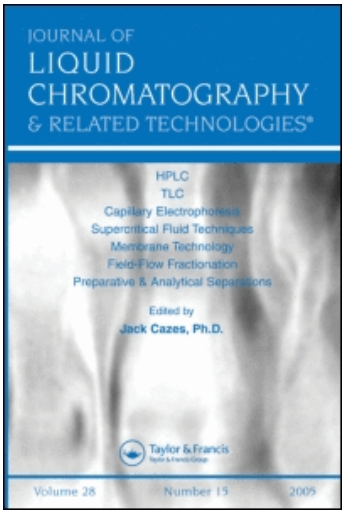

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Method Development Strategy and Applications Update for CHIROBIOTIC Chiral Stationary Phases

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To cite this Article Beesley, Thomas E. and Lee, Jauh-Tzuoh(2009) 'Method Development Strategy and Applications Update for CHIROBIOTIC Chiral Stationary Phases', Journal of Liquid Chromatography & Related Technologies, 32: 11, 1733 — 1767
To link to this Article: DOI: 10.1080/10826070902959489
URL: http://dx.doi.org/10.1080/10826070902959489

Journal of Liquid Chromatography & Related Technologies®, 32: 1733–1767, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070902959489

Method Development Strategy and Applications Update for CHIROBIOTIC Chiral Stationary Phases

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Abstract: Obtaining enantioselective separations using bonded macrocyclic glycopeptides has increased dramatically over the last several years. The products, trademarked CHIROBIOTIC phases, have advanced based on a host of publications shedding light on the mechanisms of interaction for a variety of diverse chiral analytes. This presentation will cover a brief history of these CSPs development, identify the mechanisms of interaction, and describe the mobile phases that drive those interactions. Details of operating conditions and a proposed method development protocol will be presented with validation statistics. Referenced publications will cover the period from 2004–2008 and will cover both chiral drug assays in the pharmaceutical area and chiral clinical assays utilizing a variety of detection methods. Column testing, storage, and trouble shooting tips have also been included to complete a thorough evaluation of what is known for successful operating conditions.

Keywords: Chiral drugs, Chiral separations, CHIROBIOTIC phases, Clinical LC/MS methods, Enantioseparations

INTRODUCTION

Current government FDA policy[1] regarding evaluation of drug stereoisomers specifies control procedures should be used to assure stereoisomeric

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composition of a product, with respect to identity, strength, quality, and purity. In addition, it is specified that techniques to quantify individual stereoisomers in pharmacokinetic samples should be available early. If the pharmacokinetic profile is the same for both isomers or a fixed ratio between the plasma levels of enantiomers is demonstrated in the target population, an achiral assay should suffice for later evaluations. This regulation has put tremendous pressure on the early creation of analytical chiral assays in drug development. Current chiral analytical tools have to provide broad potential to deal with the diversity of structures being developed and to accomplish this task in an efficient manner.

A comprehensive review of all chiral separation methods was recently published in 2008.[2] Macrocyclic glycopeptides were covered under three headings; capillary electrophoresis, high performance liquid chromatography and preparative. In this chapter, we will expand on the publications cited in that article and broaden the list while focusing primarily on the latter two techniques since they represent the more significant application areas.

Presented in 1994, by Dr. Daniel Armstrong,[3] the CHIROBIOTIC phases created from the bonding of various macrocyclic glycopeptides (antibiotics) offered the first truly innovative CSP, fully functional, as a result of the variety of interactive sites, in diverse mobile phase conditions, both aqueous and non-aqueous. The introduction was faced with two mental roadblocks; the notorious potential instability of antibiotics and the possibility for contamination of processed drug samples with antibiotic. Both of these anticipated problems proved inconsequential. In the first case, manufacture of the CSP by coupling to silica in 150°C solvents proved efficacious in producing a highly stable CSP through multiple linkages that has proven over the years to be as stable as the best C_{18}. In the second case, an LC/MS detection method, developed for the appropriate antibiotic, could never establish the presence of antibiotic under the most rigorous extraction treatment with no detectable amount down to 1ppb. Within the pH range of 3.0 to 7.0, the CSP remained stable for >2000 injections with no detectable bleed. The LC/MS method for the detection of the CHIRBIOTIC V and T is available from Supelco Inc.

