentration resulted in an increase in retention time. Enantioselectivity and resolution slightly improved with the decrease in acetonitrile concentration. However, the tailing factor and the run time increase by decreasing the composition of organic modifier. Using 70% organic in the mobile phase resulted in lower tailing and a shorter run time, with acceptable resolution. The retention of (-)-oxyphene with 70% organic is close to the solvent front. Therefore,

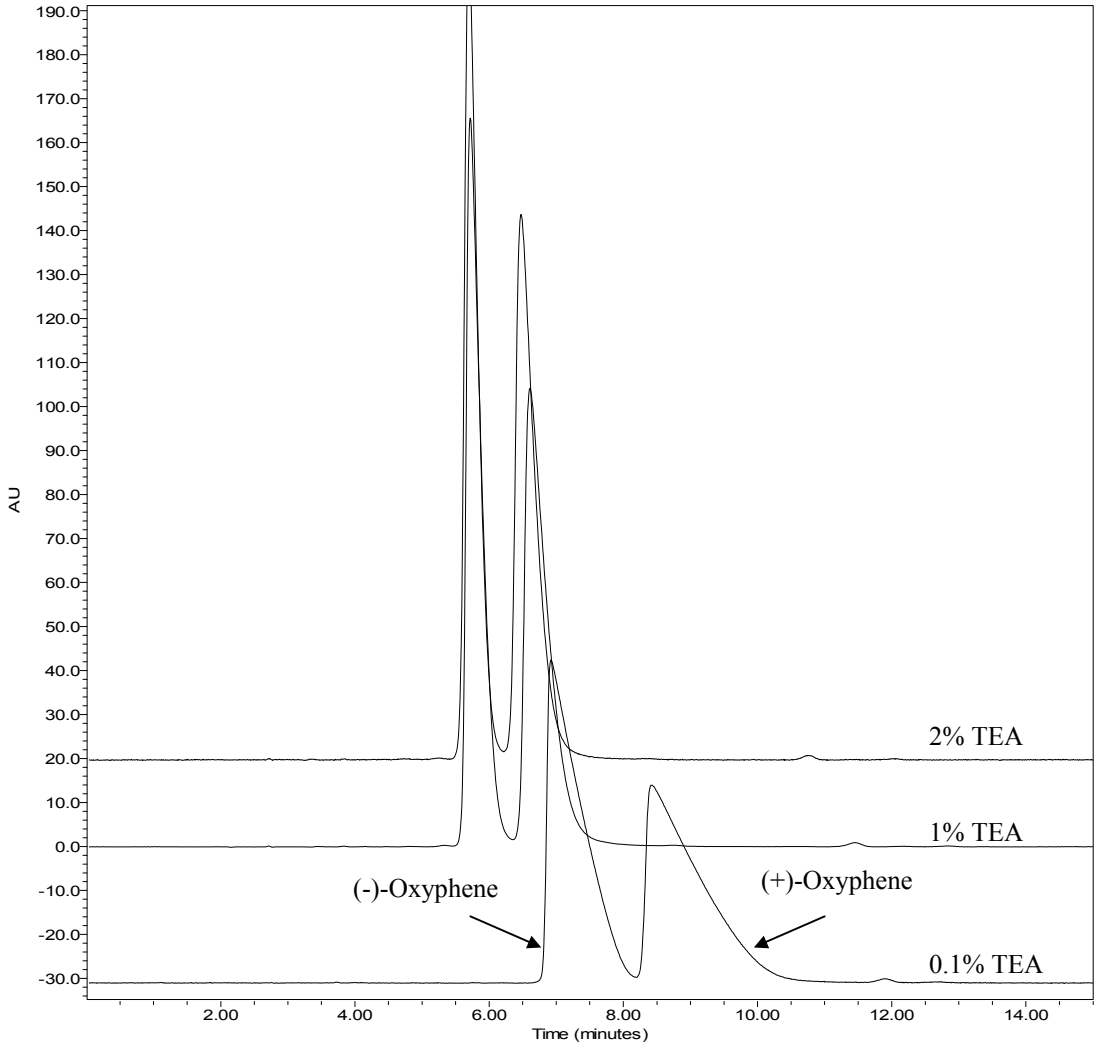


Figure 6. Chromatograms of the racemic mixture showing the influence of mobile phase buffer concentration

Table 4. Influence of TEA concentration on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a ß-cyclodextrin column

Buffer Concentration (TEA)	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
0.1%	1.31	1.0	5.2	4.6
1%	1.24	1.6	2.5	2.1
2%	1.22	1.6	2.1	1.7

Table 5. Influence of organic modifier composition on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a ß-cyclodextrin column

Organic Modifier Composition (acetonitrile)	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
40%	1.22	1.8	2.6	2.1
50%	1.24	1.6	2.1	1.7
60%	1.21	1.5	1.9	1.5
70%	1.19	1.5	1.7	1.3

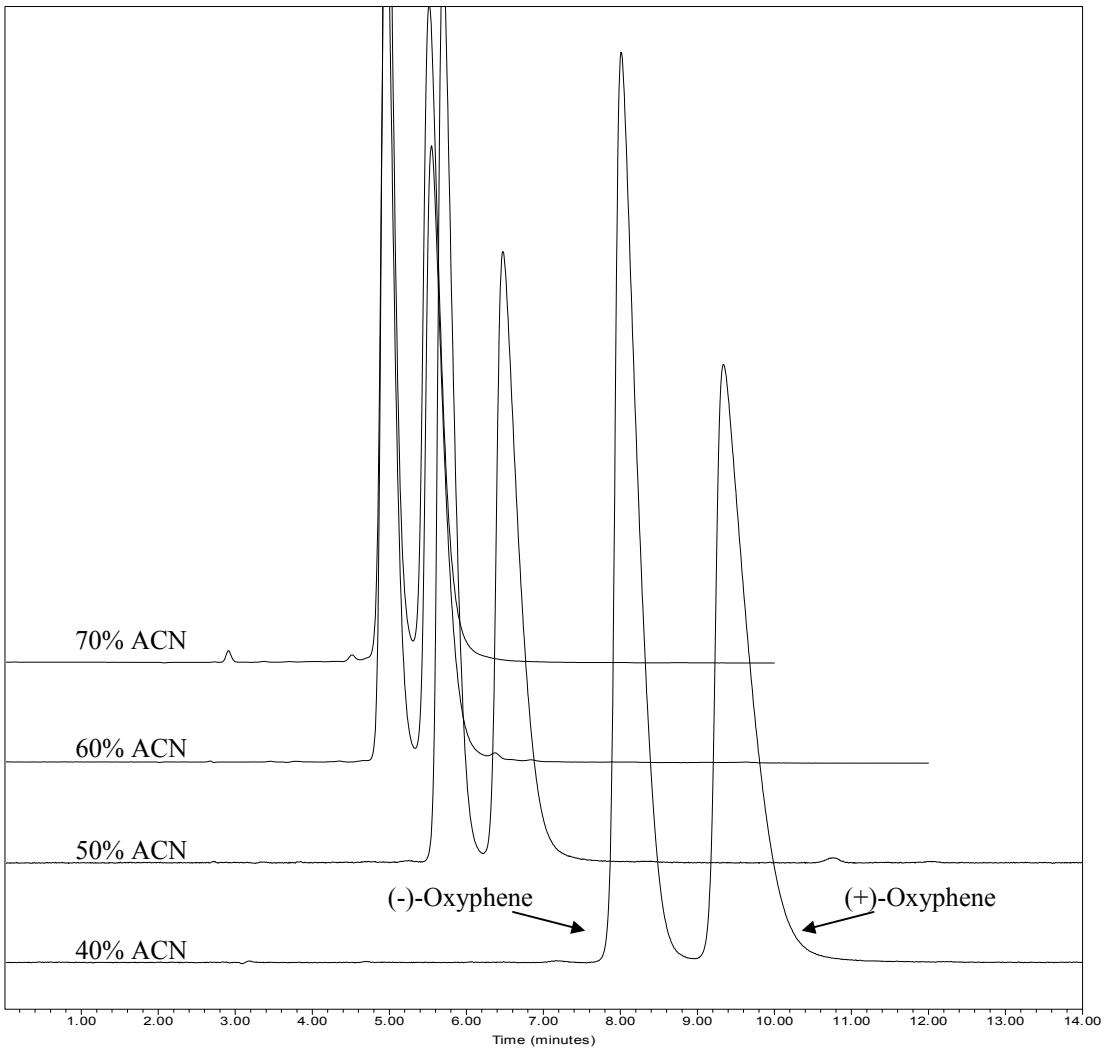


Figure 7. Chromatograms of the racemic mixture showing the influence of mobile phase organic modifier composition

organic modifier compositions higher than 70% were not evaluated. The optimal mobile phase organic modifier composition was 70% acetonitrile.

Influence of Flow Rate

All experimental studies up to this point have used a flow rate of 1.0 mL/min, sample concentration of 3 mg/mL for each isomer, and injection volume of 15 µL. Since peak area is inversely proportional to flow rate, sample concentrations and injection volumes were adjusted proportional to the flow rate in order to keep the same sensitivity. For flow rate of 0.5 mL/min, the sample concentration was reduced to 1.5 mg/mL, and 15 µL was injected. For 2.0 mL/min flow rate, the sample concentration was 3 mg/mL and 30 µL was injected. The influence of flow rate on retention, enantioselectivity, resolution, and tailing is shown in Table 6. The chromatographic effect of changing the flow rate is shown in Figure 8. As expected, a decrease in the flow rate resulted in an increase in retention time. Separation is significantly improved by reducing the flow rate. Although decreasing the flow rate below 0.5 mL/min will improve the separation, it will also increase the run time. Therefore, a flow rate of 0.5 mL/min was selected as the optimal flow rate due to the acceptable separation between the peaks, acceptable resolution values, and reasonable run time.

Influence of Column Temperature

Column temperatures of 5º C, 25º C, and 45º C were evaluated. According to the column manufacturer recommendations for this particular column, temperatures higher than 45º C should not be used. The influence of the column temperature on retention, enantioselectivity, resolution, and tailing are shown in Table 7. The

Table 6. Influence of flow rate on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a ß-cyclodextrin column

Flow Rate, mL/min	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
0.5	1.20	1.8	1.8	1.4
1	1.19		1.7	
2	1.18			

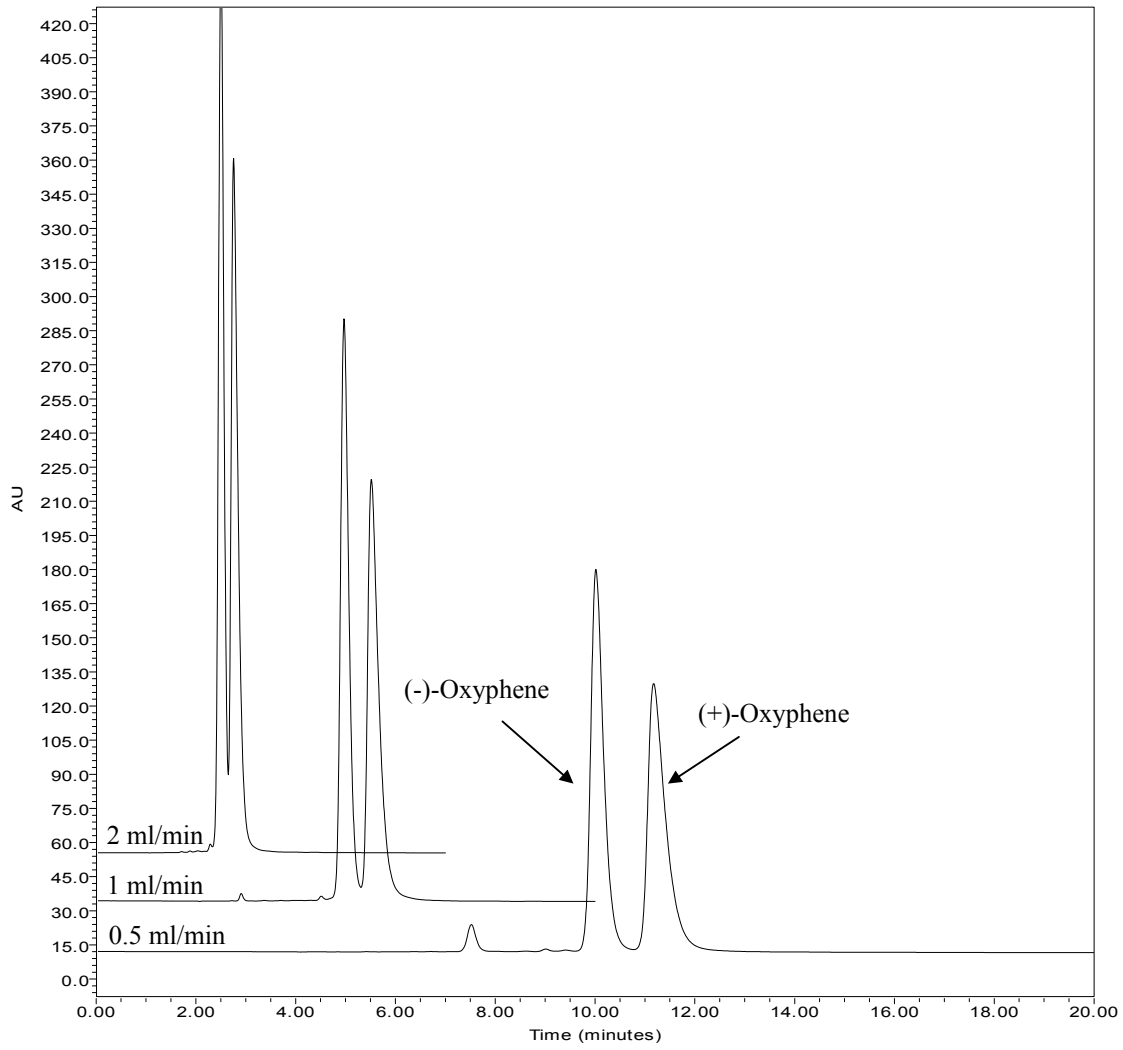


Figure 8. Chromatograms of the racemic mixture showing the influence of flow rate

Table 7. Influence of column temperature on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a ß-cyclodextrin column

Column Temperature (º C)	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
5	1.18	1.7	1.9	1.5
25	1.20		1.8	
4 5	1.20			

chromatographic effect of the column temperature is shown in Figure 9. In general, higher column temperature results in better enantioselectivity, higher resolution, lower tailing, and shorter retention times. Higher column temperatures result in better separation, as illustrated in the table. Therefore, a column temperature of 45º C was selected as the optimal column temperature.

