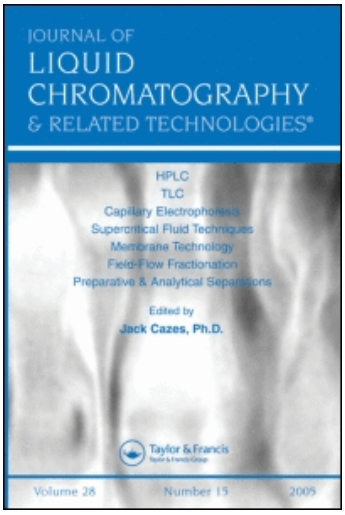

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Evaluation of an HPLC Chiral Separation Flow Scheme for Small Molecules

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Abstract: An HPLC chiral separation flow scheme was developed for identification of suitable enantioseparation conditions for small molecules. This flow scheme employs various chiral stationary phases (CSPs) and separation modes with the aim of improving efficiency by leading the scientist toward a reliable enantioseparation through a reduced number of experiments. In cases where a partial chiral separation is achieved with a particular CSP, guidance in the flow scheme is provided to improve resolution. Using prior knowledge, literature references, and data from 60 nonproprietary compounds analyzed in this study, the flow scheme was developed with separation mode (solvent compatibility) versatility in mind.

Keywords: Chiral separation, Flow scheme, HPLC, Chiral stationary phase, Enantiomer, Racemic

INTRODUCTION

The technological ability to separate the enantiomers of chiral drugs has increased dramatically over the last few decades. In tandem with this increase in capability has grown the realization of the need to utilize these technologies to isolate or synthesize the active enantiomer for optimized drug development. With chirally pure compounds available, the stereospecific action of such compounds can be assessed both in vitro and in vivo. The pharmacokinetic and pharmacodynamic profile of a chiral compound can now be studied. The observation of widely differing pharmacological action between

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enantiomers of a given chiral drug in vivo has been well established.[1] Since many racemic pharmaceutical candidates derive their pharmacological action from one isomer only, the submission of racemic entities in such cases for regulatory approval may be considered illogical. In the worst case, a racemic compound could be considered to contain 50% impurity levels. Regulatory opinion provided by the FDA first in 1987, and through formal guidelines in 1992, generally favors the submission of chirally pure drug candidates.[1–3]

Until relatively recently, asymmetric synthesis or formation and subsequent resolution of a diastereomeric salt were the two viable routes to obtain an isolated chiral drug at larger scales. The emergence of robust chromatographic techniques can now, many times, provide an alternative to the more traditional approaches. Chromatography can prove less expensive than asymmetric synthesis or classical resolution methods, as evidenced by the growing number of companies opting for chromatographic purification to obtain both synthetic intermediates and final compounds.[1,4] In addition to the potential cost savings in equipment and materials, chromatographic purification can also save time during the drug development process.

The primary chromatographic technique of scale capable of meeting material demands to support preclinical and clinical testing is simulated moving bed (SMB) chromatography. SMB can be utilized to obtain pure isomers from gram to multikilogram scale, many times with optical purities superior to what is obtained using traditional non-chromatographic resolution techniques. Examples of chiral active pharmaceutical ingredients (APIs) separated at larger scales include tramodol, formoterol, and propranolol.[4]

Enantiomeric purity is also important very early in the drug development process. When a racemic compound is observed to be biologically active, the logical course of action is to test each enantiomer to determine which isomer elicits the desired response. The material requirements for such testing can range from a few milligrams to a gram, allowing for multiple avenues to resolve the chiral material. Chromatographic choices for this scale include supercritical fluid chromatography (SFC) and batch preparative HPLC.

No matter what technique is employed at preparative scale, the fractions obtained must be analyzed at analytical scale to determine percent enantiomeric purity. The available options for such analyses are even greater and include techniques such as capillary electrophoresis (CE), HPLC, SFC, and gas chromatography (GC). The in-process analysis needs to be robust and relatively fast. Advantages exist for analytical techniques that mimic the larger scale separations.

Chiral purity analysis of process intermediates, drug substance, alone and in various formulations, and in biological matrices is also a requirement during the drug development process. The various separation techniques above (CE, SFC, HPLC, GC, and others) represent an arsenal of applications, each possessing advantages and disadvantages pertaining to speed, efficiency, and sample compatibility. In addition, certain techniques are more or less

effective depending on structure, physical, and chemical properties of the analyte.

This report focuses on the use of HPLC in various modes to develop the chiral separation. One of the greatest advantages of using this technique is the availability of instrumentation. Since the HPLC instrument is a mainstay of chromatography, one usually only needs to invest in the actual chiral columns to pursue an HPLC chiral separation. Admittedly, the columns can be costly, but not as expensive as the financial outlay that might be required to set up dedicated CE or SFC instrumentation.

Another advantage offered by HPLC is its general familiarity among scientists. Most analytical chemists understand basic HPLC and can quickly employ their familiar instrumentation toward the chiral separation. The proportion of scientists in a typical lab setting who are skilled in the competing techniques (mentioned above) is almost always notably less as compared to those familiar with traditional HPLC. In light of this, many pharmaceutical companies have set aside specialty labs consisting of scientists whose skills lie in chiral separation techniques such as SFC, CE, GC, as well as HPLC. This is perhaps the ideal setting for employing these promising ancillary techniques.

At times, competing separation technologies can offer advantages over the more traditional HPLC. Capillary electrophoresis and SFC are techniques that have been shown capable of processing racemic compounds more rapidly during a screen in search of the ideal chiral separation. In addition, waste streams from CE, SFC, and GC are of less volume, reducing waste disposal costs and up front solvent expenses.

A certain problem is presented for companies that take the approach of using a laboratory dedicated toward enantioseparation development. As we alluded to earlier, such labs owe their strength to the breadth of knowledge and techniques that are inherent in such a lab. Doubtless, one or more acceptable chiral separations using multiple approaches might be quickly identified in a well equipped laboratory. The question remains, though, whether an enantioseparation method using a technique other than HPLC will be relied upon for analysis through compound registration. For example, a search of the current USP resulted in no SFC methods and only two methods for CE.[5]

An efficient approach to meeting the chiral separation demands imposed across the drug development arena should involve the various techniques aforementioned, where and when they offer advantage. The further a racemate progresses along the drug development pipeline, however, the likelihood increases that traditional chiral HPLC will surface as the analysis method of choice at compound registration. The HPLC chiral separation flow scheme presented here is designed, in part, to meet the likely eventual need of a robust HPLC enantioseparation.

Various HPLC chiral screens have recently been reported in the literature.[6,7] Anderson and colleagues reported notable success separating multiple racemates using a combination of two screens, one employing the

popular polysaccharide CSPs and another utilizing macrocyclic antibiotic phases.[6] Matthijs and colleagues recently reported a screen focusing on the use of polysaccharide CSPs in both the normal and reversed phase modes.[7] These screens were shown to be quite successful, resolving large percentages of the racemates tested in their respective studies.

Chiral screens are very beneficial in showing the separation success of a particular class of columns with respect to an analyte set. This study, likewise, begins with the screening of a set of 60 non-proprietary compounds across several CSP classes and modes. However, this report moves beyond the screening process. Using detailed analysis of this large data set (2100 injections), combined with column manufacturer recommendations and experience, a thorough but basic HPLC chiral separation flow scheme has been developed for use by the scientist of limited experience in the area of chiral separations. The decision tree (incorporating five "steps" and eight CSPs) is designed to lead the scientist through a reduced set of experiments, with the aim of obtaining a chiral separation in a shorter period of time using only more historically promising CSPs. The steps assume the desire to obtain a separation irrespective of separation mode. If the scientist needs a specific separation system (i.e., aqueous method for drug product analysis or normal phase method for samples in a lipid-based matrix), then appropriate steps in the flow scheme can be easily located.

EXPERIMENTAL

HPLC grade solvents were obtained from various sources. Methanol (MeOH) and acetonitrile (ACN) were purchased from EMD of Gibbstown, NJ. Isopropanol (IPA) was acquired from Mallinckrodt-Baker, Phillipsburg, NJ, absolute ethanol from AAPER, Shelbyville, KY, and n-hexane from Acros Organics, Geel, Belgium. Mobile phase modifiers included acetic acid (HOAc) purchased from Mallinckrodt-Baker and triethylamine (TEA) from Sigma-Aldrich of St. Louis, MO. Aqueous buffers were prepared using ammonium acetate (NH_4OAc) and dibasic potassium phosphate (K_2HPO_4) from Sigma-Aldrich and monobasic potassium phosphate (KH_2PO_4) from Mallinckrodt. Mobile phase pH was adjusted using phosphoric acid, 85%, from Mallinckrodt. The 60 racemic analytes (Table 1) were obtained from Sigma-Aldrich (St. Louis, MO & Milwaukee, WI), Riedel de Haen (Seelze, Germany), and Fluka (Steinheim, Germany).