Over the last 14 years, a growing base of applications, has further demonstrated the unique breadth and versatility, in a variety of mobile phase conditions, of these unique CSPs. This chapter will review progress over the last 5 years (2004–2008) in methods development, operational details for successful clinical assays as well as the applied results in published applications both in the literature and internally at the manufacturing facilities, method development laboratories. Of particular interest will be the complimentary nature of these phases in screening protocols against some of the most popular CSPs to extend successful 'hit' rates. Mining of a large database of chiral separations was conducted

by Alberto Del Rio and associates[4] from ChirBase, in an attempt to characterize the separation potential of several CSPs including cellulose and amylose phases, Whelk-01, Chiral-AGP and the CHIROBIOTIC T. The conclusion for the CHIROBIOTIC T was that this CSP is more specific and especially adaptive to more restrictive families of compounds. However, the test protocol that was used was reversed phase only and to that end, the conclusion is correct. Testing in a variety of mobile phase conditions is what produces the breadth of separations for these functionally complex stationary phases as opposed to the cellulose and amylose phases. In fact, more diversity would have been apparent just from utilizing the polar ionic mode. A second survey of the ChirBase database was much more useful in providing analytical conditions for optimal enantio-separation of 13 therapeutic classes of drugs.[5] Compounds were classified according to the Anatomical Therapeutic Chemical classification and published in two parts. Part I includes commercial CSP descriptions and the first six ATC classes; alimentary tract and metabolism to cardiovascular system. The analyst can also find at a glance the optimal CSP, mobile phase conditions and the commercial supplier.

STRUCTURE OF THE CHIROBIOTIC PHASES

Six patented, commercially available CSPs are produced from three basic antibiotics; vancomycin, trademarked CHIROBIOTIC V, teicoplanin, trademarked CHIROBIOTIC T and ristocetin A, trademarked CHIROBIOTIC R. There are two additional bonded versions of the vancomycin and teicoplanin referred to as CHIROBIOTIC V2 and CHIROBIOTIC T2 and a chemically modified version of the teicoplanin where the sugars have been removed, trademarked CHIROBIOTIC TAG.

The most distinctive feature of these phases is their chiral ionic character that incorporates both anionic and cationic functional groups. This strong, potential chiral mechanism especially for ionizable analytes, operates in both an anhydrous condition, the mobile phase referred to as polar ionic mode (PIM) and aqueous condition which is a not so typical (to standard RP) reversed phase system. For neutral molecules, a peptide chain consistent in all macrocyclics, supplies both hydrogen donor and acceptor sites, up to six sites of each type. In addition, chiral sugars of varying complexity are attached to each CSP in unique ways offering supplemental chiral hydrogen bonding sites. In the CHIROBIOTIC TAG, the sugars are removed to further enhance enantiorecognition for certain classes of compounds, especially those involving anionic and cationic sites. In addition, certain normal phase interactions are enhanced from the number of aromatic groups promoting π-π interactions.

The mobile phases that are useful to promote these interactions include the polar organic mode and typical normal phase. These mobile phases and their manipulation will be described in detail later. Shallow inclusion pockets aid the separation of chiral analytes in an aqueous mode in two ways; anchoring the molecule in the RP mode for chiral interaction with functional groups at the mouth of the cavity and recognition of structural changes in the included portion of the molecule to assist in recognition of metabolites and/or degradation products. A publication on the resolution of heterohelicenium dyes in the reversed phase mode on CHIROBIOTIC TAG exemplifies this point.[6] The elution pattern for this series of dyes indicates that the retention is not driven by hydrophobicity alone but that the size and shape of the analytes, that controls their affinity for the stationary phase. There are other hydrogen bonding donor and acceptor sites that further enhance the potential of these complex structures. The differences between each of the commercially available CHIROBIOTIC phases are subtle, based on the number of inclusion pockets (3 or 4) and interactive site types and distances. This fact makes them also complementary, a condition that allows a direct substitution of these CSPs in exactly the same mobile phase condition resulting in baseline resolution with no change in mobile phase composition. To further understand this concept of the complementary nature of these phases, see CHIROBIOTIC Handbook.[7] An evaluation and comparison of the CHIROBIOTIC T, the aglycone, CHIROBIOTIC TAG and a methylated version of the aglycone was conducted in both reversed phase and polar ionic mode.[8] Improved separation efficiences were observed for acidic analytes by methylation of the aglycone but the ionic/dipolar interactions between the carboxylate group of the analyte and the amine groups on the CHIROBIOTIC T or TAG were the most important for chiral discrimination. The polar ionic mode was shown to be the most powerful mobile phase for enantiomeric separations on CHIROBIOTIC T based stationary phases, mainly due to improved efficiency.