The following are the final optimized chromatographic parameters for the separation of oxyphene optical isomers on a cyclodextrin bonded (β-cyclodextrin) chiral stationary phase:

Column: Chiral CD-Ph, 250 x 4.6 mm, 5 µm (Shiseido)
Mobile Phase: 30:70, 2% triethylamine, pH 4 with acetic acid:acetonitrile
Flow Rate: 0.5 mL/min
Wavelength: 260 nm
Column Temperature: 45º C

Analytical Separation

According to FDA guidelines, an impurity greater than or equal to 0.1% must be quantified. Therefore, an analytical method for determination of chiral purity must be able to separate and quantify a minor isomer as low as 0.1% in the presence of the major isomer. In order to determine whether the developed method is suitable for the purity determination of (+)-oxyphene and (-)-oxyphene, solutions of the minor isomer at 0.1% were prepared in the presence of the major isomer, and injected into the HPLC system. Chromatograms of the mixtures used for analytical chromatography with the final HPLC parameters, using a sample concentration of 1.5 mg/mL (major isomer) and a 15 µL injection volume, are shown in Figures 10 and 11. Results of the selectivity, resolution, and tailing are shown in Table 8. The chromatograms show that separation is achieved

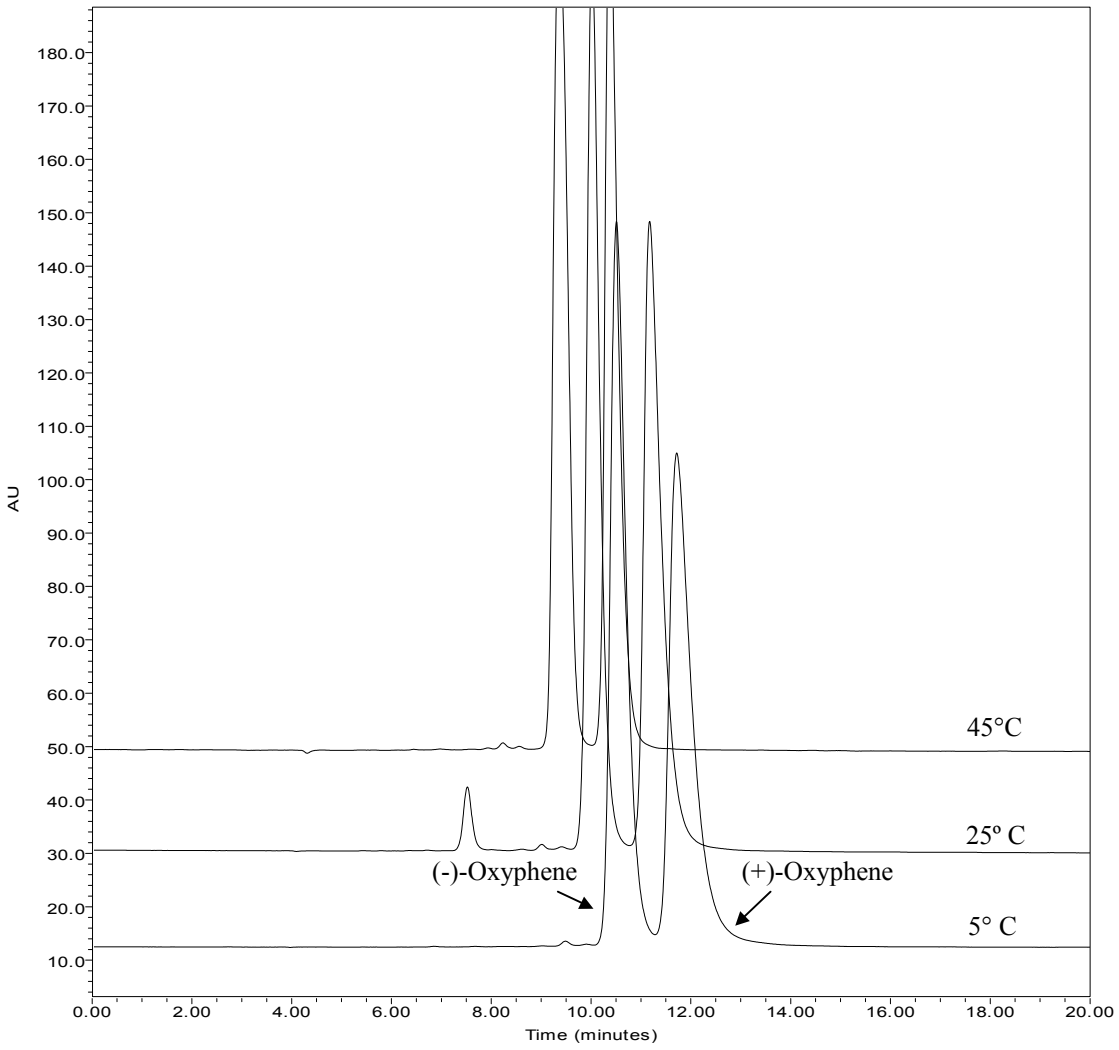


Figure 9. Chromatograms of the racemic mixture showing the influence of column temperature

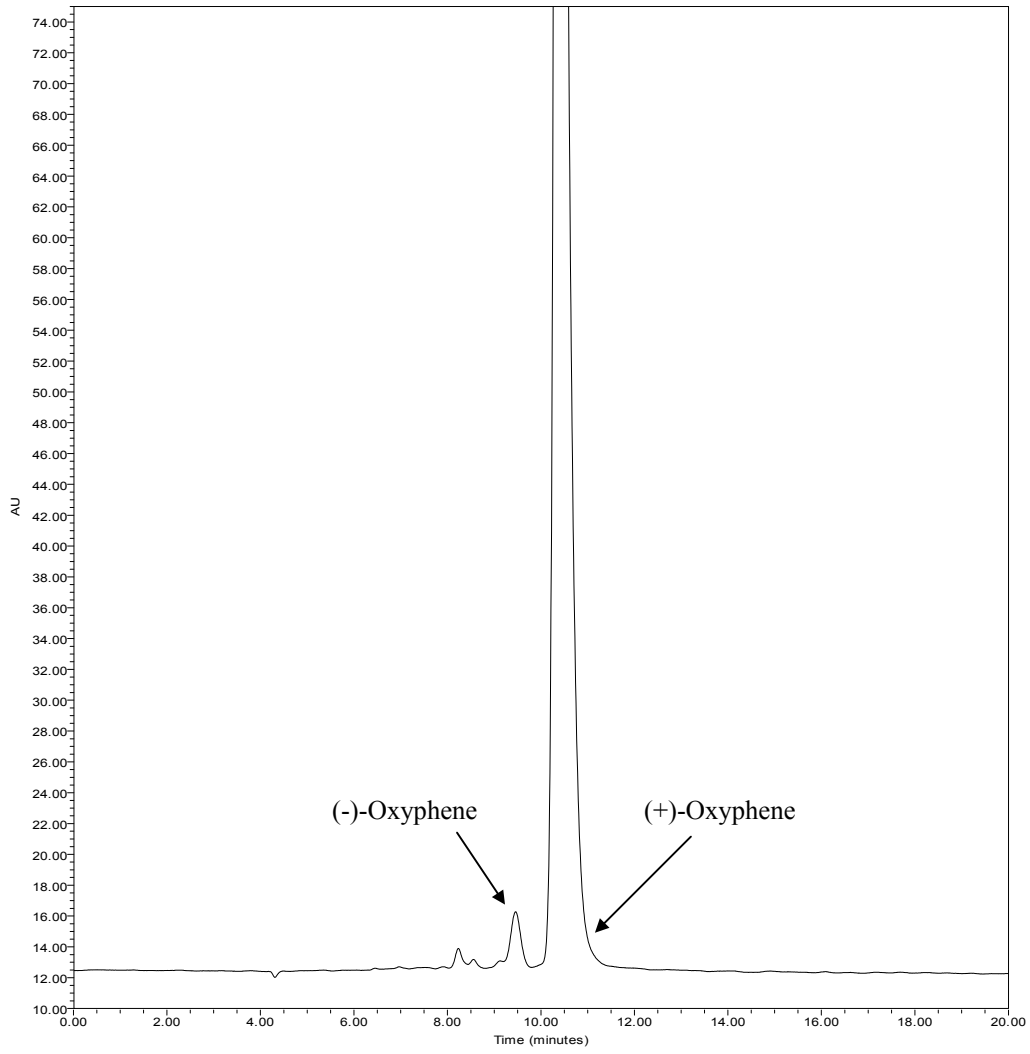


Figure 10. Chromatogram of a solution of 1.5 mg/mL (+)-oxyphene/0.0015 mg/mL (-)-oxyphene using the final HPLC parameters

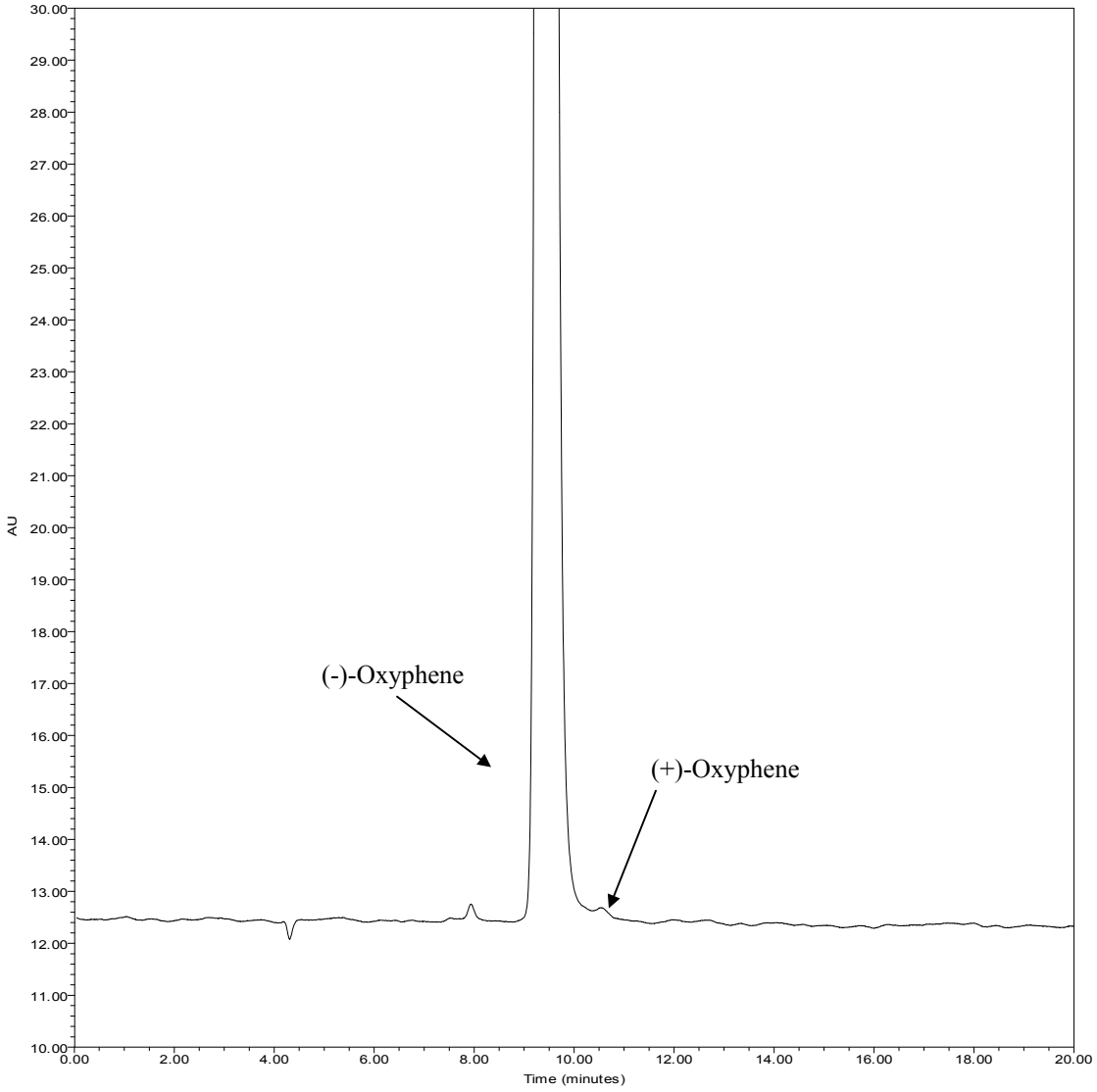


Figure 11. Chromatogram of a solution of 1.5 mg/mL (-)-oxyphene/0.0015 mg/mL (+)-oxyphene using the final HPLC parameters

Table 8. Enantioselectivity, resolution, and tailing of oxyphene isomers on a ß-cyclodextrin column using the final method parameters

Sample	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
1.5 mg/mL (+)-oxyphene/0.0015 mg/mL (-)-oxyphene	1.18	2.0	1.6	1.1
1.5 mg/mL (-)-oxyphene/0.0015 mg/mL (+)-oxyphene	1.23		1.2	1. 3

using the final, developed HPLC parameters on the Chiral CD-Ph column. Achieving separation of the isomers is difficult if the minor impurity elutes after the major compound (Figure 11). As shown in Figure 11, the minor isomer is separated from the major isomer and it can be quantified at 0.1% relative to the major isomer. Therefore, this method is appropriate for analytical chromatography, for the determination of chiral purity of both (+) and (-) isomers of oxyphene. Separation of the racemic mixture of isomers on the Chiral CD-Ph column, using the above method, is shown in Figure 12.