Assays were performed using an Agilent 1100 Series HPLC system consisting of a G1312A binary pump, G1379A mobile phase degasser, G1329A Autosampler, G1330B autosampler temperature controller, G1316A column compartment and G1315B diode array detector (Waldbronn, Germany). Chiralpak® AD-H, Chiralcel® OD-H, and Chiralcel® OJ-H columns (4.6 × 150 mm, 5 μm) were purchased from Chiral Technologies, Inc. (Exton, PA). The Chirobiotic V™ and Chirobiotic T™

Table 1. Test analytes used for chiral separation screening

Chiral compounds (Racemic)		
Acids	Neutrals	Bases
Flurbiprofen	*trans*-Stilbene oxide	Pindolol
Carprofen	Triadimefon	Nicardipine hydrochloride
N-α-dansyl-tryptophan	4,4′-Dimethoxybenzoin	Indapamide
Phenylalanine	Thalidomide	α-Methyl-α-phenylsuccinimide
Dansyl-α-aminocaprylic acid	Ketoconazole	Trimipramine maleate
Histidine	5-Methyl-5-phenylhydantoin	Propanolol hydrochloride
Fenoprofen calcium hydrate	Terfenadine	Proglumide
Dansyl-glutamic acid	Tropicamide	Terbutaline
N-CBZ-alanine	Bendroflumethiazide	Propafenone hydrochloride
p-Hydroxymandelic acid	Coumachlor	Isoproterenol
Atrolactic acid hemihydrate	Flavanone	Nadolol
Indoprofen	Warfarin	Primaquine diphosphate
β-Phenyllactic acid	Benzoin methyl ether	Prilocaine hydrochloride
m-Tyrosine	Methyl mandelate	Atenolol
Ibuprofen	1-Acenaphthenol	Metoprolol (+)-tartrate
N-CBZ-methionine	Benzoin	Carbinoxamine maleate
Dansyl-norleucine	Oxyphencyclimine hydrochloride[a]	Chlorpheniramine maleate
Ketoprofen	Mianserin hydrochloride[a]	Laudanosine
Suprofen	Bupivacaine hydrochloride[a]	Alprenolol hydrochloride
Dansyl-α-amino-n-butyric acid	Hydroxyzine dihydrochloride[a]	Sotalol hydrochloride

[a]Weakly basic tertiary amines were classified as neutral for this study.

columns (4.6 × 250 mm, 5 μm) were acquired from Advanced Separation Technologies, Inc. (Whippany, NJ). The Ultron ES-OVM and Ultron ES-Pepsin columns (4.6 × 150 mm, 5 μm) were obtained through Agilent. The (R,R) Whelk-O 1® (4.6 × 250 mm, 10 μm) column and Chiral-AGP™ (4.0 × 150 mm, 5 μm) columns were provided by Regis Technologies, Inc. (Morton Grove, IL).

Samples were analyzed using detector wavelengths of 220 nm when practical, and 254 nm when mobile phase background absorbance was increased due to additives. A flow rate of 1.0 mL/min was employed for 250 mm columns and 0.6 mL/min for 150 mm columns. Samples were prepared in the respective mobile phases at a concentration of 0.1 mg/mL. Sample injection volume was maintained at 5 μL. Column temperature was not controlled (ambient).

RESULTS AND DISCUSSION

The 60 test analytes were screened using nine columns and 35 different mobile phases, resulting in 2100 total injections. Chromatographic data for all injections including resolution (Rs), retention factor (k_2 and k_1), and selectivity (α) were compiled in a spreadsheet format. Selectivity was calculated using the equation ($\alpha = k_2/k_1$) and resolution using the standard USP equation.[5]

The 60 analytes chosen for analysis are readily available and affordable non-proprietary compounds, containing only one stereogenic center. The set includes 20 acidic, 20 basic, and 20 neutral compounds possessing various functional groups shown to be important in chiral recognition. In fact, many of the chosen analytes were cited in the chiral column manufacturers' literature. The compounds (shown in Table 1) are grouped as acids, neutrals, or bases, solely by the functional groups present on the analyte. For the purposes of this study, a small number of weakly basic tertiary amine salts were included as neutrals. The structural diversity within this compound set should prove adequate for the determination of the impact of analyte class when choosing the optimal mobile phase/CSP combination.

The sheer number of chiral CSPs available on the market does not warrant a comprehensive screen of each column and mobile phase combination. Our screen is large, but certainly not exhaustive. The 35 CSP/mobile phase combinations chosen for analysis can be grouped into five general categories covering four CSP classes. The CSP classes shown in Figure 1 represent proven and widely used chiral selector technology. The polysaccharide phases are perhaps the most widely used chiral selector class employed today, and were thoroughly studied as part of our screening process. These CSPs are comprised of various derivatized polysaccharide phases coated on a macroporous silica support. Three CSPs from this class were screened in our experiments. They include the Chiralpak® AD-H, Chiralcel® OD-H, and Chiralcel® OJ-H columns. A second chiral selector class evaluated in

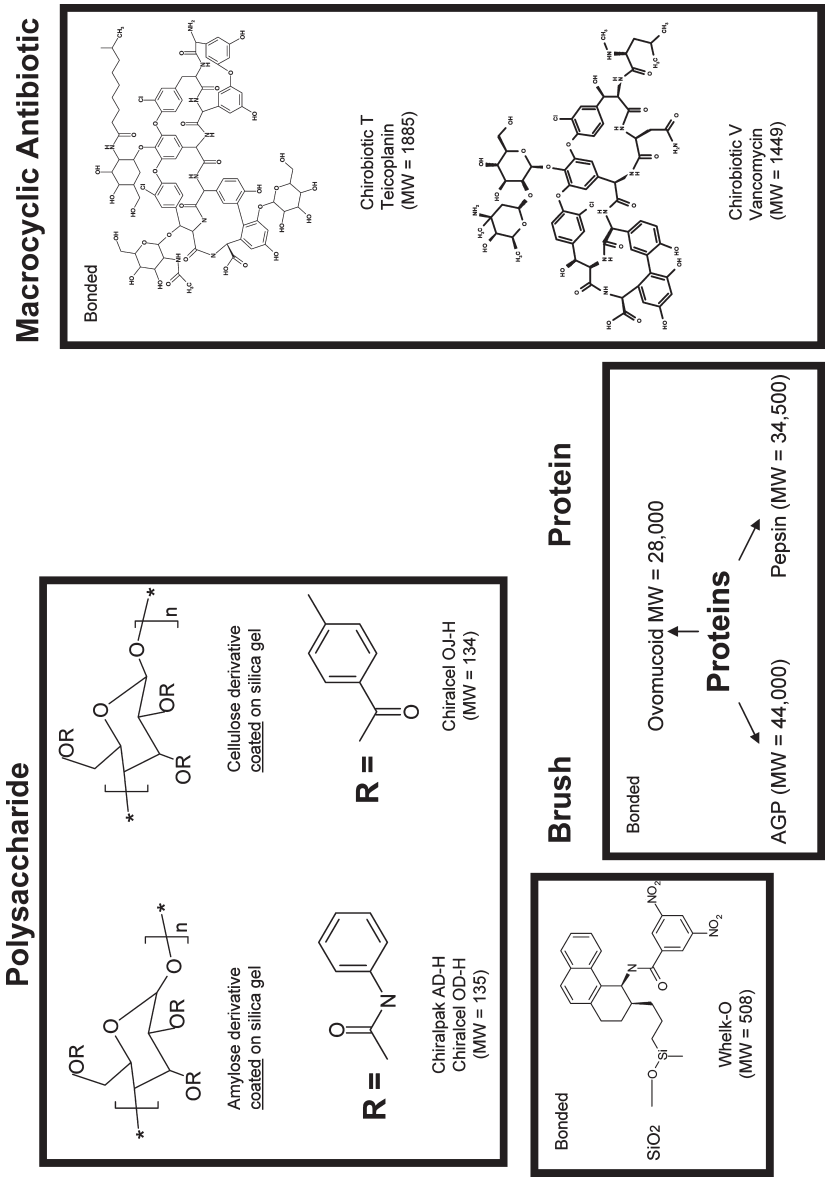


Figure 1. Overview of the four CSP classes assessed.

the screen employs macrocyclic antibiotics. These macromolecules are bonded to silica and, therefore, possess greater stability and mobile phase versatility than the coated polysaccharide CSPs. In addition to stereospecific hydrogen bonding, steric, and π-π interactions, these phases also separate, to a limited extent, via inclusion complexation. Specifically, the Chirobiotic™ V and Chirobiotic T™ columns were evaluated in this screen. Another CSP class studied in our screen is based on the "Pirkle-Concept". Also known as brush-type, this separator class capitalizes on π-electron acceptor and/or π-electron donor activity differences between isomers and the covalently bonded selector to provide a chiral separation. A unique potential benefit worth mentioning with this CSP is the ability to invert enantiomer elution order through the selective use of the R,R and S,S enantiomers of the chiral selector 1-(3,5-dinitrobenzamido)-1,2,3,4,-tetrahydrophenanthrene to produce the (R,R) Whelk-O 1® and (S,S) Whelk-O 1® columns. The (R,R) Whelk-O 1® is the representative column from the brush-type class that we chose to assess in the screen.

Protein CSPs constituted the final class evaluated during the screening process. Proteins are polymers composed of amino acids, which are chiral by nature. The unique combinations of these amino acids result in complex structures, which allow for stereoselective inclusion, hydrogen bonding, and steric interaction. The number of protein CSPs on the market is large. We focused on three of the many animal based proteins in our screen. α_1-Acid glycoprotein is a human plasma protein with a molecular weight of approximately 41,000. The column using this protein is known as the Chiral-AGP™. The second phase assessed is commercially available as the Ultron ES-OVM column. Ovomucoid is present in the white portion of a chicken egg. Its molecular weight is approximately 55,000. The third phase employed is based on the stomach enzyme pepsin. The molecule has a molecular weight of approximately 34,500 and is marketed as the Ultron ES-Pepsin column.[1]

Screen Performance as a Function of Compound Class

The chromatographic mode employed for enantioseparation development is second in importance only to the type of CSP used. In other words, the scientist could be using the right column with the wrong mobile phase. For this reason, we covered more than one separation mode when feasible for a given CSP class. The polysaccharide columns, for instance, were evaluated in the polar organic mode followed by the normal phase mode. Polar organic mobile phases consist of ACN or alcohols such as MeOH, EtOH, or IPA, either used neat or as mixtures. Polar solvents can sometimes provide alternative separation mechanisms compared to the more traditional normal and reversed phase solvent systems. Use of this separation mode many times results in sharper peak bands, not to mention improved analyte solubility

observed often with these solvents. Eleven polar organic mobile phase/polysaccharide CSP combinations were screened in this study.

Table 2 lists these polar organic mobile phase/polysaccharide CSP combinations, and summarizes the performance of these columns and conditions relative to the 60 compounds assessed. In order to limit peak tailing, a 0.1% concentration of TFA was added to the eluent when analyzing acidic analytes. Likewise, a 0.2% concentration of TEA was added to the mobile phase for the analysis of bases. Table 2 lists separation results as percentage separated, first for the entire analyte set, and then for each subset of acids, bases, and neutrals. This format allows for a general assessment of how each separation system performs relative to analyte class. The overall separation results clearly show that the Chiralpak® AD-H column provided baseline chiral separations for a greater percentage of analytes across all polar organic mobile phases tested. A closer look at the data reveals that this improved performance over the cellulose based CSPs is independent of analyte class. After the Chiralpak® AD-H, the Chiralcel® OD-H was preferred for bases and neutral compounds, while the Chiralcel® OJ was more successful for separating chiral acids.