New versions of the CHIROBIOTIC V AND T were created to further enhance chiral selectivity and sample capacity for certain classes of compounds. The new versions are designated CHIROBIOTIC V2 and CHIROBIOTIC T2 since they are exactly the same ligand but are the result of changes in the silica backbone and the linkage chemistry. From a statistical viewpoint, the CHIROBIOTIC V2 with a readily available chiral carboxyl group, shows greater selectivity for many chiral amines and is the preferred CSP for any screening protocol over the standard CHIROBIOTIC V. However, there are a sufficient number of cases that can be reviewed in the chiral data base where the CHIROBIOTIC V demonstrated higher selectivity. To accomodate this situation, after the screening protocol is conducted, the corresponding CSP is evaluated in the optimization step to assess the selectivity response. So far, no case

has been observed where one of these versions showed no selectivity while the other version did. It is simply a matter of degree but in a great number of cases a significant degree. The CHIROBIOTIC T, on the other hand, remains the main focus in the screening protocol and the CHIROBIOTIC T2 then is evaluated in the optimization protocol. A case for this latter point is the citation of the CHIROBIOTIC T2 as the preferred CSP for the analysis of single amino acid differences or differences in the chirality of a single amino acid in a peptide.[9] Figures 1 and 2 demonstrate examples of the extent of change in selectivity.

Tesarova and co-workers[10] evaluated the performance of the CHIROBIOTIC V2 and V for a variety of 6 beta blockers and 8 profens in both the polar ionic mode and reversed phase modes. Higher retention and enantioselectivity was observed for all beta blockers in both solvent modes. In fact, baseline resolution of four of the beta blockers (8 enantiomers) was achieved in less than 15 minutes in the polar ionic mode on the CHIROBIOTIC V2. For the profens, however, the results did not follow the same pattern. The CHIROBIOTC V demonstrated better results, especially for flobufen, undoubtedly due to conformational changes of the CSP resulting from different bonding chemistry.

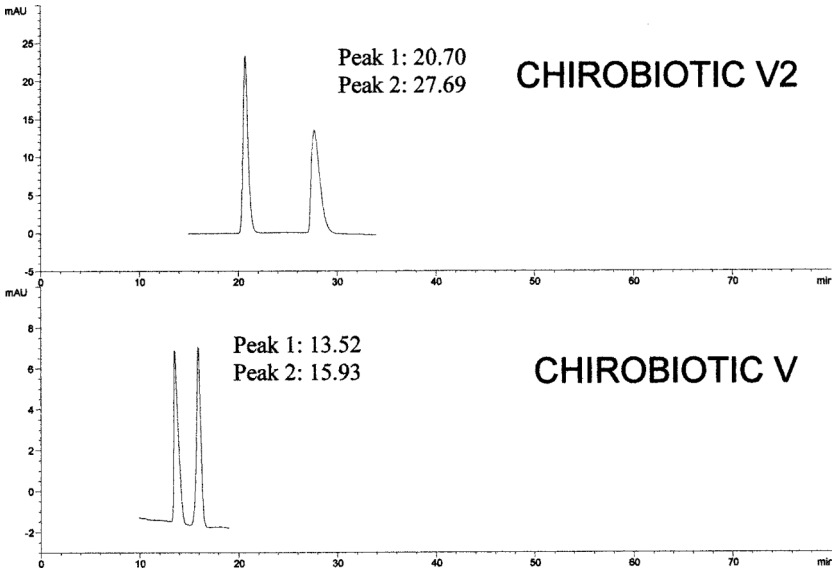

Figure 1. Enantio-separation of ritalin by CHIROBIOTIC V2 (250×4.6 mm) (top) and CHIROBIOTIC V (bottom), respectively. Mobile phase is 95/5, MeOH/20 mM NH_4OAc, pH 4.1 and the flow rate is 1 mL/min (ambient temperature).