Immobilized Protein Chiral Stationary Phase

Background

Protein chiral stationary phases immobilized on porous silica particles are good analytical tools for the separation of a wide variety of chiral pharmaceuticals. Selective molecular recognition of such proteins has been utilized in the chromatographic resolution of racemic compounds (Allenmark, 1991). The chiral recognition mechanism of protein chiral stationary phases is based mainly on a combination of hydrophobic interactions and hydrogen bonding (Narayanan, 1992). Diasteromeric complexes are formed between enantiomeric analytes and the chiral selector of the stationary phase proteins, such as α_1 acid glycoprotein (AGP), bovine serum albumin (BSA), and ovomucoid (OVM). Protein chiral stationary phases have a very low column loading capacity, and therefore they are not suitable for preparative chiral separation. However, they are useful for analytical separations. The α_1 acid glycoprotein (AGP) stationary phase has been used to separate enantiomers from many different classes of compounds (Narayanan, 1992). For enantioselective separation on an AGP chiral stationary phase,

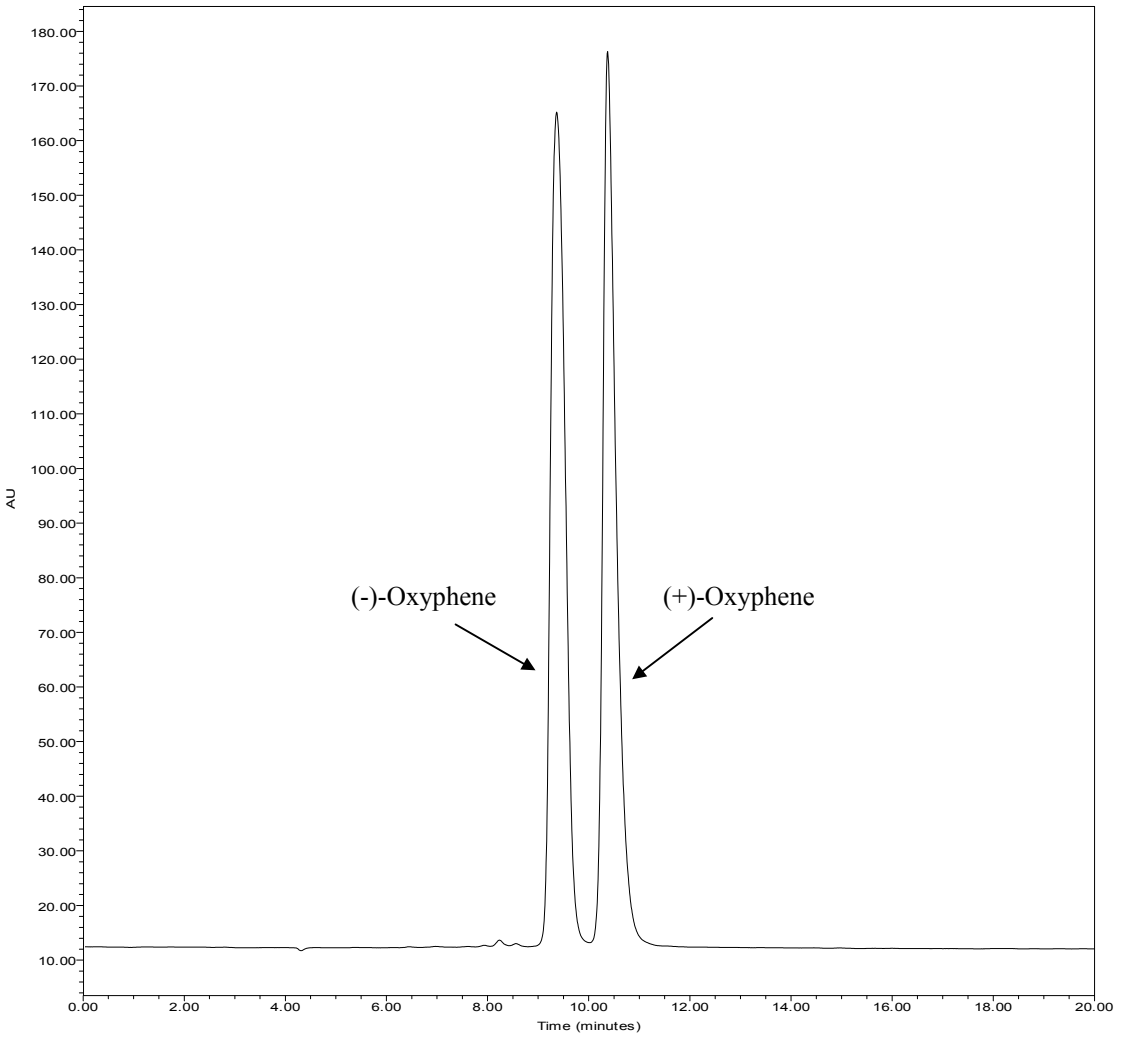


Figure 12. Chromatogram of the racemic mixture using the final method parameters on a cyclodextrin column

the analyte should have at least one binding group and a ring structure near the chiral center in order to be resolved (Hermansson, 1988; Enquist, 1990). The distance between the binding groups and the chiral center is also important to determine the degree of enantioselectivity. Hydrogen bonding groups are best when they are not more than two atoms away from the chiral center (Narayanan, 1992; Schill, 1986; Hermansson, 1988). However, because of their complex structures, the exact mechanism of chiral recognition by proteins is not known.

Method Development

All method development and optimization experiments involving the immobilized protein chiral stationary phase method utilized a Chiral AGP, 4.0 mm x 100 mm, 5 μm column. The racemic mixture, 50:50 of each oxyphene optical isomer, each at a concentration of 0.3 mg/mL, was used for the method development. In order to evaluate and compare the separation, the enantioselectivity, resolution, and tailing factors for each separation were calculated and compared. Due to the high concentration of the isomers in the racemic mixture, the peaks were not fully resolved. Therefore, tailing is only calculated for the second peak, (-)-oxyphene. As will be discussed later, the peaks were well resolved in the racemic mixture of isomers at low concentrations.

Optimization of Organic Composition

Protein columns are used with mobile phase consisting of a mixture of aqueous buffer and an organic modifier. The enantioselectivity and retention times can be regulated by changing the mobile phase composition. Ethanol at various concentrations

was evaluated as the organic modifier. Mobile phase consisting of 0.02 M, pH 6.8 acetate buffer was evaluated with ethanol at 10%, 15%, and 25%. The chromatographic effect of mobile phase consisting of ethanol at the various compositions can be observed in Figure 13. Table 9 shows influence of ethanol on enantioselectivity, resolution, and tailing. The first peak elutes close to the solvent front with 25% ethanol in the mobile phase. As a result, organic compositions of ethanol higher than 25% were not evaluated. Based on the reduced run time and lowest tailing factor, 25% ethanol was selected.

Selection of Organic Modifier Type

The type of organic modifier used in the mobile phase was optimized for the separation of (+) and (-)-oxyphene. When the organic modifier is changed, the protein conformation is changed. As a result, the separation is affected. Typically, the choices of organic modifier are isopropyl alcohol, acetonitrile, ethanol, or methanol. Mobile phases having different organic compositions of each of the aforesaid modifiers were evaluated. As shown in Figure 14, separation of the two isomers was not achieved using either acetonitrile or methanol as the organic modifier. Isopropyl alcohol at 25% was evaluated with 0.02 M, pH 6.8 phosphate buffer. Due to the high viscosity of isopropyl alcohol, the flow rate was lowered with the increasing isopropyl alcohol composition to prevent overpressure of the column. Separation of the isomers was achieved when 25% isopropyl alcohol was used in the mobile phase. As a result, mobile phase compositions of 25% isopropyl alcohol and 25% ethanol were compared, and the resulting chromatograms are shown in Figure 15. The results of the enantioselectivity, resolution, and tailing are shown in Table 10. Based on the lower tailing factor that was achieved with ethanol, as well as the reduced run time, 25% ethanol was selected as the mobile

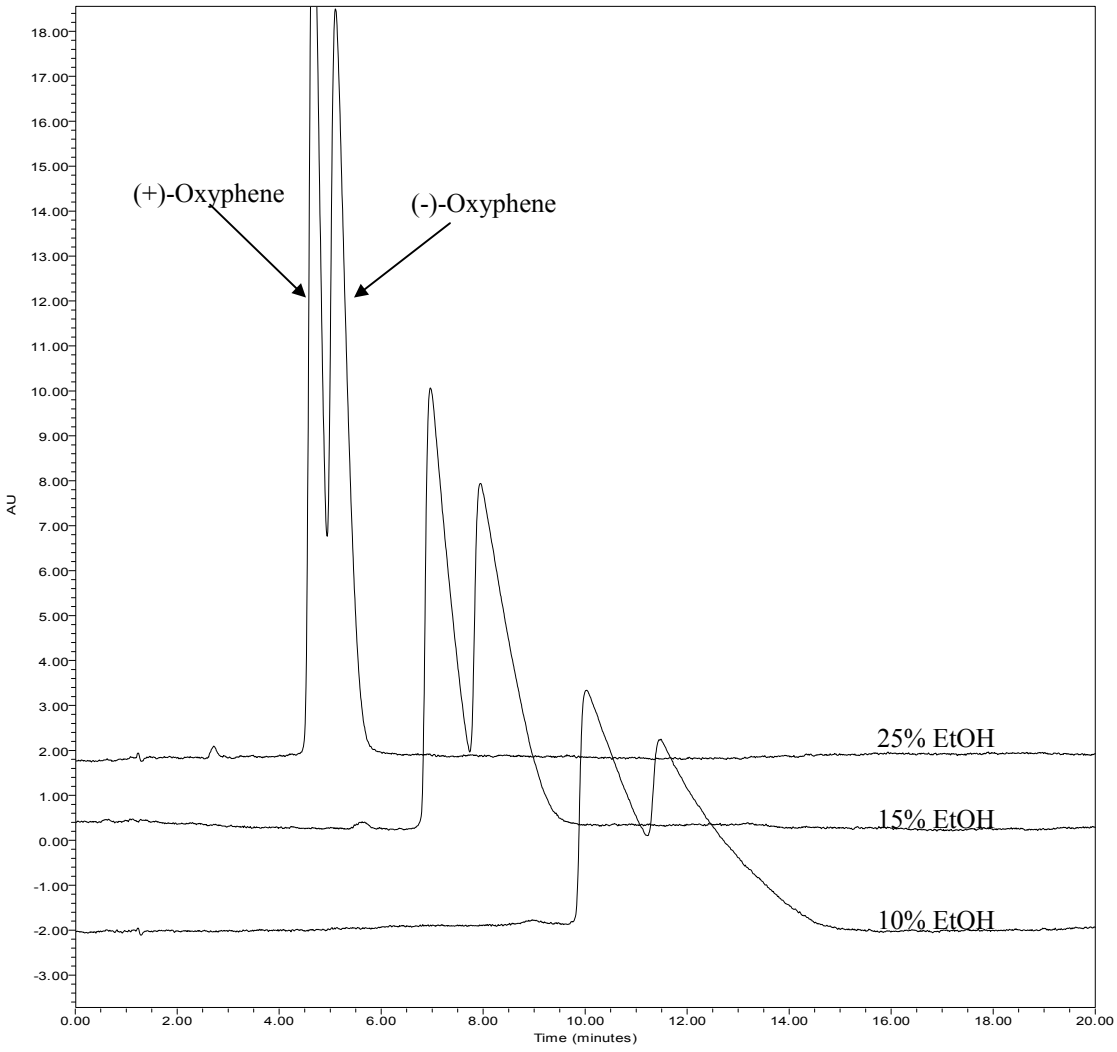


Figure 13. Chromatograms of the racemic mixture showing the influence of ethanol as the organic modifier

Table 9. Influence of ethanol on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a protein column

% Ethanol	Selectivity, α	Resolution	Tailing of (-)
10	1.16	0.2	4.0
15	1.17	0.8	2.2
25	1.13	0.8	1.6

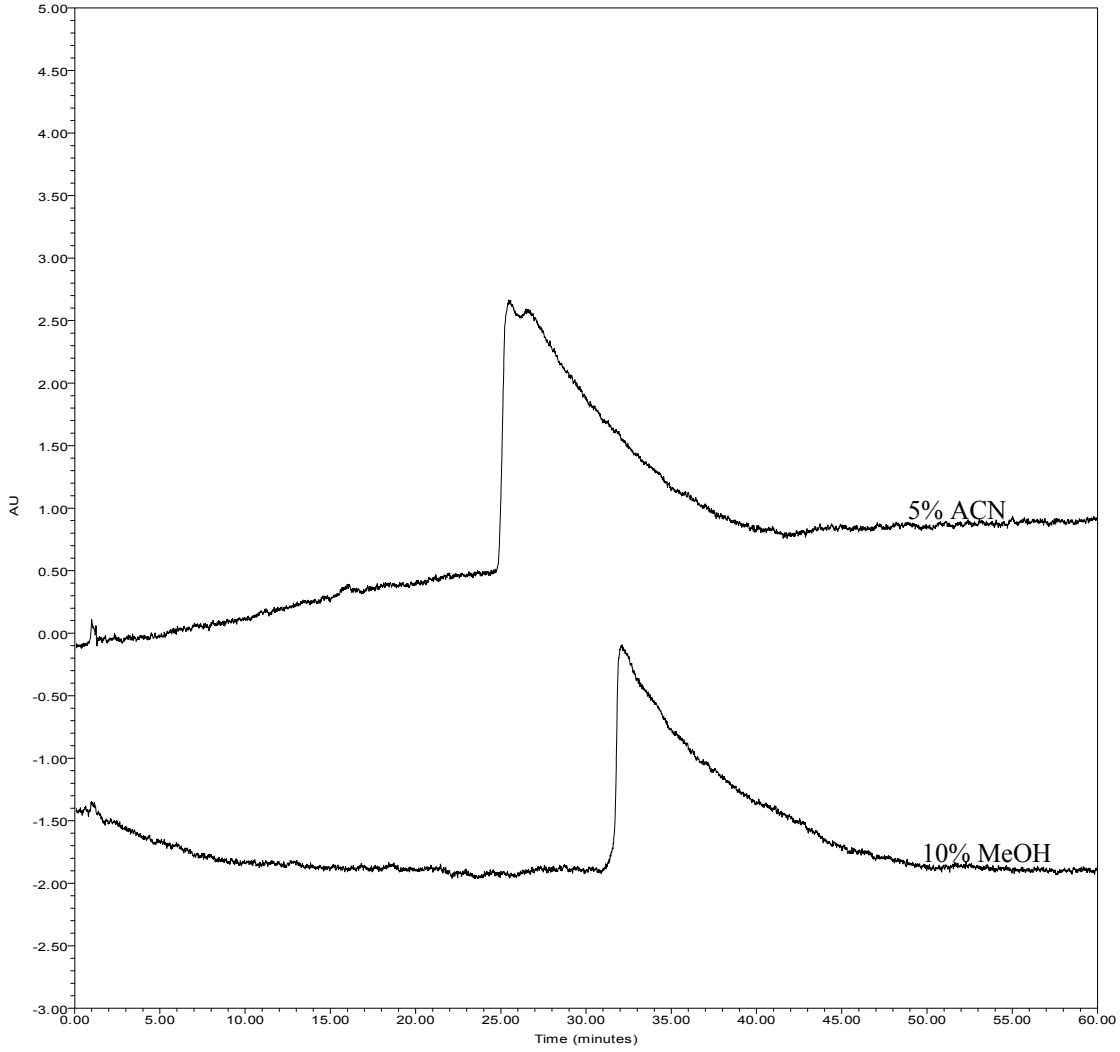


Figure 14. Chromatograms of the racemic mixture showing the influence of methanol and acetonitrile as the organic modifiers