The same polysaccharide CSPs (different columns) were next evaluated using selected normal phase systems. The normal phase chromatographic system capitalizes on the use of a mobile phase consisting in a major proportion of a nonpolar solvent, such as hexane. This solvent is modified with lesser amounts of an alcohol, such as EtOH or IPA (usually 5–30%). As the alcohol composition in the eluent increases, resolution and retention typically decrease, accompanied by sharper peak bands. Chiral separation data for six normal phase/CSP combinations is provided in Table 3. TFA and TEA were once again added to the eluents of the respective mobile phases for the analysis of acids and bases, respectively. The Chiralpak® AD-H column again demonstrated superior selectivity, providing baseline chiral separations for over 50% of the analytes. As observed with the polar organic trials, the phase is particularly adept at separating acids and bases. Neutral molecules were roughly equally well separated using the Chiralcel® OD-H column. After the Chiralpak® AD-H, the Chiralcel® OD-H column outperformed the Chiralcel® OJ-H column concerning the enantioseparation of basic and neutral analytes. Just as was observed with the polar organic experiments, the Chiralcel® OJ-H column demonstrated some success with chiral acids.

The next phase of the screening process focused on a different CSP class, the macrocyclic antibiotics. Both the Chirobiotic V™ and Chirobiotic T™ columns were screened using four mobile phases covering three separation modes. One of the eluent systems, the polar organic mode, has been previously discussed. A variation on this separation scheme employs the polar solvent MeOH in combination with varying ratios of acid and base in the mobile phase. Known as the polar ionic mode, this solvent system is reported as being very effective at separating chiral analytes with multiple "functional

Table 2. Separation results by compound class: polysaccharide columns in the polar organic mode

		Resolution percentage								
Column	Conditions	Yes (>1.5)			(0–1.5)			No		
Chiralcel OJ-H	Methanol		17%			18%			65%	
	Separation by class	15%	20%	15%	15%	20%	20%	70%	60%	65%
	90% MeOH/10% ACN		12%			18%			70%	
	Separation by class	15%	5%	15%	30%	20%	5%	55%	75%	80%
	Ethanol		20%			17%			63%	
	Separation by class	30%	15%	15%	10%	30%	10%	60%	55%	75%
	80% EtOH/20% MeOH		18%			22%			60%	
	Separation by class	20%	20%	15%	30%	25%	10%	50%	55%	75%
Chiralcel OD-H	Methanol		15%			23%			62%	
	Separation by class	20%	5%	20%	40%	10%	20%	40%	85%	60%
	Ethanol		15%			23%			62%	
	Separation by class	20%	5%	20%	35%	15%	20%	45%	80%	60%
	80% EtOH/20% MeOH		13%			25%			62%	
	Separation by class	20%	5%	15%	35%	15%	25%	45%	80%	60%

Chiralpak AD-H	Methanol		32%			32%			37%	
	Separation by class	35%	20%	40%	25%	30%	40%	40%	50%	20%
	Ethanol		38%			22%			40%	
	Separation by class	40%	35%	40%	15%	25%	25%	45%	40%	35%
	80% EtOH/20% MeOH		33%			28%			38%	
	Separation by class	40%	25%	35%	25%	35%	25%	35%	40%	40%
	70% EtOH/30% Hexane		28%			37%			35%	
	Separation by class	35%	25%	25%	25%	50%	35%	40%	25%	40%

Neutral = ■
Acidic = ■
Basic = ■

Table 3. Separation results by compound class: polysaccharide columns in the normal phase mode

		Resolution percentage								
Column	Conditions	Yes (>1.5)			Partial (0–1.5)			No		
Chiralcel OJ-H	70% Hexane/30% EtOH		37%			20%			43%	
	Separation by class	40%	55%	15%	10%	25%	25%	50%	20%	60%
	70% Hexane/30% IPA		25%			28%			45%	
	Separation by class	20%	40%	15%	40%	25%	20%	40%	35%	60%
Chiralcel OD-H	80% Hexane/20% EtOH		43%			23%			33%	
	Separation by class	55%	30%	45%	25%	30%	15%	20%	40%	40%
	80% Hexane/20% IPA		45%			22%			33%	
	Separation by class	50%	30%	55%	20%	35%	10%	30%	35%	35%
Chiralpak AD-H	85% Hexane/15% EtOH		57%			17%			27%	
	Separation by class	40%	65%	65%	30%	5%	15%	30%	30%	20%
	80% Hexane/20% IPA		50%			22%			28%	
	Separation by class	55%	55%	40%	20%	20%	25%	25%	25%	35%

Neutral = ■
Acidic = ■
Basic = ■

groups". The column manufacturer explains that the additives are necessary to preferentially interact with the many ionizable groups present on the large macrocyclic antibiotic chiral selectors.[8] The authors acknowledge that not all of the 60 analytes possess the functional groups necessary for chiral recognition using the polar ionic mode. For purposes of the screen, however, all were processed using this eluent system. Finally, the analytes were screened on both columns using an aqueous mobile phase. Though not the preferred separation mode for these CSPs, the need to provide separation conditions under aqueous conditions for drug product or biosample analysis has been previously discussed.

The screening results for the Chirobiotic V™ and Chirobiotic T™ phases are summarized in Table 4. Separation success using the neat polar organic solvents (MeOH and ACN) was inferior to both polar ionic and aqueous modes. As mentioned earlier though, this performance likely has more to do with the ionic/non-ionic nature of the compound set studied. A comparison of the two columns reveals an affinity of the Chirobiotic V™ for basic and neutral analytes and the Chirobiotic T™ for acidic analytes.

The brush type CSP class, represented by the (R,R) Whelk-O 1® column, was evaluated in two solvent systems in the normal phase mode. Though this column is quite versatile and can be employed using reversed phase solvents, the manufacturer claims superior separation performance using the normal phase mode. Table 5 displays the separation results for the 60 analytes. The column demonstrated inferior performance relative to other CSPs studied. Preferential success was observed for neutral molecules with this column. Upon reflection, perhaps a parallel aqueous mode analysis would have provided a better survey of this CSP.

The final step in our screen involved the three protein CSPs known as the ES-Pepsin, ES-OVM, and Chiral-AGP™ columns. This column class is sensitive from a column stability perspective relative to the other CSP classes analyzed. Despite this liability, the authors chose to include this CSP class due to its perceived superior performance in aqueous systems. The very large and complex proteins incorporate multiple charged groups that certainly play a role in chiral recognition.[1] For this reason, we chose to evaluate the columns using mobile phases composed of 10% EtOH with buffers of various pH's to try to take advantage of potential hydrogen bonding interactions. Table 6 displays screening results from eight CSP/mobile phase combinations.

Even a cursory examination of the data reveals that the ES-Pepsin column underperformed its competitors for the analytes and conditions tested. That said, the column did show limited success separating some basic analytes using a pH 4.5 buffered eluent system. Basic compound isomers were more frequently separated, though, using the ES-OVM and Chiral-AGP™ columns with buffers at pH $\geq$ 4.5. For neutral molecules, the ES-OVM outdistanced the competitor CSPs, especially using eluent systems buffered at pH $\geq$ 4.5. Acidic molecules were, roughly, equivalently separated with both

Table 4. Separation results by compound class: macrocyclic antibiotic columns in polar organic, polar ionic and aqueous modes

Column	Conditions	Resolution Percentage								
		Yes (>1.5)			Partial (0–1.5)			No		
Chirobiotic V	Methanol		12%			22%			67%	
	Separation by class	20%	5%	10%	0%	45%	20%	80%	50%	70%
	Acetonitrile		8%			3%			88%	
	Separation by class	15%	0%	10%	5%	5%	0%	80%	95%	90%
	0.1% AcOH/0.1% TEA/MeOH		20%			27%			53%	
	Separation by class	20%	0%	40%	0%	40%	40%	80%	60%	20%
	80% 20 mM NH4OAc/20% MeOH		13%			32%			55%	
	Separation by class	25%	5%	10%	15%	45%	35%	60%	50%	55%
Chirobiotic T	Methanol		2%			7%			92%	
	Separation by class	5%	0%	0%	5%	5%	10%	90%	95%	90%
	Acetonitrile		2%			8%			90%	
	Separation by class	5%	0%	0%	10%	0%	15%	85%	100%	85%
	0.1% AcOH/0.1% TEA/MeOH		23%			17%			60%	
	Separation by class	5%	25%	40%	10%	25%	15%	85%	50%	45%
	80% 20 mM NH4OAc/20% MeOH		22%			27%			52%	
	Separation by class	15%	35%	15%	35%	40%	5%	50%	25%	80%

Neutral = ■
Acidic = ■
Basic = ■

Table 5. Separation results by compound class: brush-type column in the normal phase mode

		Resolution percentage								
Column	Conditions	Yes (>1.5)			Partial (0–1.5)			No		
Brush										
(R,R) Whelk-O 1	50% Hexane/50% EtOH		12%			12%			77%	
	Separation by Class	30%	0%	5%	15%	20%	0%	55%	80%	95%
	50% Hexane/50% IPA		13%			5%			82%	
	Separation by Class	35%	0%	5%	10%	5%	0%	55%	95%	95%

Neutral = ■
Acidic = ■
Basic = ■

Table 6. Separation results by compound class: protein columns in the aqueous mode

		Resolution percentage								
Column	Conditions	Yes (>1.5)			Partial (0–1.5)			No		
Protein (Low pH)										
ES-OVM	90% 20 mM KH_2PO_4/ 10% EtOH (pH 3)		20%			20%			60%	
	Separation by xlass	20%	25%	15%	30%	20%	10%	50%	55%	75%
ES-Pepsin	90% 20 mM KH_2PO_4/ 10% EtOH (pH 3)		2%			2%			97%	
	Separation by class	5%	0%	0%	0%	0%	5%	95%	100%	95%
Protein (pH 4.5)										
ES-OVM	90% 20 mM KH_2PO_4/ 10% EtOH		28%			13%			58%	
	Separation by class	50%	5%	30%	20%	10%	10%	30%	85%	60%
ES-Pepsin	90% 20 mM KH_2PO_4/ 10% EtOH		12%			13%			75%	
	Separation by class	10%	0%	25%	15%	0%	25%	75%	100%	50%

Chiral AGP	90% 20 mM KH_2PO_4/ 10% EtOH		23%			18%			55%	
	Separation by class	20%	25%	25%	15%	15%	20%	65%	65%	50%
Protein (High pH)										
ES-OVM	90% 20 mM K2HPO_4/ 10% EtOH (pH 7)		23%			10%			67%	
	Separation by Class	40%	10%	20%	15%	5%	10%	45%	85%	70%
ES-Pepsin	90% 20 mM K2HPO_4/ 10% EtOH (pH 6)		2%			2%			97%	
	Separation by Class	0%	0%	5%	0%	0%	5%	100%	100%	90%
Chiral AGP	90% 20 mM K2HPO_4/ 10% EtOH (pH 7)		18%			25%			57%	
	Separation by Class	20%	5%	30%	35%	25%	15%	45%	70%	55%

Neutral = ■
Acidic = ■
Basic = ■

the ES-OVM and Chiral-AGP™ columns when employing mobile phase buffers of pH 3.0 and pH 4.5, respectively.