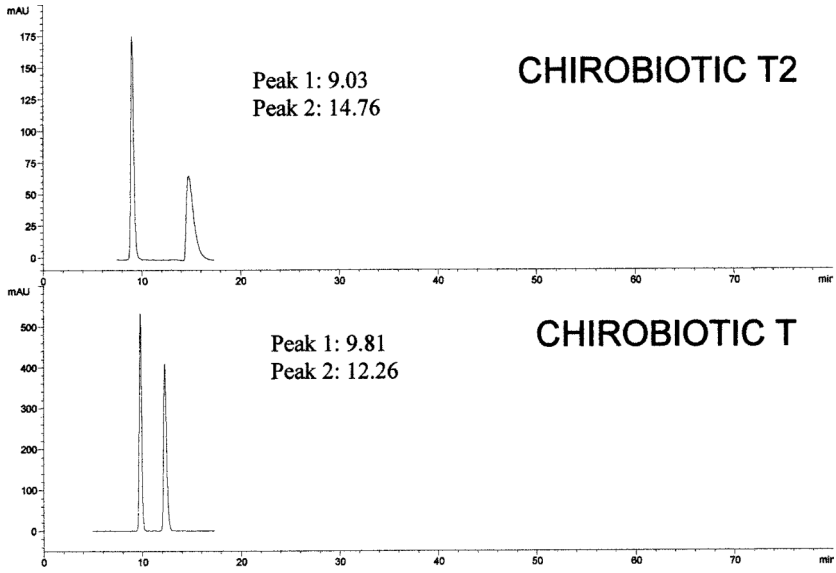

Figure 2. Enantio-separation of terbutaline by CHIROBIOTIC T2 (250×4.6 mm) (top) and CHIROBIOTIC T (bottom), respectively. The mobile phase composition is 100/0.1 w%, MeOH/NH_4TFA at 1 mL/min (ambient temperature).

CHIRAL RECOGNITION AS A FUNCTION OF MOBILE PHASE DESIGN

The functional group complexity of the CHIROBIOTIC phases offers unique opportunities for obtaining stable and reproducible chiral selectivity for a wide variety of compound classes suitable to their solubility and the specific needs of the assay. Pirkle's concept of a minimum of three points of interaction, combinations of either attractive or repulsive forces has been repeatedly demonstrated with these phases. In order to form a suitable diastereomeric complex the molecule must first be anchored nonchirally to a site within van der Waals radii of the chiral complexation site. In the Pirkle concept, this could be π-acid:π-base interaction like a dinitrophenyl group with a naphthylethylamine structure in a hydrocarbon-based solvent like heptane. With the CHIROBIOTICS for neutral compounds, the anchor options relate to inclusion into a hydrophobic cavity in a reversed phase mode or aromatic ring stacking to the phenyl rings (especially the chlorinated rings) in normal phase or hydrogen bonds to the peptide backbone in the polar organic mode. For ionizable molecules, the ionic functionalities of these CSPs offer very strong mechanisms that can function in both an aqueous mode and in

reagent grade methanol. In methanol, with small amounts of acid and base, this mobile phase focuses on the ionic nature of these CSPs and has been trademarked the polar ionic mode (PIM). The methanol can be replaced with typical normal phase solvent like heptane/IPA but the retention is then extended dramatically due to the influence of other hydrogen bonding groups that are typically masked by the methanol. In another example, for the separation of amino acids, the carboxyl group of the amino acid must be free for separation to occur. As an ester, the amino acid enantiomers will not be separated even though retention occurs under reversed phase conditions.

As can be seen, to drive these various interactions, a variety of mobile phase types had to be developed to promote the formation of an effective and efficient diastereomeric complex based on where the analyte is drawn. The following is a brief summary of the basic composition of these mobile phase types that drive appropriate chiral recognition mechanisms. The interactions cited are the minimum that have been identified and do not fully represent the complexity of what occurs but do represent the forces that are most dominant. It should also be noted that these mobile phases can be used sequentially on any of these stable CSPs in any screening protocol.