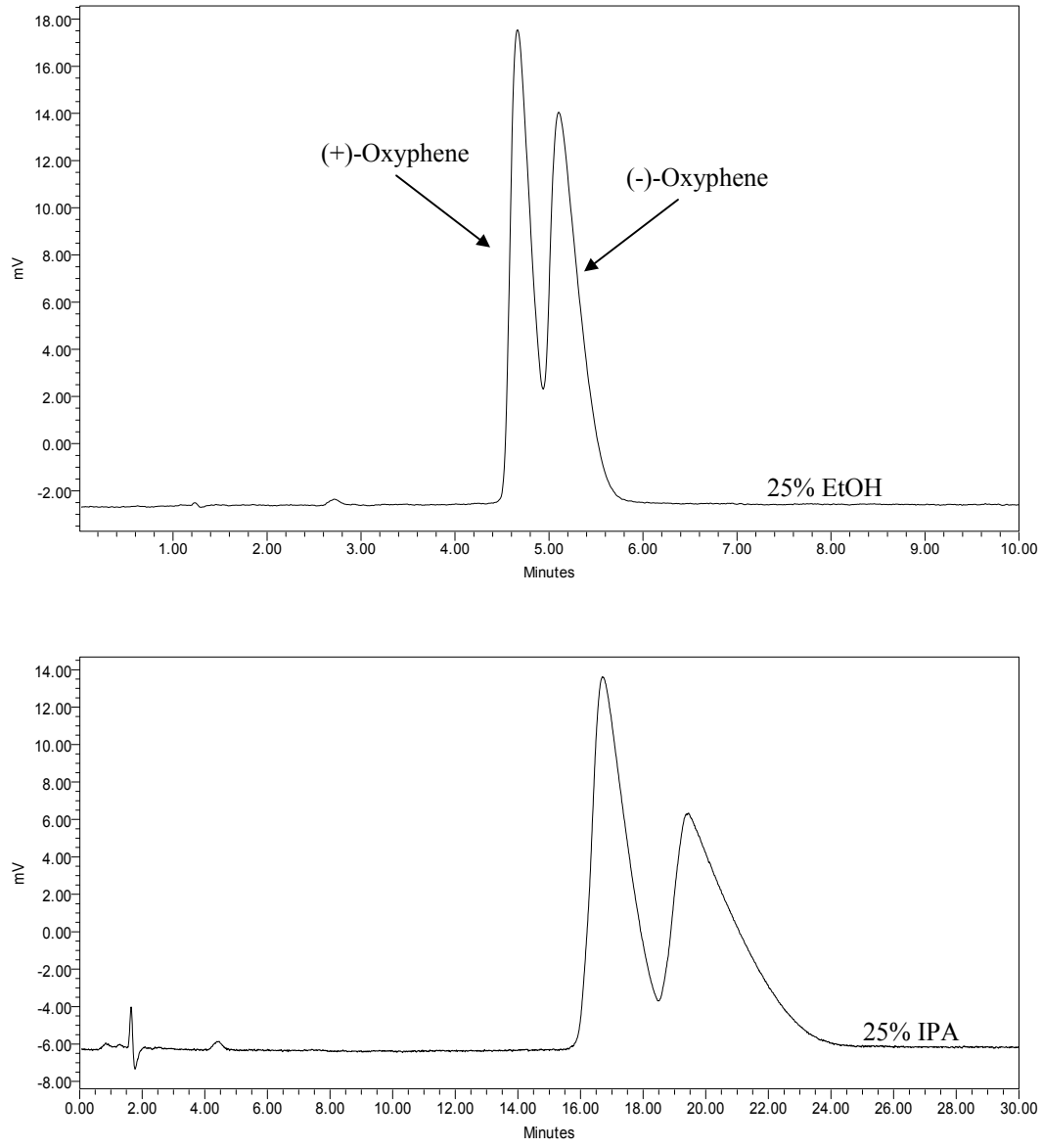


Figure 15. Chromatograms of the racemic mixture comparing 25% isopropyl alcohol and 25% ethanol

Table 10. Influence of isopropyl alcohol on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a protein column

Organic Modifier, 25%	Selectivity, α	Resolution	Tailing of (-)
Ethanol	1.13	0.8	1.6
Isopropyl Alcohol	1.18	0.8	1.8

phase organic modifier.

Influence of Buffer pH

In order to study how the pH of the buffer in the mobile phase affects the separation of the isomers, mobile phase consisting of a mixture of 0.02 M acetate buffer and ethanol in the volumetric ratio 75:25 was evaluated when the acetate buffer was pH 6.8 and 7.0. The resulting chromatograms can be observed in Figure 16, and the results of the selectivity, resolution, and tailing are shown in Table 11. The chromatograms show that there is little chromatographic difference in the pH. Based on the lower tailing factor at pH 6.8, and the fact that pH 7.0 is at the maximum of the pH tolerance range for this column, pH 6.8 was selected.

Influence of Buffer Type

Since phosphate has better buffer capacity at pH 6.8, a comparison was made between pH 6.8 phosphate buffer and pH 6.8 acetate buffer. The chromatographic effect of pH 6.8 phosphate and acetate buffer is shown in Figure 17. Table 12 shows the results of the selectivity, resolution, and tailing for pH 6.8 phosphate and acetate buffer. Phosphate buffer at pH 6.8 was selected due to the more favorable resolution and selectivity. In addition, due to better buffer capacity, phosphate buffer was selected.

Influence of Column Temperature

According to the manufacturer recommendations for this particular column, temperatures higher than 25º C should be avoided. Therefore, the maximum temperature was limited to 25º C. Column temperatures of 5º C and 25º C were evaluated. The

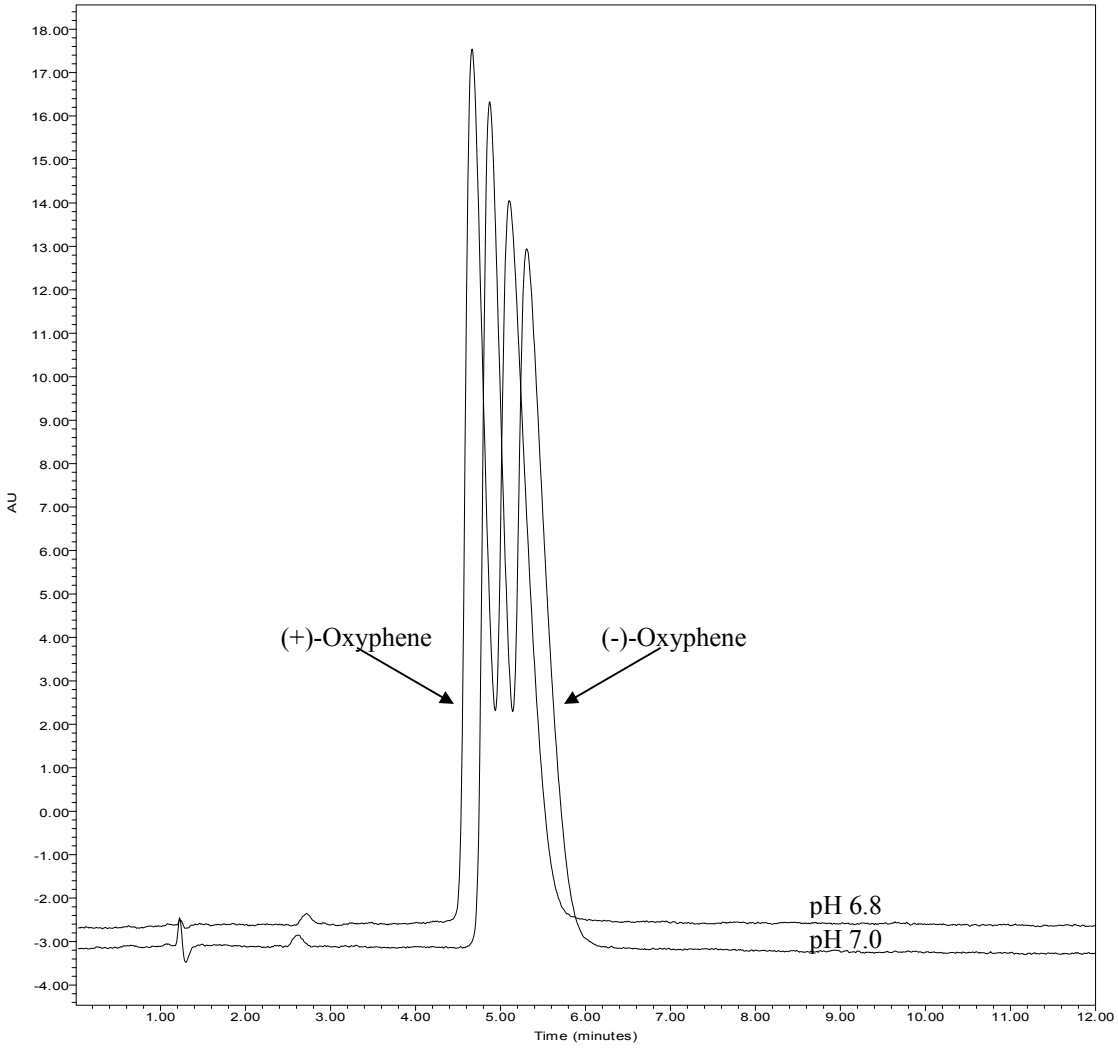


Figure 16. Chromatograms of the racemic mixture using 0.02 M acetate buffer at pH 6.8 and pH 7.0

Table 11. Influence of acetate buffer pH on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a protein column

pH	Selectivity, α	Resolution	Tailing of (-)
6.8	1.13	0.8	1.0
7.0	1.18	0.8	1.5

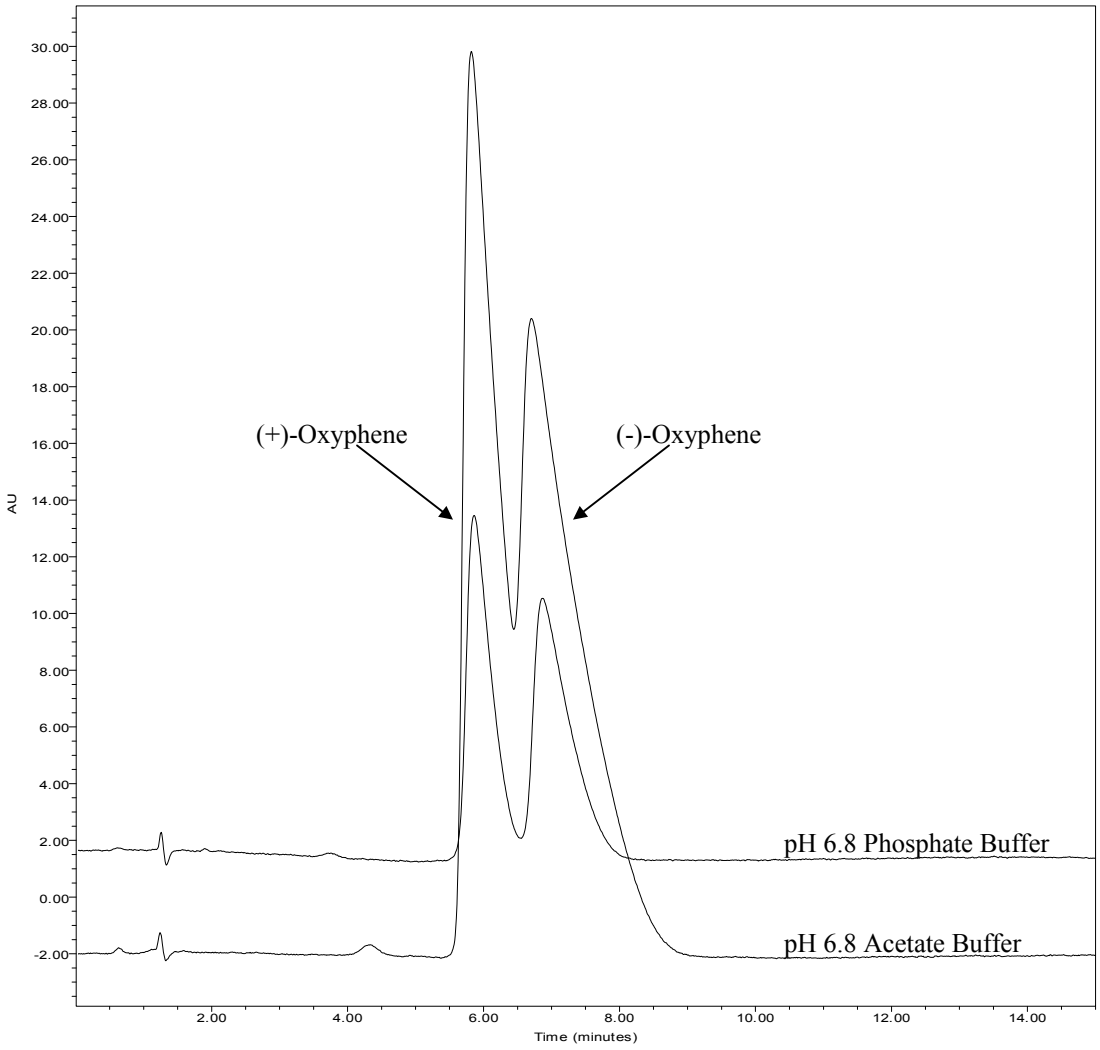


Figure 17. Chromatograms of the racemic mixture showing a comparison of pH 6.8 phosphate buffer and pH 6.8 acetate buffer

Table 12. Influence of pH 6.8 phosphate and acetate buffers on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a protein column

Buffer, pH 6.8	Selectivity, α	Resolution	Tailing of (-)
Phosphate	1.22	1.1	1.5
Acetate	1.13	0.8	1.0

chromatographic effect of column temperature can be observed in Figure 18. The results of enantioselectivity, resolution, and tailing are shown in Table 13. The lower column temperature resulted in better selectivity and resolution. Therefore, a column temperature of 5º C was selected.

Influence of Buffer Concentration

With this type of protein chiral stationary phase, retention of the isomers can be manipulated by varying the ionic strength of the buffer (Narayanan, 1992). All experimental studies on the Chiral AGP column, up to this point, have used a buffer concentration of 0.02 M. Phosphate buffer concentrations of 0.004 M and 0.01 M were evaluated. Figure 19 illustrates the chromatographic effect of the buffer concentration. The influence of the buffer concentration on retention, enantioselectivity, resolution, and tailing is shown in Table 14. Although the results for 0.004 M and 0.01 M concentrations are very similar, 0.004 M was selected as the optimal buffer concentration based on the lower tailing factor.