The data in Tables 2 through 6 demonstrate various relationships between analyte class and enantioseparation success, given a specific CSP/mobile phase combination. For instance, when using the polysaccharide CSPs, the need for acidic and basic mobile phase modifiers when attempting to separate acidic and basic modifiers is paramount. Though this fact has been well documented in the literature, our data demonstrates the importance of employing said modifiers even when screening for an enantioseparation, as opposed to employing the additive after a suitable separation has been identified. Analyte class appears even more important when considering the macrocyclic antibiotic CSPs. The screen data actually supports the choice of which CSP to begin with, depending upon whether the compound is neutral, acidic or basic. The same can be said of the protein CSPs, where a relationship appears to exist between analyte class and both column and mobile phase pH.

Screen Performance as a Function of Individual Compounds

Now that the importance of analyte class in chiral separation development is established/verified, the 2100 separation results were organized by individual compound. The idea behind this presentation format was to look for separation uniqueness and redundancy. The separation results for the 20 acidic analytes using this format are shown in Table 7. This table categorizes the five major CSP classes along with the specific column tested. The number in parenthesis next to the column name represents the number of mobile phases employed using that specific CSP. The detached column at right displays summary information, including the number of baseline and partial chiral separations observed for a specific analyte. This presentation format allows for analysis of both separation "hits" and "misses" for each compound/CSP class. Flurbiprofen enantiomers, for instance, are baseline separated using all four polar organic mobile phases with the Chiralpak® AD-H column. The summary data shows that the analyte enantiomers were baseline separated using 7 of 35 conditions, other successes occurring with the Chiralcel® OJ-H column in polar organic and normal phase modes, along with an aqueous system employing the Chiral-AGP™ column. The data in Table 7 also reveal the fact that histidine isomers are not separated using any of the conditions attempted. N-alpha-dansyl-DL-tryptophan enantiomers, on the other hand, were separated using 15 different column/eluent combinations. Table 7 also clearly highlights the ineffectiveness of certain columns, namely the (R,R) Whelk-O 1® and ES-Pepsin phases, at separating the acidic enantiomers using these conditions. In summary, 19 of 20 of the acidic compounds were resolved using at least one set of HPLC conditions.

Table 8 lists chiral separation summary data for the 20 basic compounds screened in the study. All analytes in this class were separated using at least

Table 7. Separation results by compound: acidic analytes

Acid separation summary																										
	Polys[a] polar organic						Polys[a] normal phase						Macrocyclic				Brush		Protein							
Compound CSP (# of possible "hits")	AD-H (4)		OD-H (3)		OJ-H (4)		AD-H (2)		OD-H (2)		OJ-H (2)		V (4)		T (4)		Whelk (2)		OVM (3)		Pepsin (3)		AGP (2)		Totals by compound	
	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P
Flurbiprofen	4				1	3		2		1	1	1		1		1							1		7	9
Carprofen	3	1		3	2	2	2		2		2			1		1		1		1					11	10
N-alpha-Dansyl-DL-tryptophan	2	1	3		2	1	2		2		2			2					1				1	1	15	5
DL-Phenylalanine		3									1					1									1	4
Dansyl-DL-alpha-aminocaprylic acid		2		2			1	1		2				3	1								1		3	10
DL-Histidine		1																							0	1
Fenoprofen calcium hydrate salt	3	1				2	2			2	2			1						1					7	7
Dansyl-DL-Glutamic acid		1				1				2	1	1		1		1				1				1	1	9
N-CBZ-DL-Alanine	2	2					2		2		1	1	1	2	2					1					10	6
DL-p-Hydroxymandelic acid		2					2		2		1	1		1	2	1		1							7	6

(*continued*)

Table 7. Continued

Acid separation summary																										
	Polys[a] polar organic						Polys[a] normal phase						Macrocyclic				Brush		Protein							
Compound CSP (# of possible "hits")	AD-H (4)		OD-H (3)		OJ-H (4)		AD-H (2)		OD-H (2)		OJ-H (2)		V (4)		T (4)		Whelk (2)		OVM (3)		Pepsin (3)		AGP (2)		Totals by compound	
	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P
DL-Atrolactic acid hemihydrate		3			2	2	2			2	2		1		2			1	1						10	8
Indoprofen	1			3		1		1	2			2		1		2								1	3	11
DL-beta-phenyllactic acid							2				1	1		1	1	1									4	3
DL-m-Tyrosine														1	1				1						2	1
Ibuprofen					1	3						2							2				1	1	4	6
N-CBZ-DL-Methionine		2				2	2		2		1			3	2				1				1		9	7
Dansyl-DL-Norleucine	2	2					2			2		1		3	1	1		1	2				1	1	8	11
Ketoprofen	3	1				1	2				2			2		1				1				1	7	7
Suprofen	1	2			4		2			2	2			1		2				2				2	9	11
Dansyl-DL-alpha-amino-n-butyric acid		4					1	1						3		2		1					1	1	2	12
Totals	21	28	3	8	12	18	24	5	12	13	19	10	2	27	12	14	0	5	8	7	0	0	7	9	120	144

Resolution $>$ 1.5; 0 $<$ Resolution $<$ 1.5 Each compunded injected 35 times using different conditions.

[a]polysaccharide.

Table 8. Separation Results by compound: basic analytes

	Base Separation Summary																									
	Polys[a] Polar Organic						Polys[a] Normal Phase						Macrocyclic				Brush		Protein							
Compound CSP (# of possible "hits")	AD-H (4)		OD-H (3)		OJ-H (4)		AD-H (2)		OD-H (2)		OJ-H (2)		V (4)		T (4)		Whelk (2)		OVM (3)		Pepsin (3)		AGP (2)		Totals By Compound	
	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P
Pindolol		1	3			2	1	1	2			1	1	1					1	1			1		9	7
Nicardipine hydrochloride						2		1		2		1	2			2			1		1	1			4	9
Indapamide		3	3		4		1	1	2		1	1				1	2		2	1			2		17	7
alpha-Methyl-alpha-phenylsuccinimide	4			3	4		1		2		2			1	1	2			3				2		19	6
Trimipramine maleate salt		3				1		1					2									2	1		3	7
(+/−)-Propanolol hydrochloride	3	1				3	1		2			1	3		1	1			1	1			1		1	11	9
Proglumide		1					1		1			1	1		1	1							2		6	3
Terbutaline		1				1								2	1	1			1						2	5
Propafenone hydrochloride	3	1					2			1				1		1					1			1	6	5
Isoproterenol		1	1				1				1	1		3	2										5	5
Nadolol	3	1	1	1			2		1					2	1					1		1		1	8	7
Primaquine diphosphate	1			1					1										1		2		1	1	6	2

(*continued*)

Table 8. Continued

Base Separation Summary																												
	Polys[a] Polar Organic						Polys[a] Normal Phase						Macrocyclic				Brush		Protein									
Compound CSP (# of possible "hits")	AD-H (4)		OD-H (3)		OJ-H (4)		AD-H (2)		OD-H (2)		OJ-H (2)		V (4)		T (4)		Whelk (2)		OVM (3)		Pepsin (3)		AGP (2)		Totals By Compound	
	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P
Prilocaine hydrochloride	1							2		1		1		1						1					1	6		
Atenolol		1		2				1	2				1		1										4	4		
(+/ −)-Metoprolol (+)-tartrate salt	4			3			1		2			1	1		1				1			1		1	10	6		
Carbinoxamine maleate salt	3	1					2		1	1				2					1		1		1		9	4		
(+/ −)-Chlorphenir-amine maleate salt		3					2		1			2		1					1		1		1	1	6	7		
DL-Laudanosine	2	1	3		4		2		1		2		1	1											15	2		
Alprenolol hydrochloride	3	1		3			2		2				1	2	1							1	1	1	10	8		
(+/ −)-Sotalol hydrochloride	1	3					2						1	2	1										5	5		
Totals	28	23	11	13	12	9	21	7	20	5	6	10	14	19	11	9	2	0	13	5	6	7	12	7	156	114		

Resolution > 1.5; 0< Resolution < 1.5 Each compunded injected 35 times using different conditions

[a]Polysaccharide

one CSP/eluent combination. While the enantiomers of prilocaine were baseline separated using only the Chiralpak® AD-H column and one polar organic mobile phase, alpha-methyl-alpha-phenylsuccinimide isomers were resolved with 19 column/eluent systems.

Partial Enantioseparations

For the relatively experienced chromatographer, achieving a partial chiral separation often indicates that a baseline enantioseparation can be developed with minor mobile phase modification. A closer look at the aforementioned prilocaine hydrochloride data highlights the importance of including such partial separation results. From Table 8, it is apparent that the analyte isomers may well be separable using multiple other CSPs, other than Chiralpak® AD-H column with modified eluent systems.