Description of Four Basic Mobile Phase Types

There have been four mobile phase types that have been characterized to drive certain mechanisms to achieve chiral recognition. They are:

1. *Polar Ionic Mode* (*PIM*): composed of 100 parts reagent grade methanol and 0.01 to 0.50 parts of anhydrous acid and base, typically, in equal parts. The acid is usually acetic acid and the base triethylamine. These components can at the point of optimization be transferred to specified percentage, by weight, of volatile salts like ammonium acetate or ammonium formate in methanol.
2. *Polar Organic Mode* (*POM*): combinations of methanol/ethanol or acetonitrile and either alcohol. Simple methanol is the best starting point.
3. *Normal Phase* (*NP*): composed of a hydrocarbon like hexane, heptane or isohexane and a polar constituent like isopropanol or ethanol. Another normal phase possibility is MtBE/ACN/MeOH for more polar neutral molecules. However, for the CHIROBIOTIC phases heptane/ethanol is usually the best starting point.
4. *Reversed Phase* (*RP*): composed of an organic solvent like acetonitrile or methanol and an aqueous buffer with pH control between 3.0 and 7.0. Unlike standard reversed phase, there are many cases methanol

demonstrated higher efficiency then acetonitrile for chiral separations on these CSPs. Both methanol and acetonitrile therefore must be tested. The choice of buffer is also important because it influences the inclusion of the hydrophobic portion of the molecule in the cavities of the CSP. Ammonium acetate has been one of the most useful buffer salts as well as the acetic acid and triethylamine as used in the PIM mode. The reversed phase mode also exhibits selectivity at both high and low organic solvent composition. This U shaped plot permits a choice of mobile phase composition that suits the needs of the assay and/or the solubility of the analyte.[11]

Description of Primary Mechanisms Driven by Four Mobile Phase Types

1. Polar Ionic Mode (PIM): ***Ionizable compounds only*** **(acids and bases):**
   a. Ionic
   b. Hydrogen bonding
   c. Steric/π-π
2. Polar Organic Mode (POM): ***Polar neutral molecules***
   a. Hydrogen bonding
   b. π-π
   c. Steric/Dipole
3. Normal Phase (NP): ***Nonpolar neutral molecules***
   a. Hydrogen Bonding
   b. π-π
   c. Steric/Dipole
4. Reversed Phase (RP): ***All compounds***
   a. Ionic
   b. Hydrogen bonding
   c. Steric/Inclusion/Hydrophobic

Resolution of Ionizable Chiral Analytes Only: Polar Ionic Mode (PIM)

This unique PIM mobile phase was developed to take maximum advantage of the chiral ionic character of these phases in an anhydrous condition and was designed to be most suitable for drug and clinical assays, especially on LC/MS platforms as well as preparative applications. Methanol is the basic component for this mobile phase, an ideal solvent for a wide variety of pharmaceutical compounds. To reagent grade methanol is added, in small amounts, anhydrous acid and base to affect interactions that occur with the anionic and cationic sites of the CSP. Acetic acid was found to offer the broadest window of selectivity along

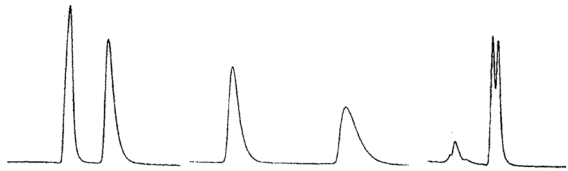

Figure 3. Acid/base ratio effects on the selectivity/retention in polar ionic mode (MeOH/HOAc/TEA). The analyte is mianserin and the column is CHIROBIOTIC V (250 × 4.6 mm).

with the base triethylamine. A typical composition, useful for screening purposes, is 100/0.1/0.1; v/v/v; MeOH/HOAc/TEA. Adjustment of the ratio of acid to base, often, further enhances selectivity see Figure 3. Favoring the acid is beneficial to enhancing the selectivity for chiral amines while favoring the base generally improves selectivity response to chiral acids like the profens. Once an ideal ratio is established, the typical range being from 4:1 to 1:4, then the total concentration of acid + base can be adjusted to affect retention as shown in Figure 4. As the

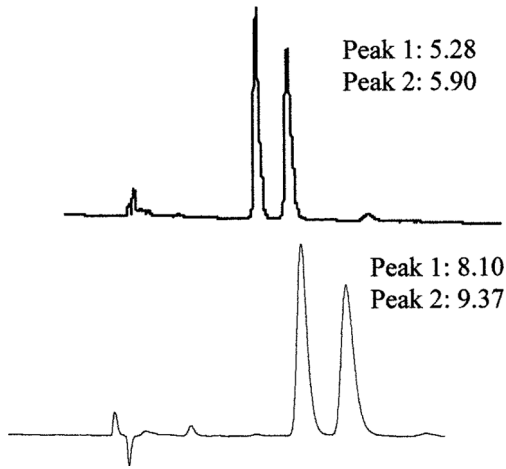

Figure 4. Additive concentration effects on retention in polar ionic mode. Higher ionic strength of additive gives shorter retention times and better efficiency. Top: 100/0.5/0.5, MeOH/HOAc/TEA. Bottom: 100/0.01/0.1, MeOH/HOAc/TEA. The column is CHIROBIOTIC V2 and the compound is oxamniquine.

concentration of the acid/base ratio is increased retention will decrease, conversely when the concentration is decreased retention will increase. This adjustment has a minimal effect on selectivity but can increase peak efficiency further enhancing enantioresolution.