The following are the final developed, and optimized chromatographic parameters for the separation of oxyphene optical isomers on a protein chiral stationary phase:

Column: Chiral AGP, 4.0 mm x 100 mm, 5µm column (ChromTech)
Mobile Phase: 75:25; pH 6.8, 0.004 M phosphate buffer:ethanol
Flow Rate: 0.8 mL/min
Wavelength: 260 nm
Column Temperature: 5º C

Chromatograms of the racemic mixture at the highest and lowest concentrations that resulted in peaks that could be quantitated, using the above method, are shown in Figure 20. The results of the enantioselectivity, resolution, and tailing are shown in

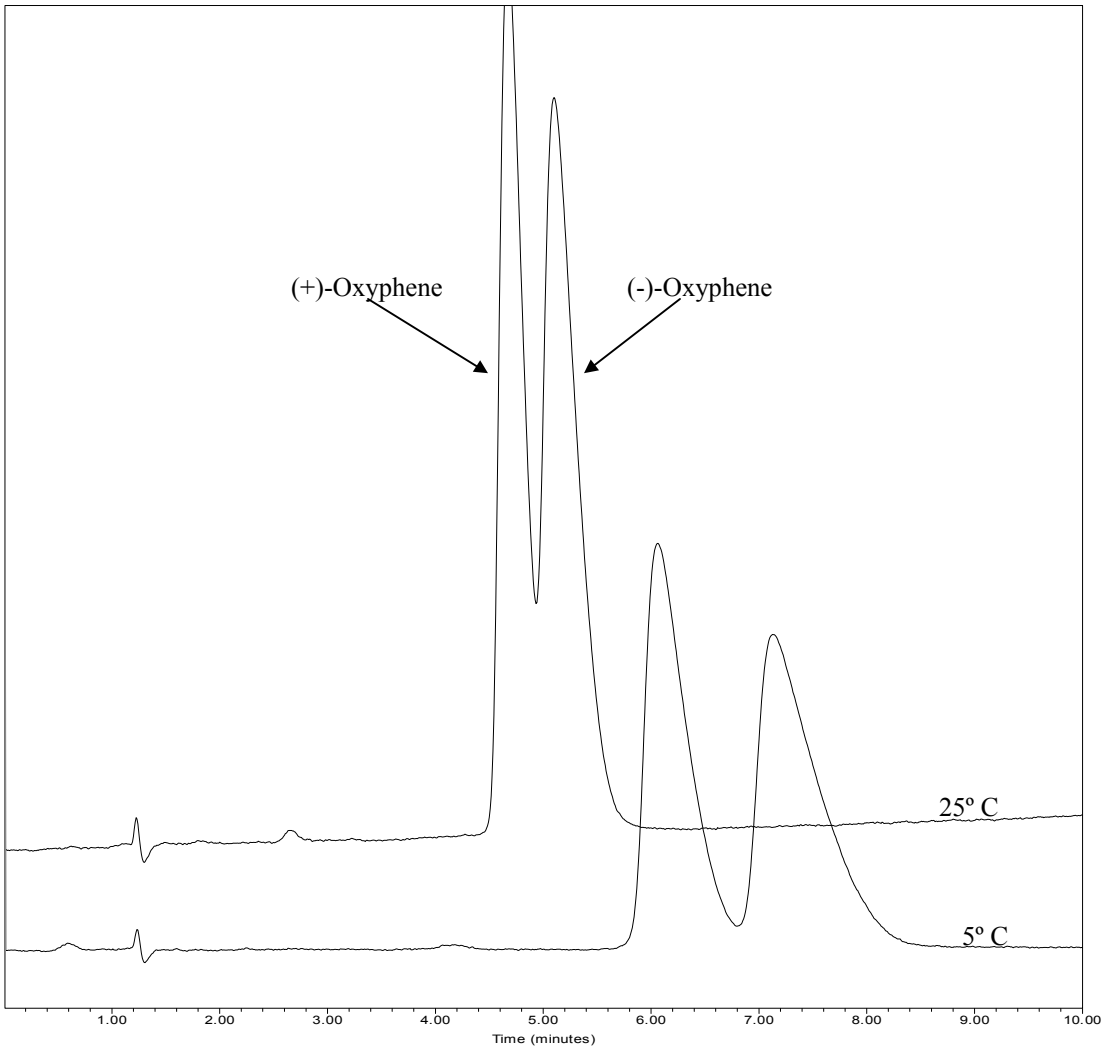


Figure 18. Chromatograms of the racemic mixture showing the influence of column temperature

Table 13. Influence of column temperature on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a protein column

Column Temperature	Selectivity, α	Resolution	Tailing of (-)
5º C	1.22	1.1	2.0
25º C	1.13	0.8	1.1

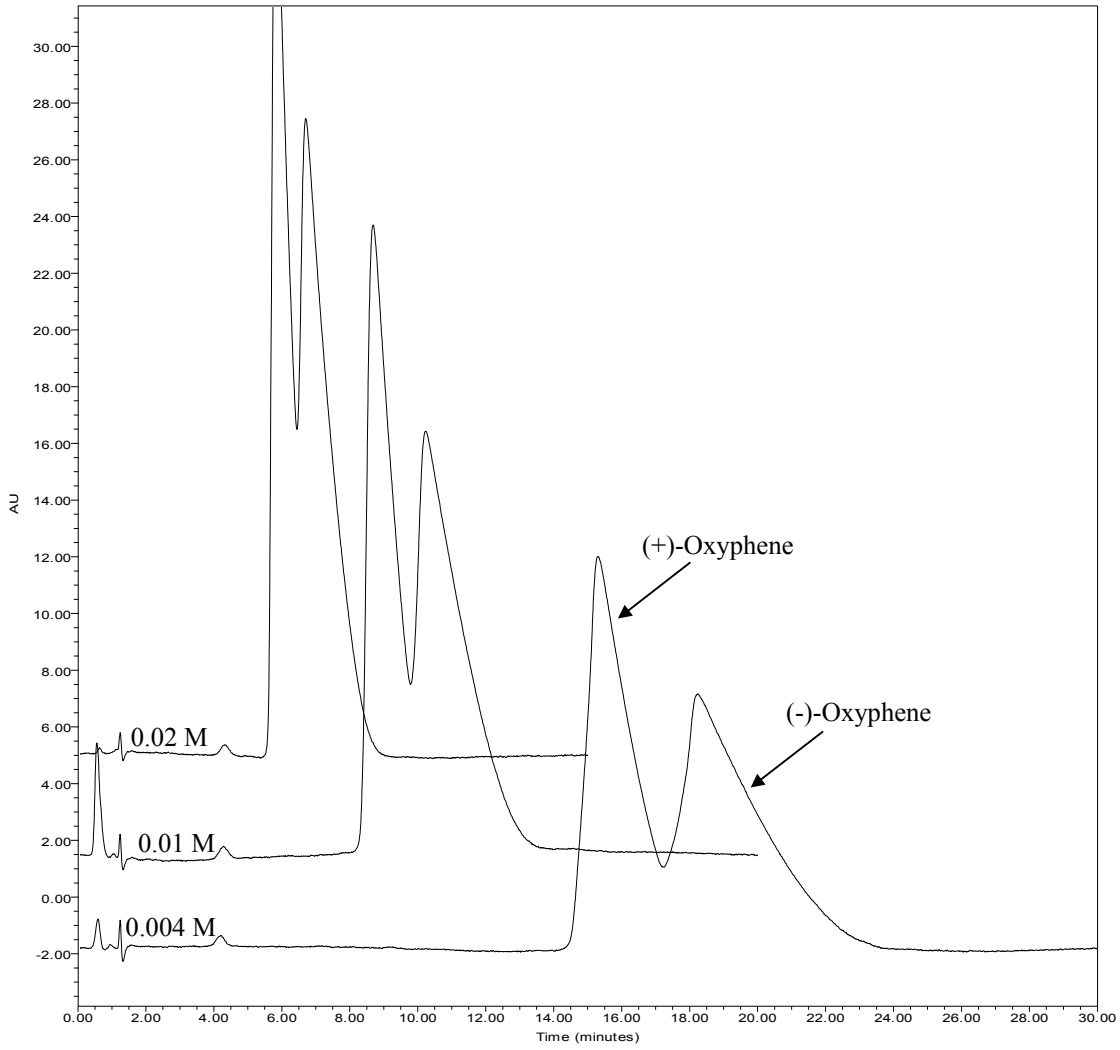


Figure 19. Chromatograms of the racemic mixture showing the influence of buffer concentration

Table 14. Influence of buffer concentration on retention, enantioselectivity, resolution, and tailing of oxyphene isomers on a protein column

Buffer Concentration	Selectivity, α	Resolution	Tailing of (-)
0.004 M	1.21	0.7	1.6
0.01 M	1.21	0.7	2.0
0.02 M	1.19	0.5	2.3

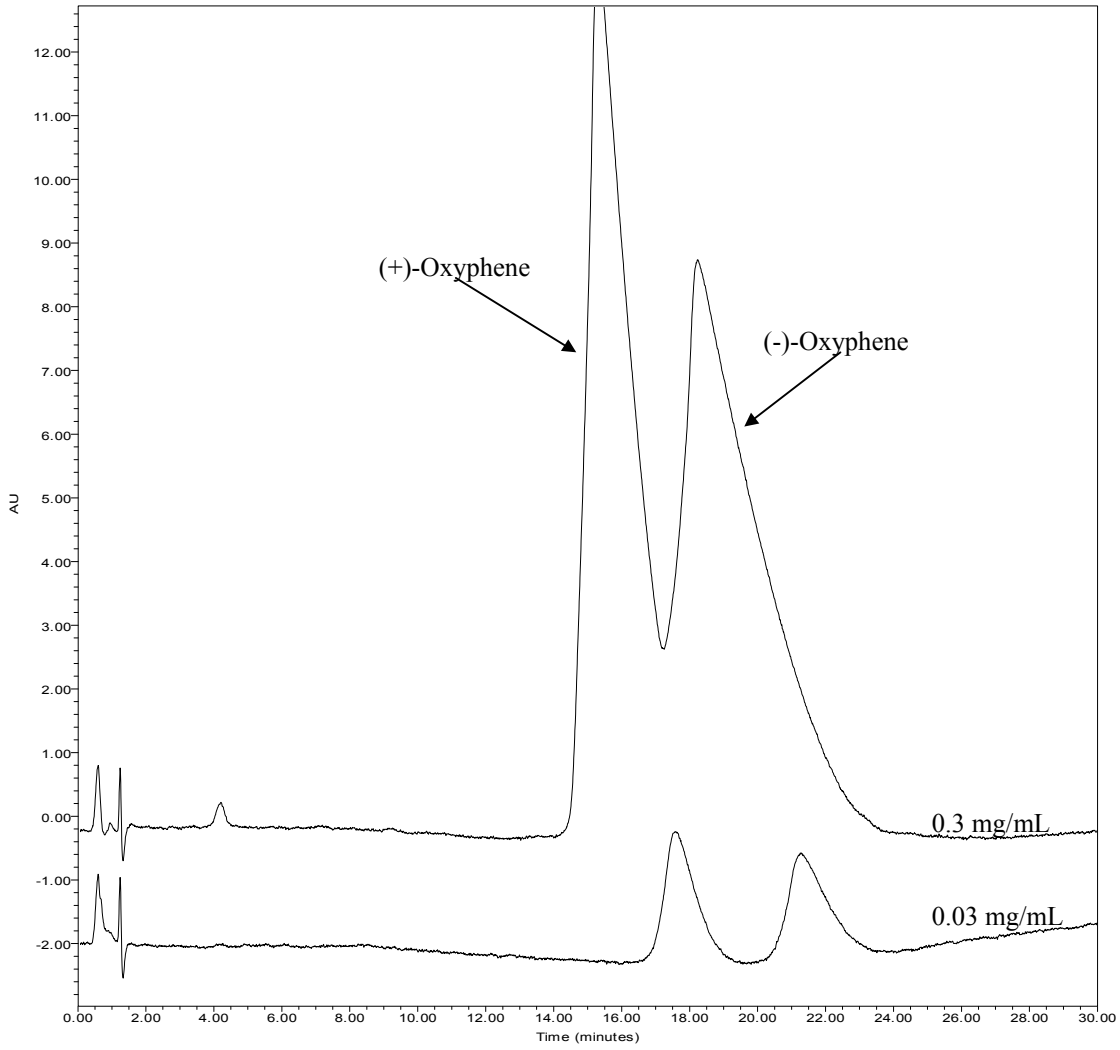


Figure 20. Chromatograms of the racemic mixture at high and low concentrations using the final method parameters for the protein column

Table 15. The data indicates that adequate baseline separation is achieved on the analytical scale, and the resolution between the peaks decreased by increasing the sample concentration. This conclusion was expected based on the low loading capacity of the protein chiral stationary phase.

Synthetic Polymer Chiral Stationary Phase

Background

The most widely used synthetic polymeric based chiral stationary phases are based on derivatized polysaccharides (Chiral Technologies, 2004). This chiral stationary phase consists of cellulose derivatives coated onto silica. The chiral recognition mechanism of synthetic polymer chiral stationary phases is based on attractive interactions (H-bonding, pi-pi interaction, and/or dipole stacking) and inclusion complexes (Wainer, 1987). This type of chiral stationary phase provides for a very broad range of applications due to the use of polysaccharide backbones combined with a variety of side chains and the use of various mobile phases.

A method in the literature currently exists for the simultaneous determination of the enantiomeric excess of dextropropoxyphene and α-(+)-oxyphene by chiral high-performance liquid chromatography (Fadnavis, 2001). Fadnavis et al. reports analytical conditions that provide good separation of (+) and (-)-oxyphene using a Chiralcel OD chiral stationary phase. However, due to solubility issues with propoxyphene and oxyphene salts in the mobile phase, they had to convert the salt to the free base in order to make them soluble in the mobile phase. Fadnavis et al. injected 2 – 200 μg of the compound by injecting 20 μL of 0.1 – 10 mg/mL of free base sample solutions. The Fadnavis method also shows the chromatographic separation of four compounds,

Table 15. Enantioselectivity, resolution, and tailing of oxyphene isomers on a protein column using the final method parameters

Sample	Selectivity, α	Resolution	Tailing of (-)
0.3 mg/mL (+)-oxyphene/0.3 mg/mL (-)-oxyphene	1.21	0.7	1.6
0.03 mg/mL (+)-oxyphene/0.03 mg/mL (-)-oxyphene	1.22	1.8	1.4

(-)-propoxyphene, (+)-propoxyphene, (+)-oxyphene, and (-)-oxyphene. The goal of this research was to develop a method using a Chiralcel OD column for the direct separation of (+) and (-)-oxyphene salts.

Method Optimization

All of the method optimization experiments for the synthetic polymer chiral stationary phase utilized a Chiralcel OD, 250 mm x 4.6 mm, 10 μm column. The analytical conditions in the Fadnavis method were used as the starting chromatographic parameters for the method optimization. The method employed a non-polar mobile phase of 1% isopropyl alcohol in hexanes with 0.2% diethylamine (DEA), flow rate of 0.3 mL/min, and detection wavelength of 260 nm. The racemic mixture, with each of the isomers at a concentration of 0.6 mg/mL, and a 25 μL injection volume was used for the method optimization experiments. At this concentration oxyphene salt did not require conversion to the free base form. It was soluble in the mobile phase at a concentration of 0.6 mg/mL for each isomer. Therefore, the sample solutions in the salt form were directly injected into the HPLC system. In order to evaluate and compare the separation, the enantioselectivity, resolution, and tailing factors for each separation were calculated and compared. A chromatogram of the racemic mixture using the aforementioned chromatographic parameters can be observed in Figure 21. The chromatogram shows that there is good resolution between the peaks. However, the run time is long. A reduced run time is desired in both analytical and preparative separations to increase efficiency and reduce cost. Therefore, the chromatographic parameters were optimized.