A comparison of the data in Tables 7 and 8 with the results for the 20 neutral molecules (Table 9), reveals that the screen resulted in more "hits" with this neutral molecule subset. A total of 310 out of 700 possible baseline or partial chiral separations were observed with the neutrals, as compared with 270 and 264 for the bases and acids, respectively. Baseline chiral separations were obtained for all 20 neutral analytes. Terfenadine isomers were fully resolved using only the ES-OVM column, though partial separation occurred with the Chiralcel® OD-H and Chiralcel® OJ-H CSPs. Trans-stilbene oxide isomers, on the other hand, were separated using 24 of 35 CSP/eluent combinations.

Building the Flow Scheme from the Screen

Crafting a reliable and easy to follow flow scheme, for small molecule chiral separation development, is the ultimate goal of this research effort. While the data pool for 2100 injections is significant and provides direction toward this goal, manufacturer and literature guidance was incorporated when available. The experimental results prove most valuable in multiple ways. First and foremost, the results point toward the more successful column/eluent combinations. These would obviously be suggested as early experiments to try in the flow scheme. Alternatively, the data demonstrate that certain column/mobile phase combinations are redundant or had minimal effect. They either proved unsuccessful at separating the analytes, or offered no unique separating capability with respect to other column/eluent combinations. Falling into this category is the ES-Pepsin column. This phase was routinely outperformed by both the ES-OVM and Chiral-AGP™ columns for acidic, basic, and neutral analytes. This column is excluded from the flow scheme. Likewise, the mobile phase of 80% EtOH/20% MeOH, included in the screen with the three polysaccharide CSPs, will not be a step in the flow scheme, as it did not provide

Table 9. Separation results by compound: neutral analytes

Neutral separation summary																										
	Polys[a] polar organic						Polys[a] normal phase						Macrocyclic				Brush		Protein							
Compound CSP (# of possible "hits")	AD-H (4)		OD-H (3)		OJ-H (4)		AD-H (2)		OD-H (2)		OJ-H (2)		V (4)		T (4)		Whelk (2)		OVM (3)		Pepsin (3)		AGP (2)		Totals by compound	
	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P	Y	P
Trans-stilbene oxide	4		3		4		2		2		2					1	2		1	2	2		2		24	3
Triadimefon	2	1		3			1	1	2			2								1			2		7	8
4,4′-Dimethoxybenzoin	1	3		3	1	1	2		2		1	1				1	2		2	1					11	10
(+/−)-Thalidomide	3	1		1	4						1		4			3				1					12	6
Ketoconazole	4			1	1			2												1				1	5	5
5-Methyl-5-phenylhydantoin	4					3	2		1	1		1	4		4		2		2	1				2	19	8
Oxyphencyclimine hydrochloride							1	1	1			2	1				1	1	1				1		6	4
Tropicamide		4					2		2		1	1							2						7	5
Bendroflumethiazide		1						2	1	1	1				1			2	2	1			1	1	6	8
Coumachlor	4			3	1	3	2		2		1		1	1	1	1	1	1							13	9

Mianserin hydrochloride		2				1	1			2		1	1			1			1	1		1		2	3	11
Warfarin	4		1	2	3	1	2		2				1			2	1	1	1						15	6
Benzoin methyl ether		2		3			2		2		1			1		1			2			1		1	7	9
Methyl DL-mandelate			3			1													1	1				1	4	3
Bupivacaine hydrochloride				1						1			3						1			1		1	4	4
(+/−)-Benzoin		3	2			2	1	1	2		2			1		1	2		3				1		13	8
Terfenadine				2		1													1	1					1	4
Hydroxyzine dihydrochloride				3		2		1		2		1	1						1					1	2	10
1-Acenaphthenol		1						2		2		1					2			2				1	2	9
Flavanone	4		3		2	2	1		2		2			1		1			1						15	4
Totals	30	18	12	22	16	17	19	10	21	9	12	10	16	4	6	12	13	5	22	13	2	3	7	11	176	134

Resolution > 1.5; $0 <$ Resolution < 1.5. Each compunded injected 35 times using different conditions.
[a]Polysaccharide.

any benefits over the use of neat EtOH or MeOH eluents alone. Other CSP/eluent combinations tested in the screening experiments were shown to be of lesser importance, but were still deemed relevant. These conditions were relegated to lower priority in the flow scheme. For example, instead of including a separate step to try the Chiralcel® OJ-H column with a 90% MeOH/10% ACN eluent, the flow scheme simply lists the mobile phase as something to attempt only if broad peaks are observed using a 100% MeOH mobile phase.

For the flow scheme layout, the experimental data in Tables 7–9 was relied upon to prioritize which columns and eluent systems to attempt and in what order to try them. Then, focusing on the remaining unresolved compounds, we determined what systems separated most of the remaining isomers. Manufacturer information was incorporated also, especially as guidance for separation optimization once a partial enantioseparation occurred. Specifically, the flow scheme structure combines manufacturer recommendations concerning the macrocyclic antibiotics, protein, and brush phases with experimental data in this report.

The Flow Scheme–Step by Step

With respect to the 60 chiral compounds analyzed in the screen, the Chiralpak® AD-H column proved superior to the other CSPs tested. By employing both the polar organic and normal phase modes, this column resolved a surprising three out of four analytes in the study. The recommended first experiments to try are outlined in Figure 2 and are grouped together under the heading "Step 1", precisely because all involve the use of solely the Chiralpak® AD-H CSP. After determining if the compound is acidic, basic, or neutral, the analyst is directed to add the respective modifier to the mobile phase if necessary. The first eluents recommended are neat MeOH followed by neat EtOH. The two solvents can provide varying selectivity, as reflected in the triadimefon separations shown in Figure 3. These highly successful eluent/CSP combinations often provided baseline chiral separations along with very short retention times and sharp peaks. This could prove very beneficial with analytical methodology as it relates to the limit of detection and quantitation. Such separations are also ideal for preparative chromatography where short retention times and sharp bands can translate into reduced fraction volumes.

When a partial enantioseparation is encountered, the flow scheme recommends options to increase retention time and perhaps resolution. If no separation is observed with the polar organic trials, the analyst is next directed to attempt separation using normal phase eluents. It must be emphasized that these normal phase trials should be conducted on a separate column and not the one used for polar organic analysis. Though conversion between separation modes through either EtOH or IPA is possible with the Chiralpak®

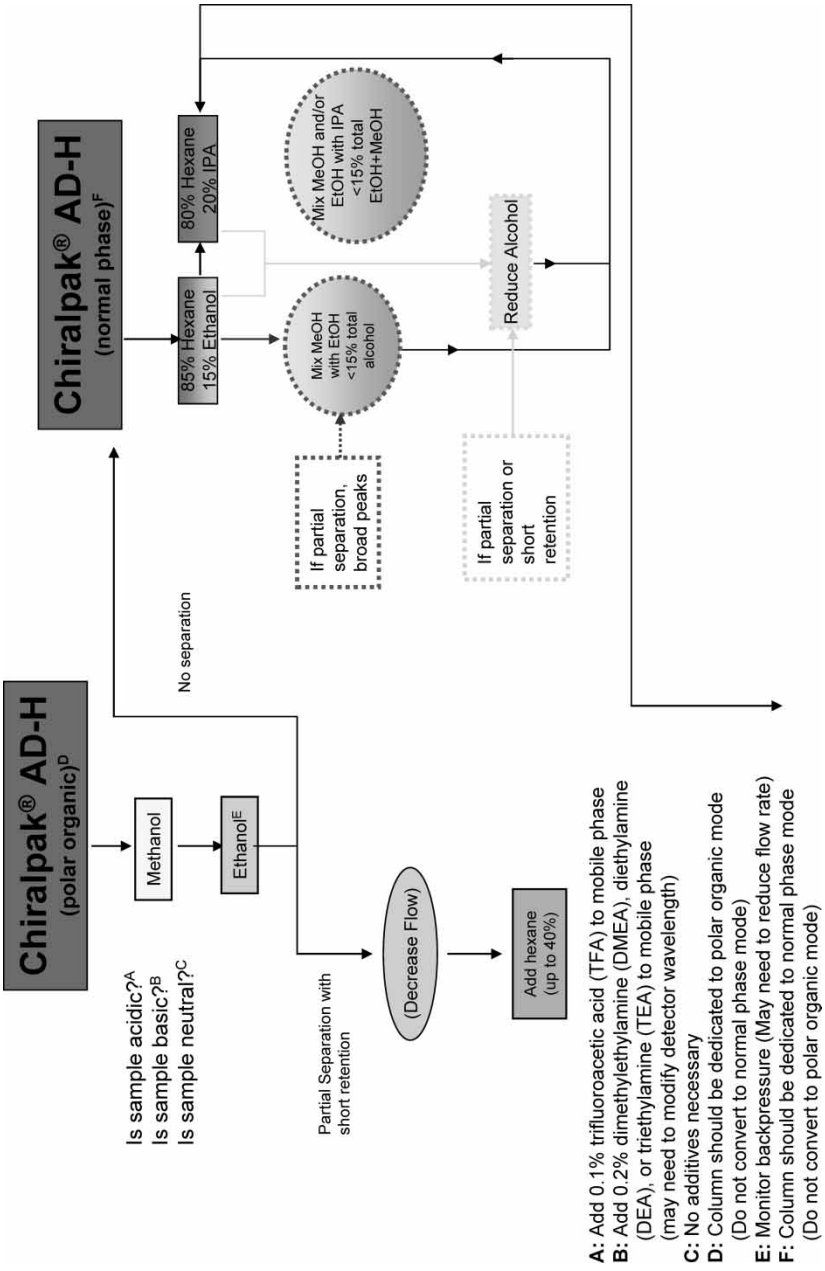


Figure 2. Step 1 of the HPLC chiral separation flow scheme.