The chiral ionic character of these CSPs has been found to be the dominant force for the enantioresolution of racemates such as profens and various chiral amines; primary, secondary or tertiary or amino alcohols (Figure 5). Since the CHIROBIOTICS are amphoteric, having both positive and negative charges, control with anions and cations is essential. While the additives, acetic acid and triethylamine have the broadest applicability for screening an appropriate concentration of a volatile salt can often be substituted for LC/MS platforms, preparative applications or other circumstances requiring a volatile salt as with an evaporative light scattering detector (ELSD). The salts found most appropriate for utilization with the MS platform are ammonium acetate that demonstrated higher selectivity for acidic racemates and ammonium formate found most useful for amines and as a generally applied salt as seen in Figure 6. Concentrations ranged from a low of 0.01 to a high of 0.5% by weight in methanol. For a quick study, a fast gradient can be used of 1% ammonium formate into methanol for most racemates.

Lehotay and co-workers,[12] working with a series of β-blockers of the aryloxyamino-propanol type established that the polarity of the CHIROBIOTIC V and T are equivalent while the CHIROBIOTIC TAG demonstrated enantioselective free energy 2–6 times higher for the compounds studied. The sugar moieties, therefore, decreased

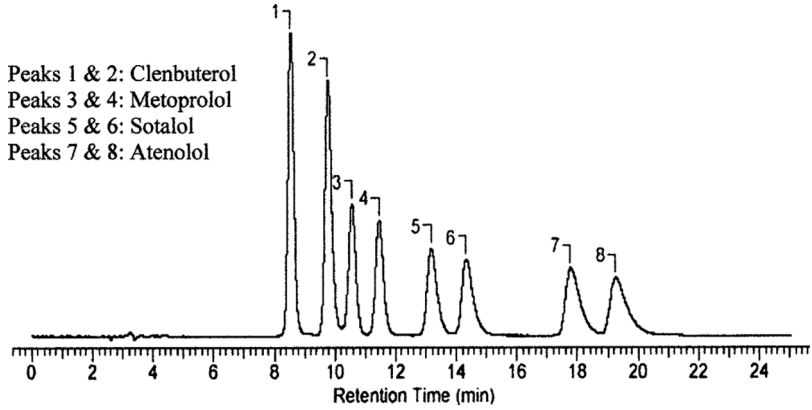

Figure 5. Simultaneous chiral separations of 4 beta blockers using CHIROBIOTIC T (250×4.6 mm in polar ionic mode. The mobile phase is 15 mM Ammonium Formate in CH_3OH, 1 mL/min (ambient temperature).

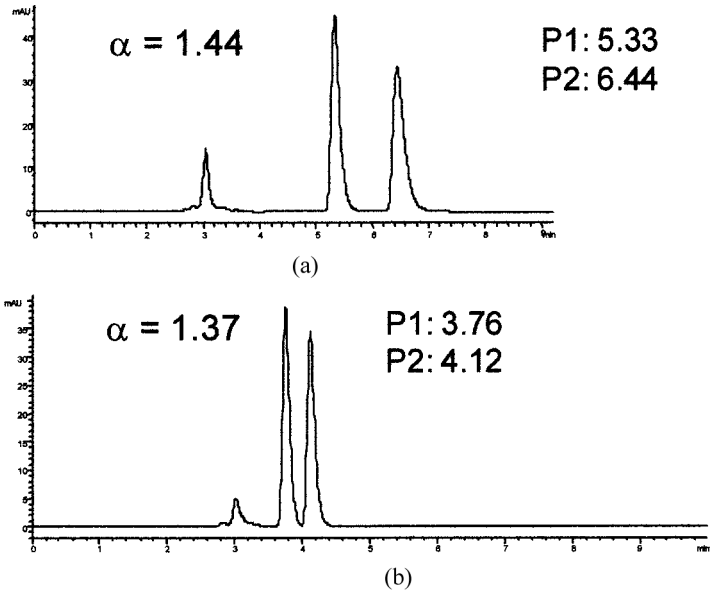

Figure 6. Salt effects on polar ionic mode using CHIROBIOTIC V2 (250 × 4.6 mm). Top: 100/0.1 w%: MeOH/NH_4TFA. Bottom: 100/0.1w%: MeOH/NH_4 formate. The sample is bupivacaine.

enantiorecognition for these types of β-agonists. The polar ionic mode was again the preferred mobile phase in this study.