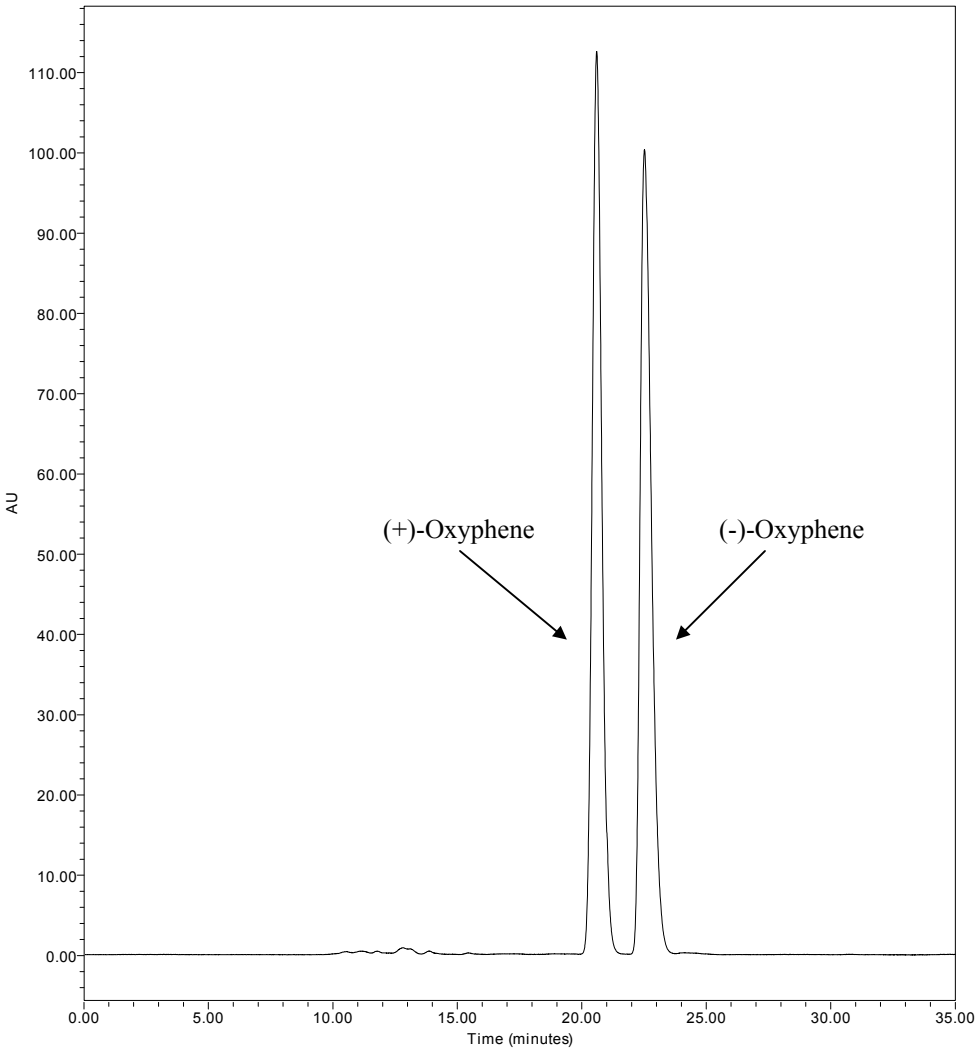


Figure 21. Chromatogram of the racemic mixture using the starting chromatographic parameters

Optimization of Organic Modifier Composition

The organic modifier in the mobile phase was optimized for the separation of (+) and (-)-oxyphene. This type of chiral stationary phase is most commonly used with a non-polar mobile phase of alkanes (hexane or heptane) and an alcohol polar mobile phase modifier, such as ethanol or isopropyl alcohol. The composition of isopropyl alcohol in the mobile phase was evaluated at 1%, 2%, and 10%. Increasing the composition of isopropyl alcohol increases the strength of the mobile phase. Therefore, the retention times should be reduced. The chromatographic effect of increasing the strength of isopropyl alcohol in the mobile phase is illustrated in Figure 22. The results of the enantioselectivity, resolution, and tailing are shown in Table 16. The increase in isopropyl alcohol from 1% to 2% did not result in significant differences in retention time and resolution. Tailing factors were slightly lower with 1% isopropyl alcohol, compared to 2%. Although the retention times are shorter with 10% isopropyl alcohol, the resolution was lower. Therefore, hexanes with 1% isopropyl alcohol and 0.2% diethylamine was selected.

Selection of Organic Modifier

Ethanol has a different selectivity than isopropyl alcohol, and therefore it was evaluated as the organic modifier. Compositions of ethanol in the mobile phase at 1% and 10% were evaluated. The chromatographic effect of ethanol in the mobile phase can be observed in Figure 23. As shown, mobile phase containing ethanol as the organic modifier resulted in no resolution. Ethanol is not suitable as the organic modifier due to the co-elution of the peaks.

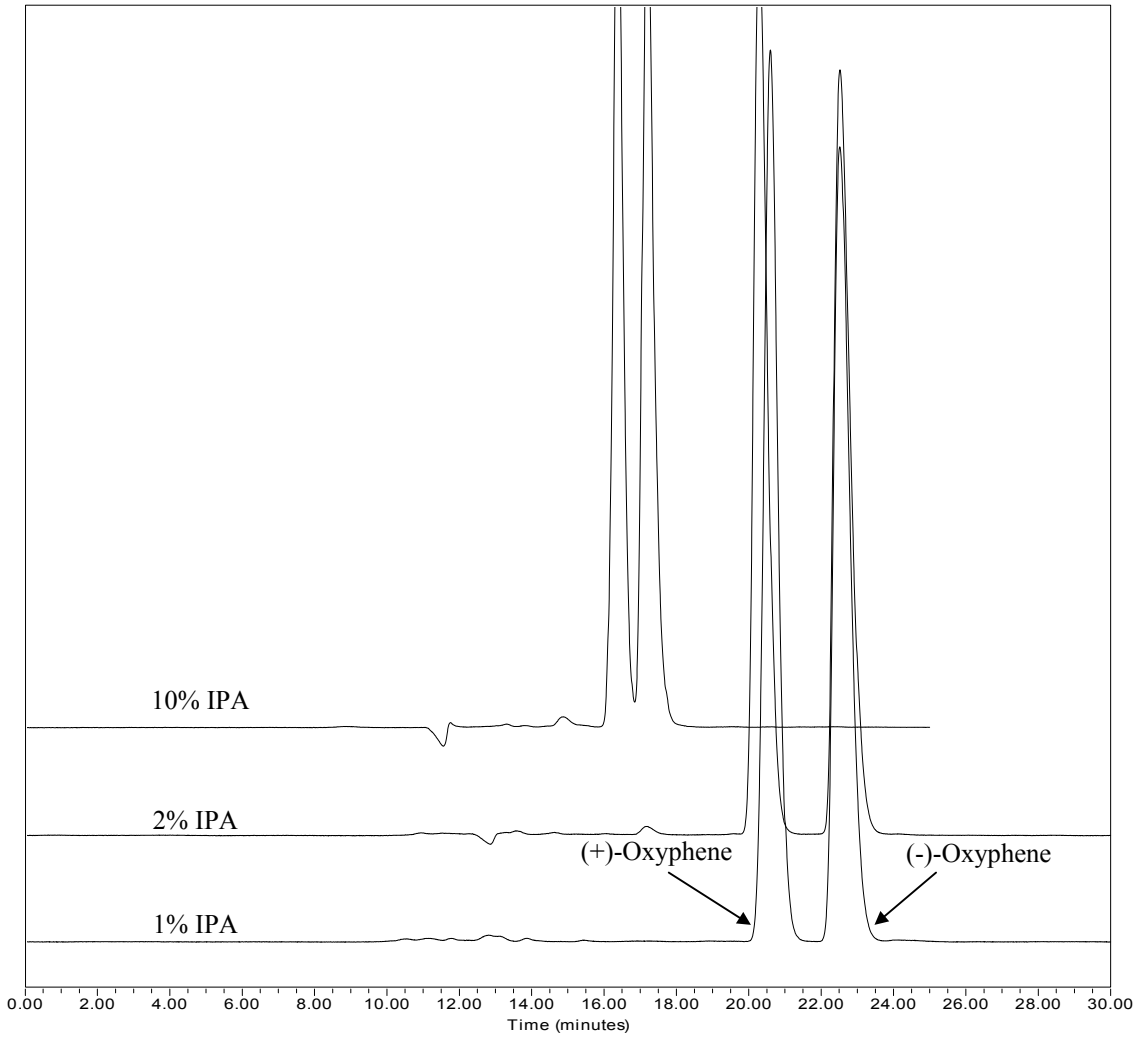


Figure 22. Chromatograms of the racemic mixture using isopropyl alcohol in the mobile phase

Table 16. Influence of isopropyl alcohol on enantioselectivity, resolution, and tailing of oxyphene isomers on a Chiralcel OD column

Isopropyl Alcohol Composition	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
1%	1.19	2.5	1.2	1.5
2%	1.24	2.7	1.4	1.6
10%	1.14	1.4	1.1	1.3

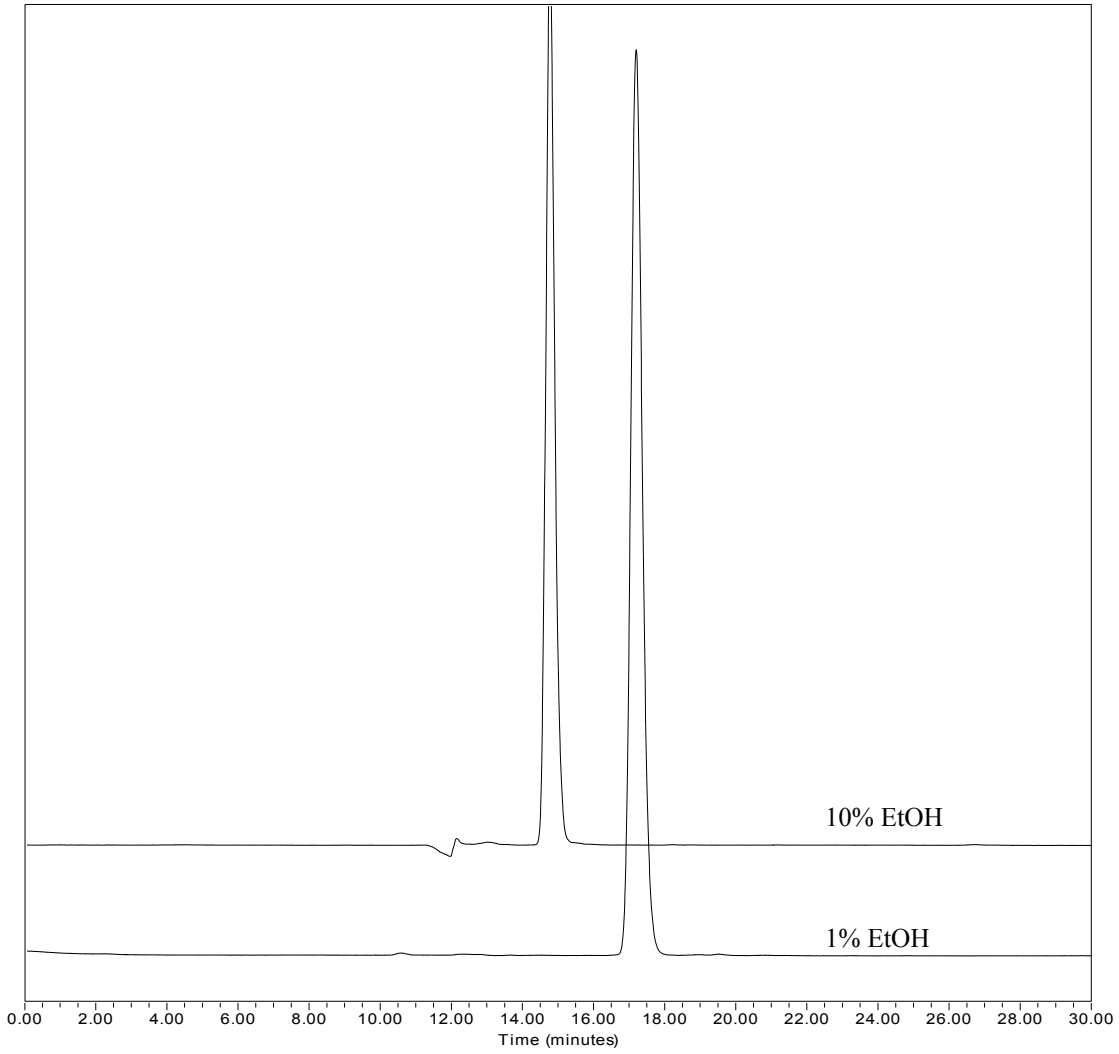


Figure 23. Chromatograms of the racemic mixture showing the influence of ethanol in the mobile phase

Optimization of Flow Rate

All experimental studies on the Chiralcel OD column, up to this point, have used a flow rate of 0.3 mL/min, and injection volume of 25 µL. Since peak area is inversely proportional to flow rate, injection volumes of a sample solution of 0.6 mg/mL for each isomer were adjusted proportional to the flow rate in order to obtain similar peak areas, and therefore similar sensitivity. Flow rates of 0.6 mL/min and 1.0 mL/min were evaluated with injection volumes of 50 µL and 75 µL, respectively. The chromatographic effect of flow rate is shown in Figure 24. The influence of flow rate on retention, enantioselectivity, resolution, and tailing is shown in Table 17. As expected, the retention times are shorter with the increase in flow rate. The 0.6 mL/min flow rate yields the best resolution combined with a reasonable run time. Therefore, a flow rate of 0.6 mL/min was selected as the optimal flow rate.

Optimization of Column Temperature

Column temperatures of 25º C and 45º C were evaluated. The chromatographic effect of column temperature can be observed in Figure 25. The influence of the column temperature on retention, enantioselectivity, resolution, and tailing are shown in Table 18. Increasing the column temperature resulted in better resolution, and reduced retention times. Since lowering the column temperature below 25º C would result in a longer run time, no other column temperatures below 25º C were evaluated. Manufacturer recommendations for this column stated that the column should not be used at temperatures greater than 45° C. Due to the improved resolution and reasonable run time, a column temperature of 45° C was selected as the optimal column temperature.