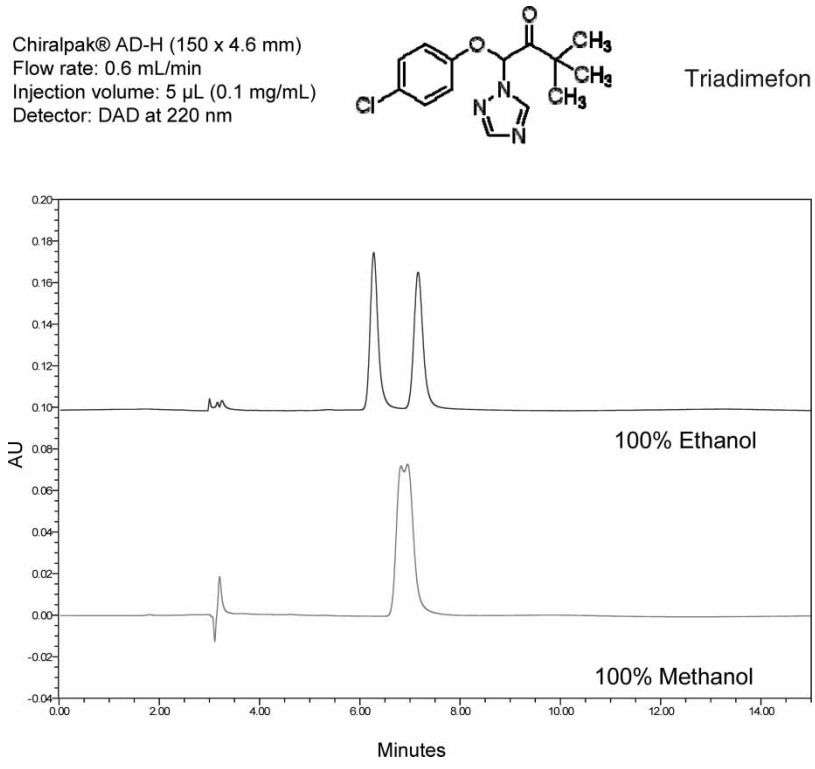


Figure 3. Effect of varying polar organic eluent on the separation of triadimefon enantiomers with the Chiral® AD-H column.

AD-H column, it is not recommended. Commonly referred to as the "memory effect" such interconversions over time tend to decrease column efficiency. It is worth mentioning here that the data from Tables 2, 3, and 7–9 show that the Chiralpak® AD-H column in normal phase mode actually separates a greater number of isomers than when using polar organic eluents. Despite this, the polar organic mode should be attempted first, due to the many aforementioned potential advantages inherent with these eluent systems.

The normal phase eluent systems in step 1 consist of the well known and widely used combinations of hexane with alcohols of varying polarity. These mobile phases are very successful at separating isomers when used in conjunction with the polysaccharide phases. The flow scheme suggests two combinations of hexane and alcohol, along with variations on alcohol identity and proportion to improve the isomer separation if necessary. Figure 4 demonstrates the importance of both alcohol identity and mobile phase additive, as it relates to the chiral separation of pindolol.

Despite the overwhelming success observed with the Chiralpak® AD-H CSP in step 1, 15 of the 60 compounds were not resolved using this

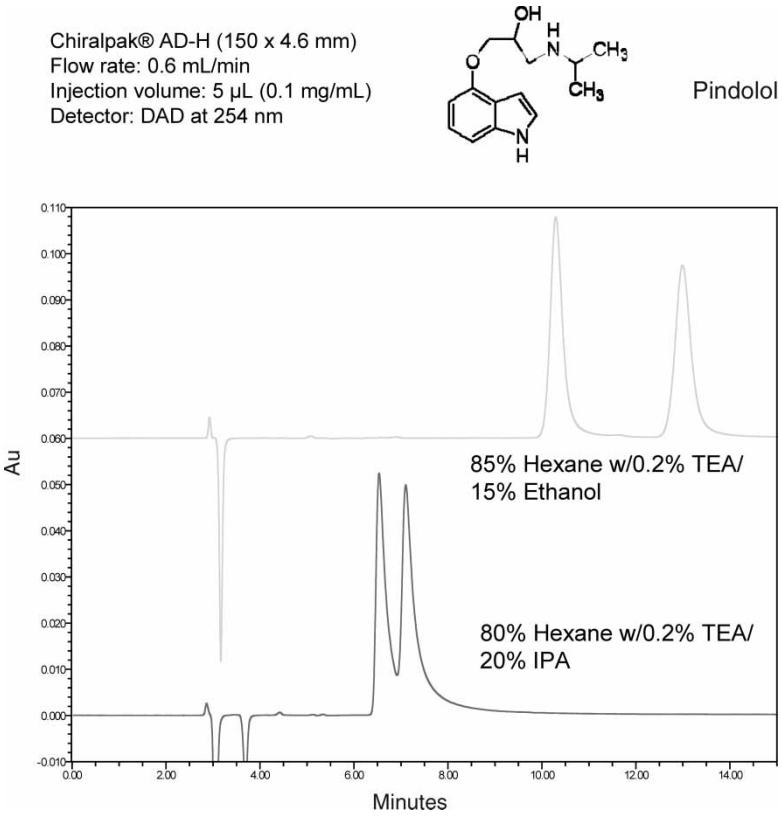
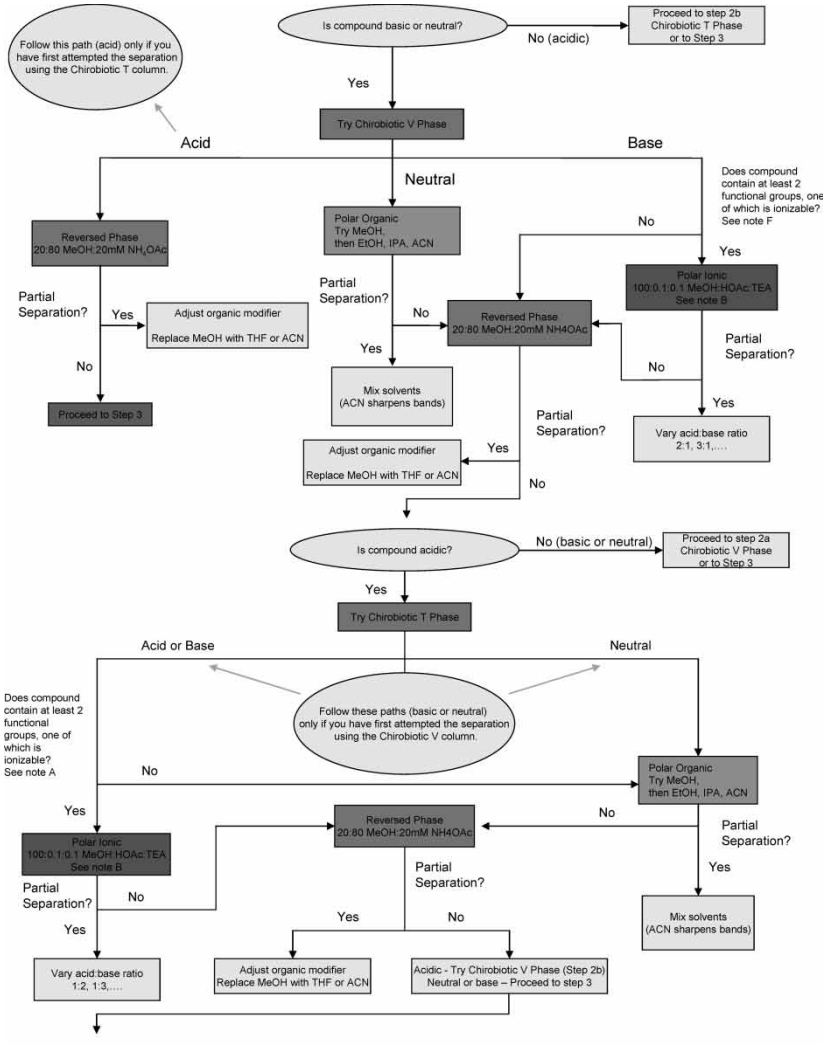


Figure 4. Effect of varying mobile phase alcohol identity on the separation of pindolol enantiomers with the Chiralpak® AD-H column.

column. In addition, since this phase is not amenable to aqueous mobile phases, step 1 would not be relevant to the analyst desiring a separation in the aqueous mode. The versatility of the macrocyclic antibiotic CSPs is precisely why the Chirobiotic V™ and T™ columns were chosen as the focus of step 2 in the flow scheme (Figure 5). These bonded phases are quite stable and can be used with reversed phase, normal phase, and polar solvents, allowing for enantioseparation development using multiple modes.

For the macrocyclic antibiotics, an initial assessment of compound structure is of importance. Step 2 begins by asking the analyst to determine if the compound to be separated is acidic, basic, or neutral. Our experimental data echoes the manufacturer recommendation that the Chirobiotic T™ is the better choice for enantioseparation development for an acidic compound. Conversely, the Chirobiotic V™ column should be used with a neutral or basic analyte.

For neutral compounds, a polar organic eluent system (similar to step 1, but with greater solvent choice) using the Chirobiotic V™ column is



A: Functional groups include alcohols, halogens (I, Br, Cl, F), nitrogen in any form, carbonyl, carboxyl, oxidized forms of phosphorous or sulfur

B: Analyte retention controlled by varying overall acid:base concentration

Figure 5. Step 2 of the HPLC chiral separation flow scheme.

recommended. Though the manufacturer emphasizes the importance of assessing several solvents in this mode, our screen was not extensive enough to sufficiently test this claim. If the polar organic mode proves unsuccessful, an aqueous based mobile phase is recommended. If the compound to be separated is basic, a closer analysis of structure is required to determine if

the polar ionic mode can be employed. According to the manufacturer, in order for the polar ionic mode to be considered, the analyte must contain at least two functional groups, one of which must be ionizable. A "functional group" can be an alcohol, halogen (I, Br, Cl, F), nitrogen, carbonyl, carboxyl, or oxidized form of sulfur or phosphorous.[8] With this separation mode, the flow scheme (and column manufacturer) recommend using equal amounts (0.1%) of acidic and basic modifiers in a MeOH mobile phase. Analyte retention is controlled by varying the total percent of acid and base present in the eluent. If resolution is observed with the starting mobile phase, isomers could potentially be further resolved by varying the acid to base ratio. Though not described in this report, the phenomenon has been observed with many analytes, sometimes using an acid:base ratio as great as 100:1. If this separation mode is unsuccessful, or if the analyte does not possess the required functional groups, the scientist is directed to try the aqueous mode. For acids, the flow scheme is similar to what is recommended for basic compounds, except for the very important change in column to the Chirobiotic T™. If the analyte possesses the required functional groups, the polar ionic mode is recommended. If not, or if this mode proves unsuccessful, the analyst is again directed to an aqueous mobile phase.

As mentioned previously, the Chirobiotic T™ is the preferred macrocyclic antibiotic CSP for acids, and the Chirobiotic V™ for basic and neutral compounds. If a chiral separation has still not been found using the above column/CSP conditions, the analyst could choose to proceed in one of two directions. One path shown in the flow scheme is to attempt a chiral separation using recommended modes on the alternative CSP (i.e., the Chirobiotic T™ for basic and neutral compounds and the Chirobiotic V™ for acids). Using this approach, the flow scheme separated 29 of 60 analytes, 8 of which were not separated with the Chiralpak® AD-H CSP in step 1. The alternative choice is to move directly to CSP/eluent systems provided in step 3.

Two polysaccharide based CSPs, the Chiralcel® OD-H and Chiralcel® OJ-H columns, are employed in step 3 of the flow scheme (Figure 6). Since these columns are very similar in nature to the Chiralpak® AD-H CSP, it is not surprising that the eluent systems recommended in step 3 to an extent mimic those suggested in step 1. Mobile phase modifiers, as demonstrated earlier, can largely affect isomer peak shape. Such modifiers should be included in the mobile phase if the analyte is either acidic or basic. Though both CSPs proved quite versatile in our screen, the Chiralcel® OD-H CSP was moderately more successful than the Chiralcel® OJ-H column at separating the chiral analytes. For the same reasons as explained previously concerning the Chiralpak® AD-H CSP (sharp bands, short retention times), the polar organic mode is recommended as the starting point for both columns. MeOH and EtOH are again the polar organic solvents of choice. Though, not typically a problem with the Chiralpak® AD-H column of step 1, peak broadening is more frequently observed with these cellulose based columns, even when operating in the polar organic mode. For this reason, the use of

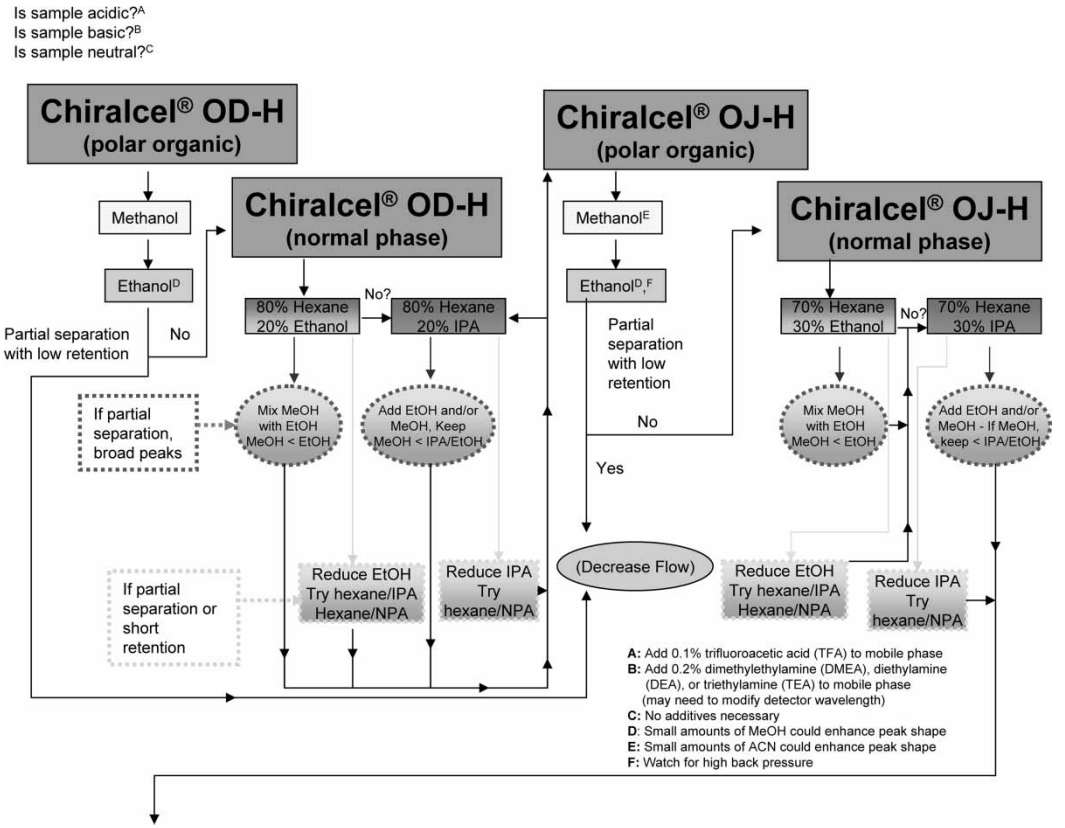


Figure 6. Step 3 of the HPLC chiral separation flow scheme.

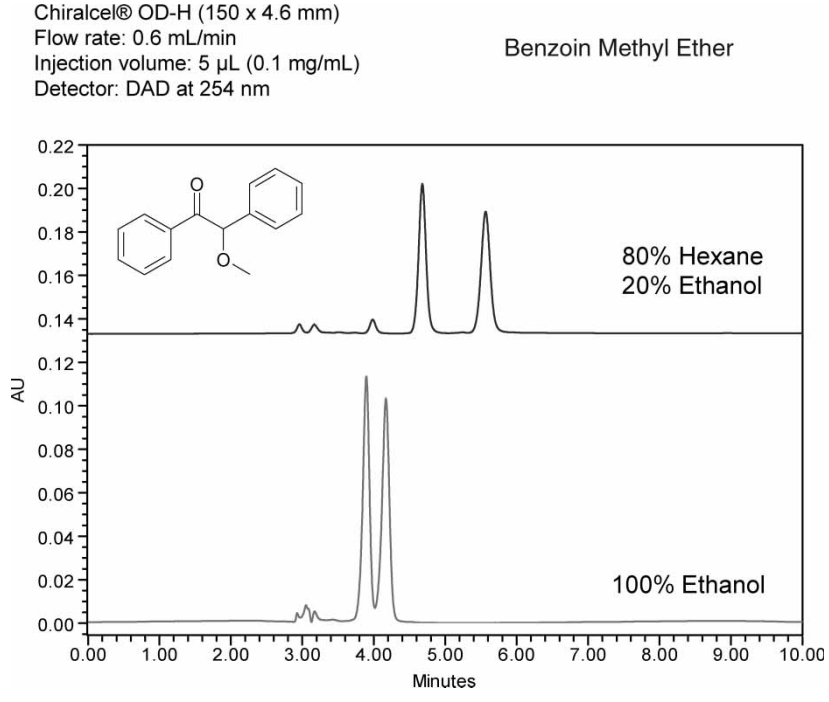


Figure 7. Comparison of a polar organic and normal phase separation of benzoin methyl ether enantiomers with the Chiralcel® OD-H column.

small proportions of a cosolvent to sharpen peaks is recommended if necessary. MeOH is typically added to a neat EtOH system, while small amounts of ACN can be added to a MeOH system (Chiralcel® OJ-H column only).

If a chiral separation is not identified using the polar organic mode, the analyst is directed to try two different normal phase systems on each CSP. Suggested mobile phase modifications to optimize a potential normal phase separation are also provided. Figure 7 shows an example of where the polar organic mobile phase identified a partial enantioseparation, but normal phase conditions ultimately separated the benzoin methyl ether enantiomers. Once again, conversion of the polysaccharide phases between polar organic mode and normal phase mode is possible, but not recommended. The column/eluent combinations of step 3 resolved 44 of the 60 analytes in our screen. This number is indeed greater than that of step 2, but the macrocyclic antibiotic CSPs provided a more unique selectivity compared to the Chiralpak® AD-H CSP of step 1, not to mention allowing for an aqueous eluent option.

If the scientist is not limited to a certain separation mode due to compound solubility or sample matrix miscibility, it is likely that step 4 will rarely be necessary for the development of a chiral separation

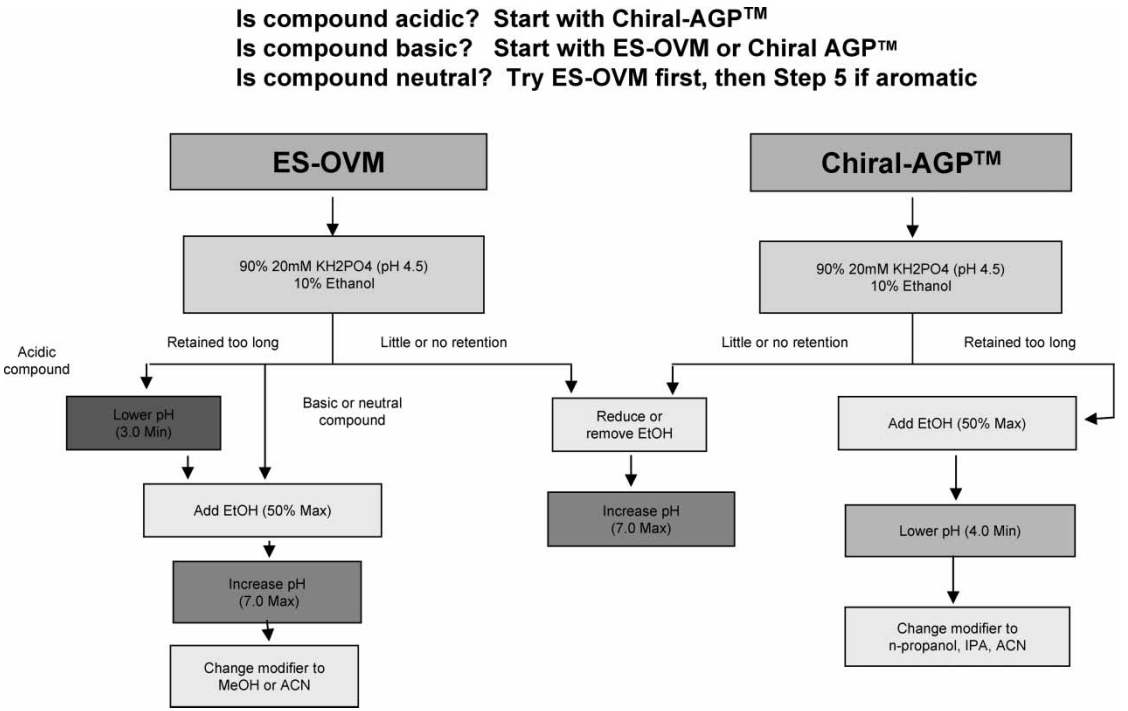


Figure 8. Step 4 of the HPLC separation flow scheme.