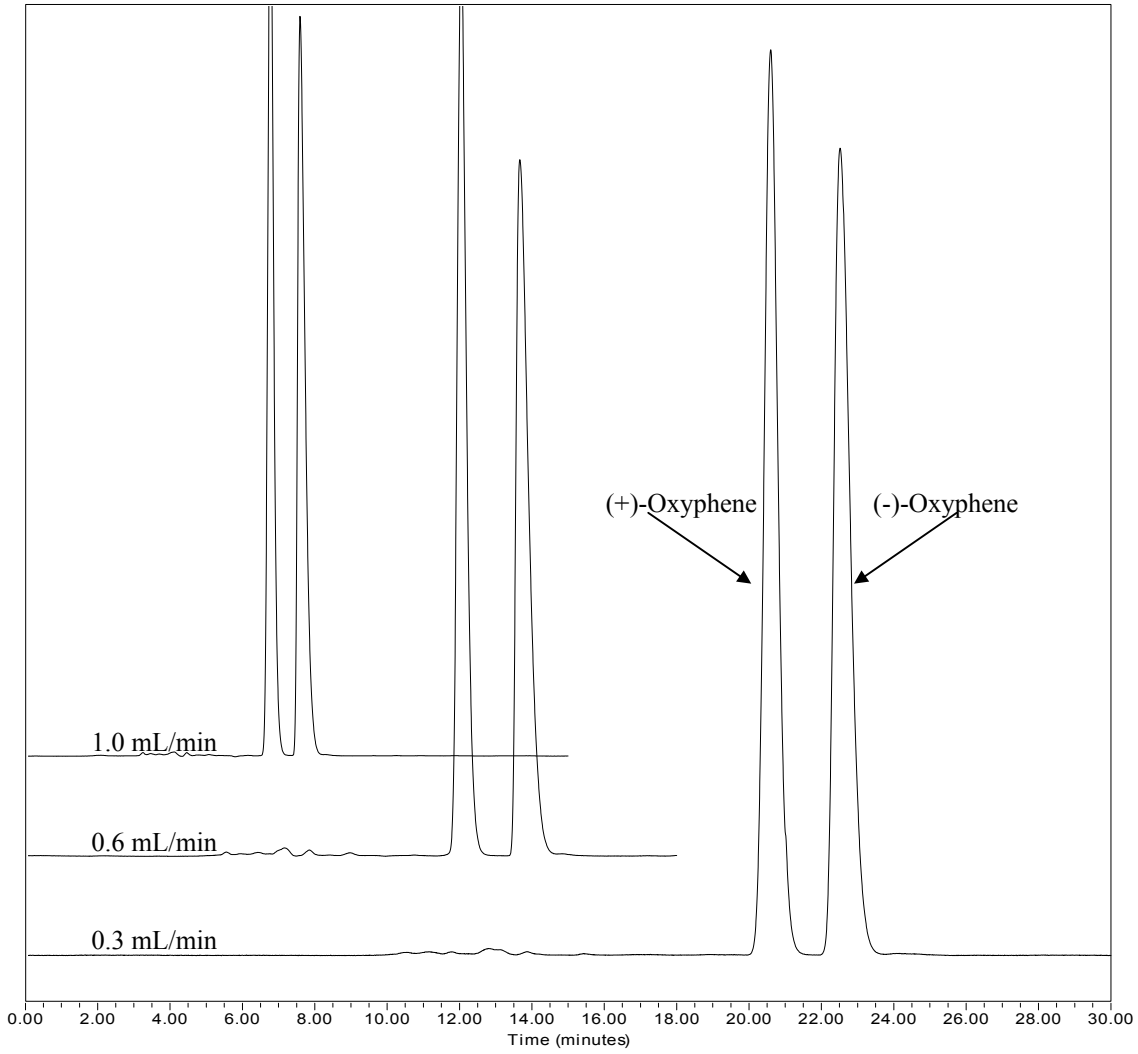


Figure 24. Chromatograms of the racemic mixture showing the influence of flow rate

Table 17. Influence of flow rate on enantioselectivity, resolution, and tailing of oxyphene isomers on a Chiralcel OD column

Flow Rate, mL/min	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
0.3	1.19	2.5	1.2	1.5
0.6	1.24	2.9	1.3	1.9
1.0	1.18	2.3	1.2	1.8

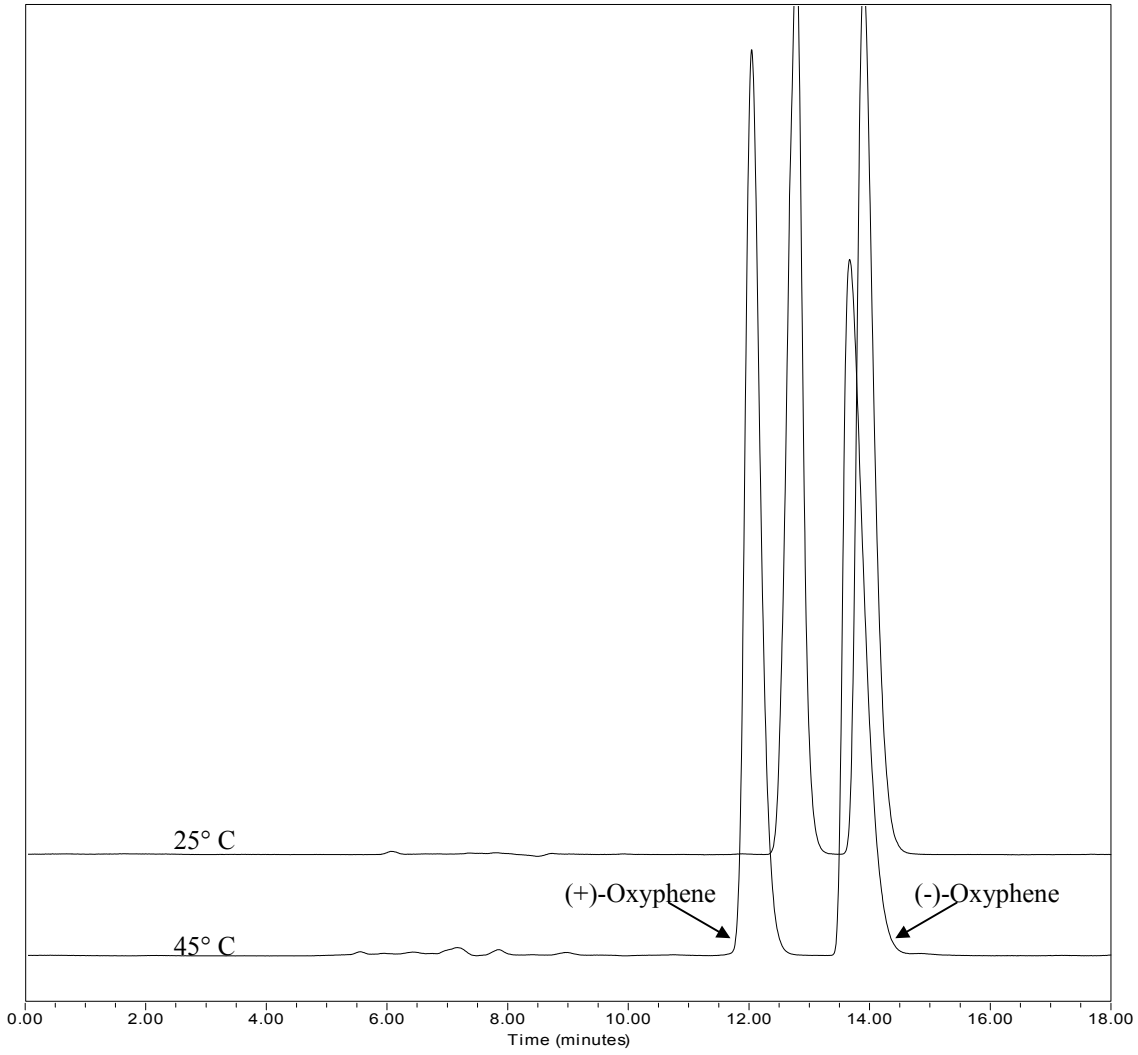


Figure 25. Chromatograms of the racemic mixture showing the effect of column temperature

Table 18. Influence of column temperature on enantioselectivity, resolution, and tailing of oxyphene isomers on a Chiralcel OD column

Column Temperature	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
25° C	1.24	2.7	1.3	1.9
45° C	1.15	2.9	1.0	1.5

The following are the final optimized chromatographic parameters for the separation of oxyphene optical isomers on a Chiralcel OD chiral stationary phase:

Column: Chiralcel OD, 250 x 4.6 mm, 10 μm (Daicel)
Mobile Phase: Hexanes with 1% isopropyl alcohol and 0.2% diethylamine
Flow Rate: 0.6 mL/min
Wavelength: 260 nm
Column Temperature: 45º C

Analytical Separation

Solutions of the minor isomer at 0.1% of the concentration of the major isomer were prepared in the presence of the major isomer, and injected into the HPLC system using the above chromatographic parameters. Chromatograms of the mixtures used for analytical chromatography with the above method, using a sample concentration of 0.6 mg/mL (major isomer) and a 50 μL injection volume are shown in Figures 26 and 27. Results of the selectivity, resolution, and tailing are shown in Table 19. A chromatogram of the racemic mixture with the above method is shown in Figure 28. As illustrated, the minor isomer is separated from the major isomer and it can be quantified at 0.1% relative to the major isomer. Therefore, this method is appropriate for analytical chromatography, for the determination of chiral purity of both (+) and (-) isomers of oxyphene.

Brush Type Chiral Stationary Phase

Background

William Pirkle was an early pioneer in the development of chiral stationary phases. Brush, also known as Pirkle type chiral stationary phases contain a small chiral selector covalently bonded to the silica surface. With brush type chiral stationary phases,

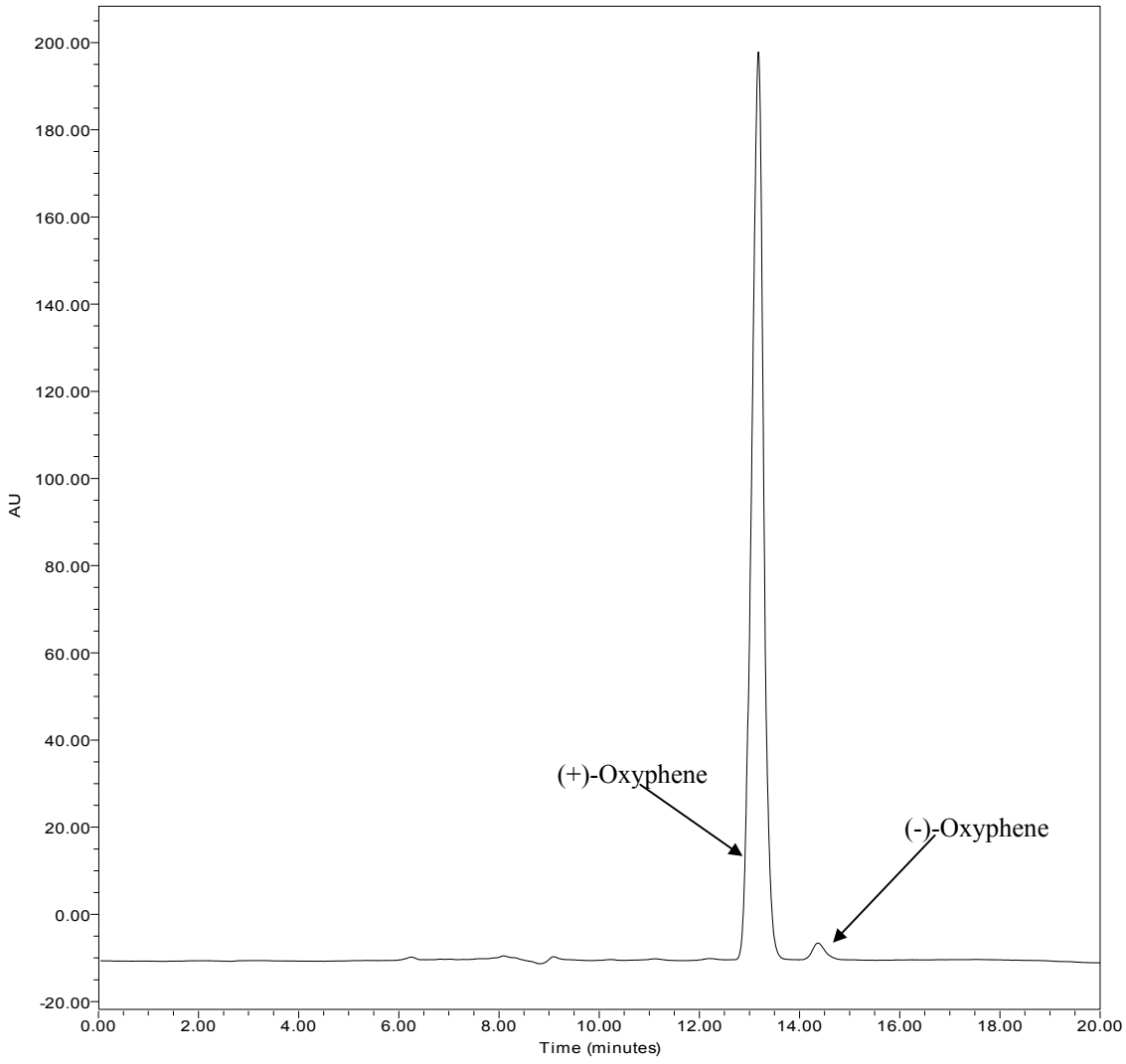


Figure 26. Chromatogram of a solution of 0.6 mg/mL (+)-oxyphene/0.0006 mg/mL (-)-oxyphene using the final HPLC parameters

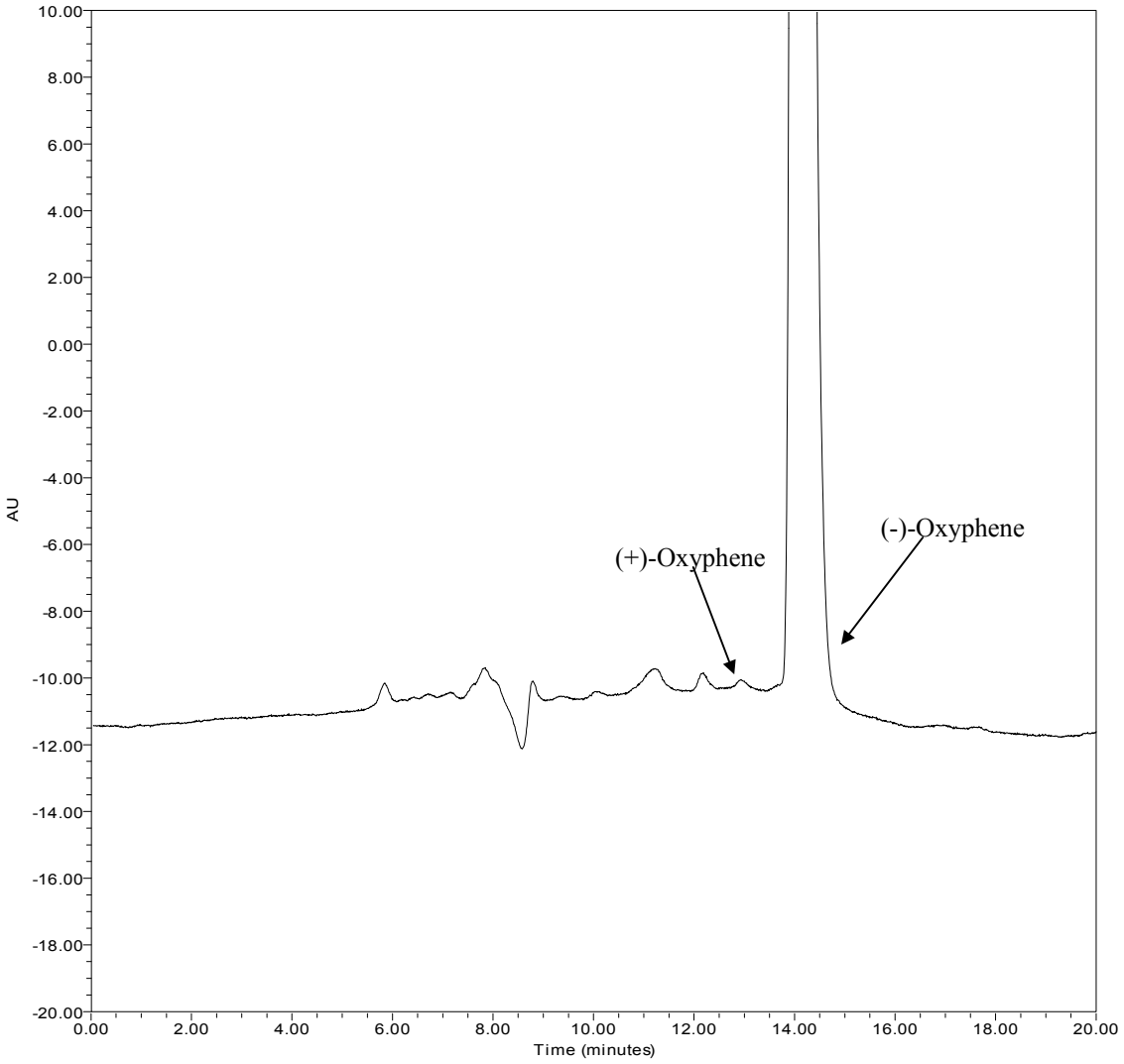


Figure 27. Chromatogram of a solution of 0.6 mg/mL (-)-oxyphene/0.0006 mg/mL (+)-oxyphene using the final HPLC parameters