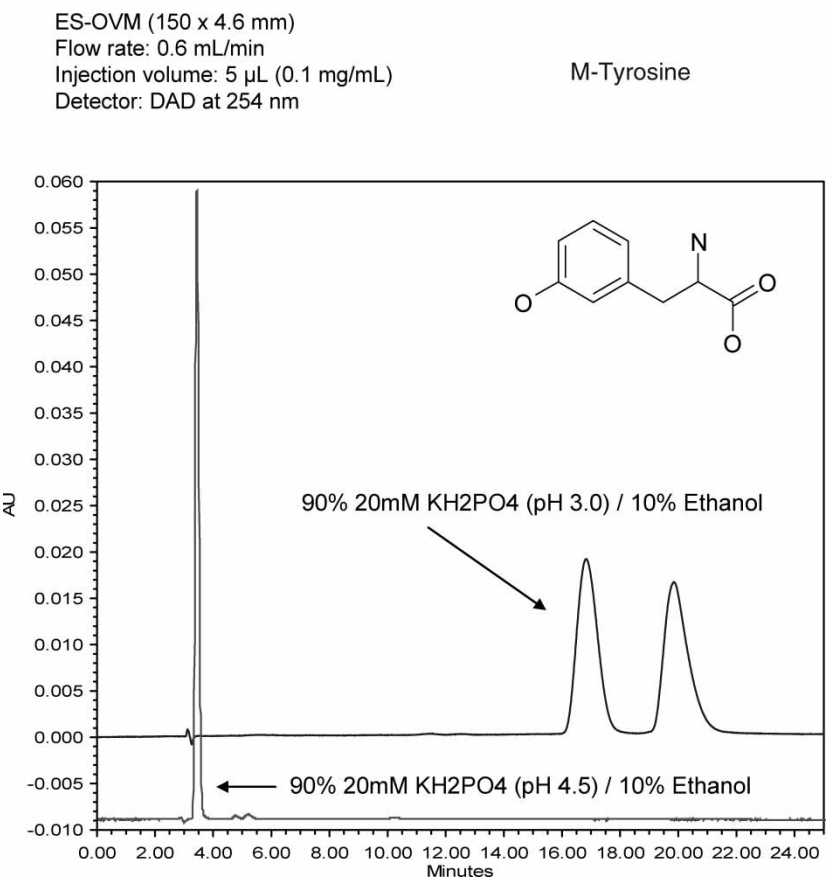


Figure 9. Effect of buffer pH on the separation of m-tyrosine isomers with the ES-OVM column.

(Figure 8). If an aqueous enantioseparation is required, however, this step incorporates two protein CSPs that provide unique selectivity. As with other steps, it is important to know if the compound is acidic, basic, or neutral. In the case of the protein CSPs, analyte class ultimately determines which column to begin with. Our screen demonstrated that the Chiral-AGP™ column showed some affinity toward acidic compounds, while neutrals were more often resolved using the ES-OVM CSP. For our sample set of 20 basic analytes, both columns performed equally well and are not differentiated. Once the CSP is chosen after answering the questions at the beginning of step 4, the initial eluent to use is ironically the same for both columns. If retention is short, the amount of EtOH in the eluent is reduced in both cases. If the retention time is too long, depending on the analyte class and CSP, the amount of organic modifier is increased and/or the pH

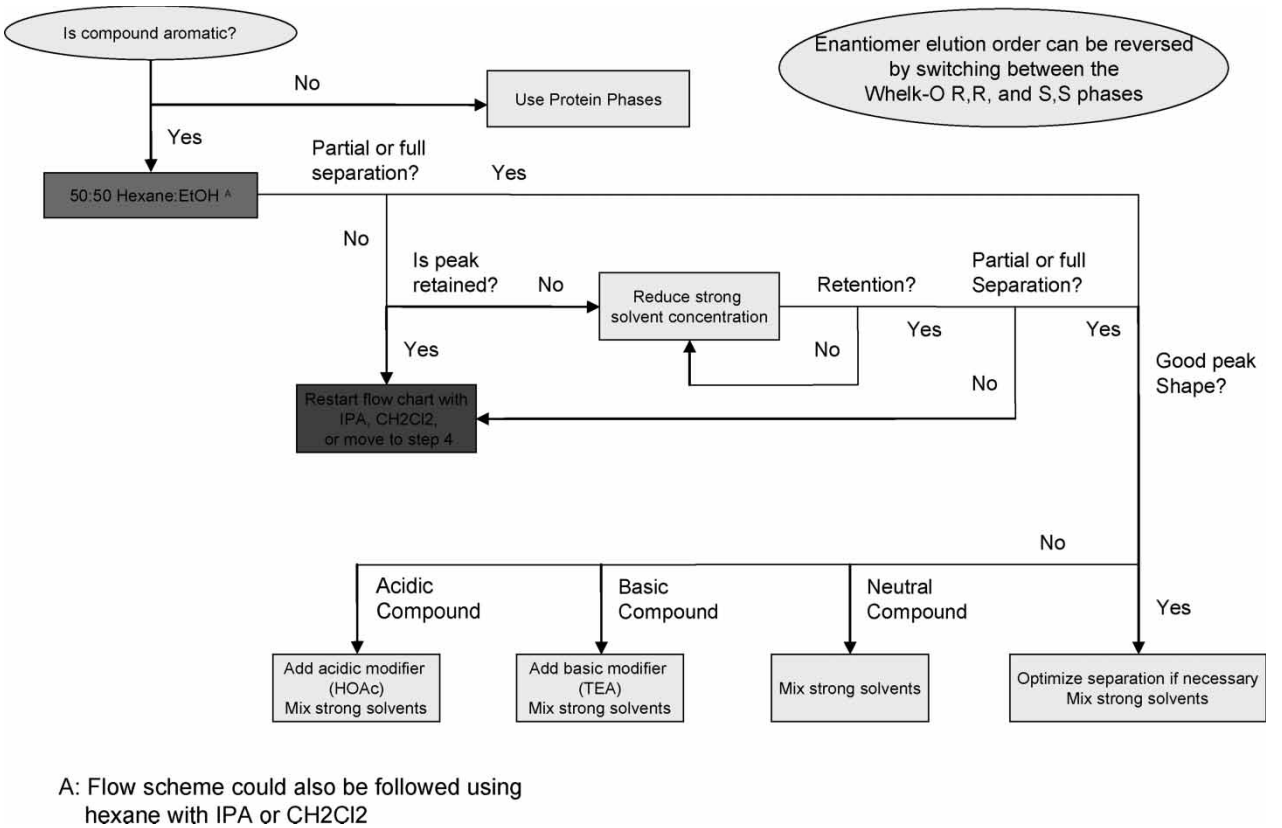


Figure 10. Step 5 of the HPLC chiral separation flow scheme.

of the mobile phase is adjusted. The separation data from our screen verified manufacturer claims that mobile phase pH has a dramatic effect on both analyte retention and isomer separation.[9,10] An example of the importance of pH can be seen in the separation of m-tyrosine isomers (Figure 9), where the only difference in the eluents is a buffer pH change from 3.0 to 4.5. The Chiral-AGP™ and ES-OVM columns successfully baseline resolved 39 of the 60 analytes tested. In many cases, the protein CSPs provided the only aqueous chiral separation in the screen.

The final step in our flow scheme employs either the (R,R) Whelk-O 1® or (S,S) Whelk-O 1® column. As mentioned previously, this brush phase and its sister column allow for the reversal of isomer elution order for a given chiral separation. Controlling enantiomer elution order is a powerful option, especially for preparative applications or for analytical methods where the measurement of the minor isomer is necessary in matrices where its enantiomer is much more prominent. Step 5 (Figure 10) begins by asking if the chiral analyte is aromatic in nature. Since the major enantioselective interaction between analyte and the Whelk-O 1® CSP is known to be between π-π electrons, the presence of an aromatic ring in the compound's structure is almost always required.[11] If the compound does indeed possess an aromatic ring, the analyst is directed to start with a mobile phase of 50% hexane/50% EtOH. If a baseline isomer separation is obtained, peak shape can be improved if necessary, with the addition of a mobile phase additive (HOAc for acids, TEA for bases). The band quality of neutral compounds can sometimes be improved with the addition of a miscible polar cosolvent in the eluent. A partial enantioseparation can sometimes be improved upon by reducing the EtOH content in the mobile phase if analyte retention is short. Additionally, the strong solvent could be changed to IPA or dichloromethane (CH_2Cl_2). Though our screen and flow scheme did not cover these modes, the Whelk-O 1® CSPs can also be employed using both polar organic and aqueous conditions. Step 5 successfully baseline resolved 9 of 60 compounds in the normal phase mode. The success rate would likely have been greater, however, if this CSP was assessed under aqueous conditions. Despite the column's limited success, the Whelk-O 1® was included in the flow scheme, due to its versatility as it pertains to multiple separation modes and elution order reversibility.

CONCLUSION

By employing a subset of the many chiral columns on the market and a limited selection of mobile phases, the successful baseline resolution of 59 of 60 nonproprietary chiral compound isomers was achieved in this study. Using experimental data from 35 CSP/mobile phase combinations (2100 injections), along with manufacturer guidance, a chiral flow scheme was crafted to provide the separation scientist with a detailed but easy to follow chiral

separation guide. The sequence of column/eluent combinations is ordered in such a way as to improve the probability of identifying a chiral separation using a reduced number of experiments. With the aid of this flow scheme, the potential exists for greatly reduced HPLC chiral separation development expense and time.

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