Table 19. Enantioselectivity, resolution, and tailing of oxyphene isomers on a Chiralcel OD column using the final method parameters

Sample	Selectivity, α	Resolution	Tailing of (+)	Tailing of (-)
0.6 mg/mL (+)-oxyphene/0.0006 mg/mL (-)-oxyphene	1.15	2.5	1.0	1.2
0.6 mg/mL (-)-oxyphene/0.0006 mg/mL (+)-oxyphene	1.15	2.5	1.2	1.5

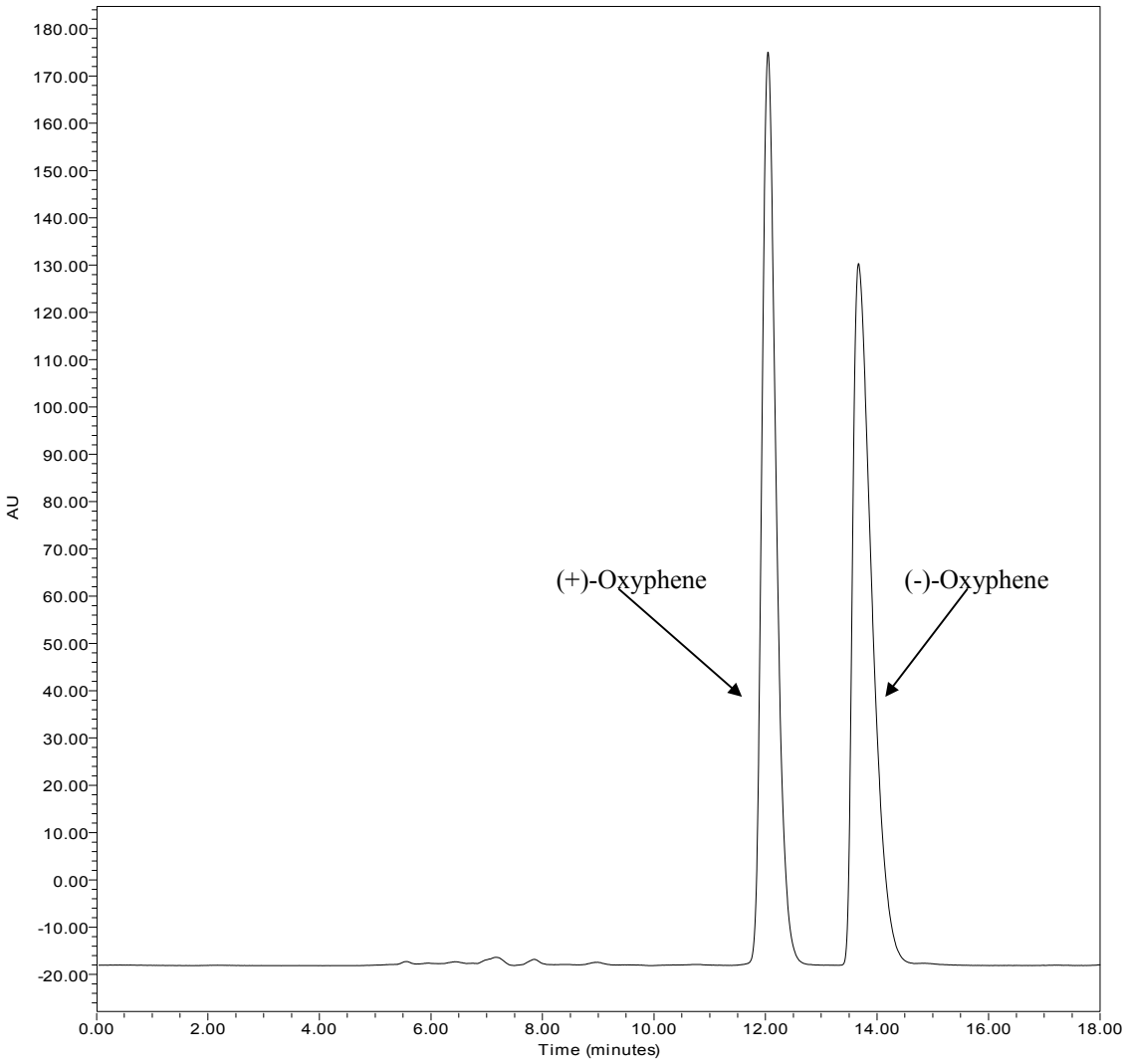


Figure 28. Chromatogram of the racemic mixture (0.6 mg/mL each isomer) using the final HPLC parameters

chiral recognition occurs at the binding sites. Major binding sites are classified as pi-basic or pi-acidic aromatic rings, acidic sites, basic sites, and steric interaction sites. Aromatic rings are potential sites for pi-pi interactions. Acidic sites supply hydrogens for potential intermolecular hydrogen bonds. Basic sites, such as non-bonding or pi electrons may also form hydrogen bonds. Repulsive (steric) interactions may also occur between large groups. Brush type chiral stationary phases generally fall into three classes: pi-electron acceptors such as phenylglycine, pi-electron donors such as naphthylleucine, and pi-electron acceptor / pi-electron donors such as Whelk-O1 (4-(3, 4-dinitrobenzamide, tetrahydrophenanthrene). Separation on these chiral stationary phases is based on a three point interaction model where enantiomers will have three possible interaction points with the chiral stationary phase. One enantiomer will interact more strongly than the other, thus it will be retained longer. The best separations occur when the solute has similar structural features to the chiral stationary phase. Tertiary alcohols, such as oxyphene, can be separated directly by a (S, S) - Whelk-O1 chiral stationary phase. The structure of (S, S) - Whelk-O1 chiral stationary phase is shown in Figure 29 (Kennedy, 1996). This chiral stationary phase has both pi-acid (p-nitrobenzyl) and pi-base (naphthyl) functionality. This dual functionality allows for the separation of a wide variety of compounds such as amides, esters, carbamates, aldehydes, ketones, carboxylic acids, and alcohols (Villani, 1995; Welch, 1997). Unfortunately, this column was not available in our laboratory. The only available brush type column in our lab was a pi-electron acceptor type L-phenylglycine (3, 5-dinitrobenzyl derivative of phenylglycine bound covalently to amino propyl silica). Separation of the underivatized oxyphene

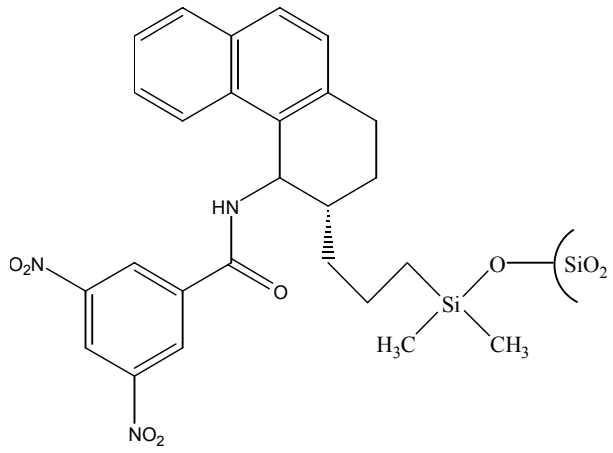


Figure 29. Structure of (S,S)-Whelk-O1 chiral stationary phase

isomers was not achieved with the available L-phenylglycine chiral stationary phase. According to Perrin, et al. this column should be able to resolve the carbamate derivative of alcohols using 1-naphthylisocyanate (Perrin, 1991). Derivatization of the alcohol (oxyphene) is needed to provide necessary points of interaction for separation on this type of chiral stationary phase.

Procedure

Derivatization of the active hydrogen of the alcohol was attempted using two different procedures. The first derivatization procedure was the reaction with 1-naphthylisocyanate to form the urethane derivative (Perrin, 1991). 1-naphthylisocyanate is a derivatizing agent that has been used to successfully convert chiral secondary alcohols to their urethane derivatives (Pirkle, 1988). The procedure followed was the acylation reaction of the chiral compound with 1-naphthylisocyanate described by Perrin, et al. (Perrin, 1991). The procedure was as follows: 5.0 mg of the chiral compound, 7 µL of 1-naphthylisocyanate, and 2 mL of toluene were added to a 5 mL reaction vial. The solution was stirred and heated at 70º C for 30 minutes. After cooling, the solvent was evaporated to dryness under a stream of nitrogen, and the residue was taken up in 3 mL of methylene chloride. The mixture was transferred to a separatory funnel and the organic layer was washed with 2 x 1 mL of a 0.1 M sodium bicarbonate solution, 2 x 1 mL of a 0.1 M hydrochloric acid solution, and 2 x 1 mL of water. The organic layer was filtered through anhydrous sodium sulfate and the solvent was evaporated to dryness under a stream of nitrogen. The residue was taken up in 4 mL of mobile phase, which consisted of hexanes, 1, 2-dichloromethane, and ethanol in the volumetric ratio 90:10:2, and injected into the HPLC system. When separation of the isomers was not achieved,

the procedure was repeated and the derivatization solution was heated for 24 hours, instead of 30 minutes. Again, it was unsuccessful. Davis et al. report that the relative rates of uncatalyzed reactions of primary, secondary, and tertiary alcohols with phenyl isocyanate had the ratios of 100:33:1, respectively (Davis, 1934). Primary alcohols react rapidly at room temperature, secondary alcohols must be warmed before reacting rapidly, and tertiary alcohols react slowly even at high temperatures (100º C). Increasing the temperature in an attempt to accelerate the reaction is not an option since higher temperatures would increase the elimination reaction, resulting in the formation of olefins instead of the desired product (Davis, 1934).

The second derivatization procedure used 1-naphthylisocyanate combined with butyllithium as a catalyst. A catalyst is needed for the derivatization of a tertiary alcohol. Bailey, et al. describes a procedure in which a small amount of lithium is added to a tertiary alcohol to generate the lithium alkoxide. The lithium alkoxide then reacts with the isocyanate carbon and form an intermediate with a negative charge on the nitrogen. The nitrogen would abstract a proton from another alcohol molecule and regenerate the alkoxide (Bailey, 1977). According to Bailey, et al., the key to the successful addition was using a small amount of lithium alkoxide in the presence of an excess of the tertiary alcohol. The tertiary derivatives should be stable under the basic conditions. This stability would prevent the elimination reaction that would form the olefin, instead of the desired carbamate. Bailey, et al. found that there are several critical aspects of the derivatization procedure. One is that lithium salts are preferred over sodium and potassium because they are more soluble in organic solvents and therefore greater concentrations of the alkoxides can be achieved by using lithium alkoxides of high

molecular weight alcohols. Also, if water is present, lithium hydroxide can form which will not interfere with the reaction. Another is that the carbamate formation is optimized by using an excess of the tertiary alcohol when the isocyanate is added dropwise. Derivatization of the tertiary alcohol was attempted via the following procedure: The dry alcohol was dissolved in dry heptane. One equivalent of n-butyllithium was added under nitrogen using a needle and syringe, and the solution was stirred for 30 minutes. One equivalent of isocyanate was added dropwise over 5 minute using a needle and syringe, and the solution was stirred for 30 minutes. The solution was washed with water, the organic phase was separated, and redissolved in the mobile phase for injection on the HPLC system. Separation of the isomers was not achieved. It is likely that this derivatization procedure was not successful due to the trace amounts of moisture. Even though an inert atmosphere was used, and care was taken to use dry equipment (glassware, syringes, needles, etc.), it would only take a very small amount of water to consume the naphthylisocyanate. The procedure was repeated on a larger scale; however the derivatization was still not successful.

Chiral Stationary Phases for Use in Preparative Liquid Chromatography

During the last two decades, the number of chiral stationary phases available for the separation of enantiomers has grown rapidly, making it possible to develop an analytical separation of almost any racemic mixture. However, the number of chiral stationary phases that are of practical use in the development of an efficient preparative separation of enantiomers is restricted due to the issues of loading capacity, chemical and/or mechanical stability, availability in large quantities at reasonable cost, and solvent limitations, which can have a great impact on both solubility and productivity. Protein

and ß-cyclodextrin columns have low column loading capacity and are not appropriate for preparative liquid chromatography for the isolation of enantiomers. Brush or Pirkle type chiral stationary phase is suitable for preparative separations. Chiralcel OD has a high column loading capacity and is suitable for preparative separation (Okamoto, 1994). In general, the synthetic polymer chiral stationary phase has a high column loading capacity and as well as a good enantiorecognition ability (Okamoto, 1994). The only disadvantage, particularly in preparative separation, is the restricted use of mobile phase systems due to the solubility of this class of chiral stationary phases in many common solvents. They are the most widely used chiral stationary phases for preparative separations.

CONCLUSION

Two new HPLC methods were developed and optimized for the separation of oxyphene optical isomers using a cyclodextrin bonded chiral stationary phase and a protein chiral stationary phase. A method that is currently in the literature for the simultaneous determination of dextropropoxyphene and α-(+)-oxyphene using a synthetic polymer chiral stationary phase was modified and optimized for the separation of oxyphene optical isomers.

Protein chiral stationary phases, such as the α_1 acid glycoprotein column, are excellent analytical tools since they have a very high selectivity. A wide variety of enantiomeric solutes can be resolved on these chiral stationary phases. Since they use aqueous mobile phases, they can be used for the analysis of biological samples. The disadvantage of these columns, especially α_1 acid glycoprotein, is that they are very delicate and unless the column is treated with extreme care, it may have a short life.

Another disadvantage of the protein chiral stationary phases is that they have very low column loading capacity and are not suitable for preparative separations.

Polysaccharide-based columns such as Chiralcel OD have broad applicability and good column efficiency. They also have high loading capacity, and they are the most widely used chiral stationary phases in preparative separations.

An advantage of cyclodextrin-based chiral stationary phases is that they can be used with all types of solvents. They can be used in the reverse phase and the normal phase modes. This type of chiral stationary phases can also be used for preparative separations. Like protein columns, they can be used for the direct analysis of biological fluids because they can be used with aqueous mobile phases. Aqueous mobile phases are compatible with the biological fluids.

This research demonstrated that separation of oxyphene isomers was achieved using synthetic polymer, cyclodextrin, and protein chiral stationary phases. The Chiralcel OD and the ß-cyclodextrin chiral stationary phases yielded the best peak shape, baseline resolution, and reasonable run times for the separation of oxyphene isomers. The synthetic polymer chiral stationary phase was best for the analytical separation because it yielded the highest resolution, and because of its good column efficiency and enantiorecognition. It is also the best for preparative separations due to its high loading capacity. Quantitation of the minor isomer, as low as 0.1%, in the presence of the major isomer was possible using both the synthetic polymer and cyclodextrin chiral stationary phases.

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