Confirmation of this strong ionic effect was also observed when the racemate was fully charged by observing an ion repulsion effect where the first, minimally interactive enantiomer eluted ahead of the column void as seen in Figure 7. In these cases, adjustment of the apparent pH or an increase in the concentration of the additive eliminates this response (Figure 8).

There are cases, when ammonium trifluoroacetate was found to be the most efficient, usually dictated by the strength of the amine interaction with the CSP (Figure 9). It should also be noted, that on occasion elution order can be reversed on different CHIROBIOTIC columns under the same mobile phase conditions as seen in Figure 10.

Resolution of Neutral Chiral Analytes; Nonpolar and Polar: Normal Phase (NP) and Polar Organic Mode (POM)

With the availability of both π-π, dipole stacking, and a plethora of varying strength hydrogen bonding sites in these CSPs, neutral molecules

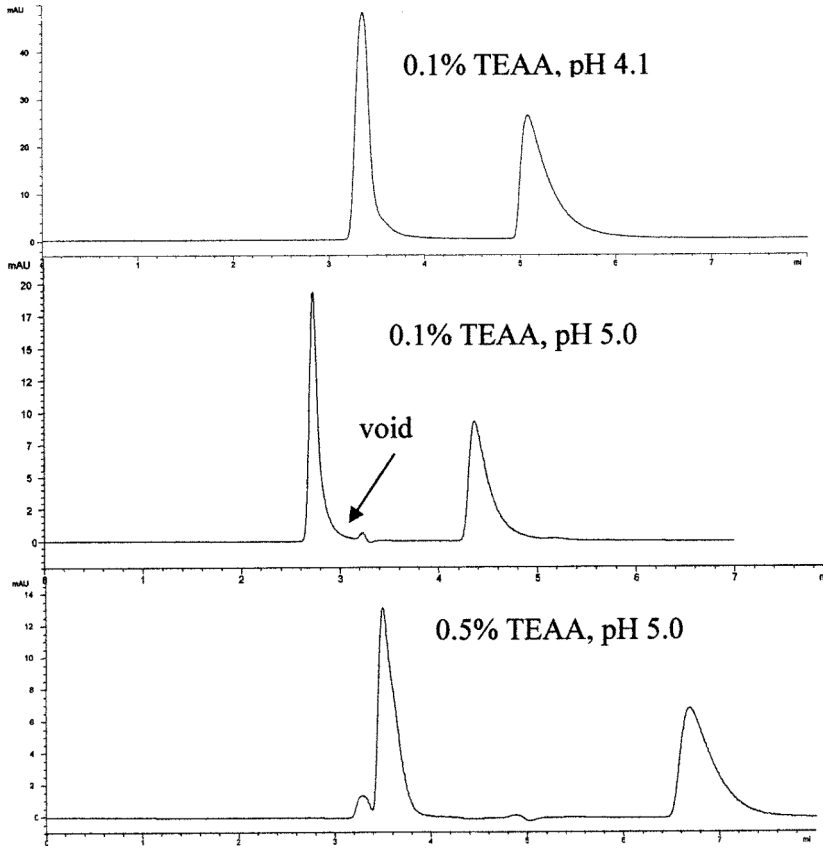

Figure 7. Ionic effects showing the repulsion phenomenon between CSP (CHIROBIOTIC T, 250 × 4.6 mm) and the analyte (mandelic acid) in 30/70, MeOH/TEAA. At pH 5 (0.1% TEAA), the first peak eluted before column void (3 mL). Higher concentrations (0.5% TEAA) of buffer can alleviate that effect.

can be resolved in two different mobile phase conditions depending on the polarity of the racemate in question. The plane of the aromatic groups within these CSPs however, does restrict the broad applicability of these phases in heptane/IPA type solvents unlike the much broader opportunities in the other mobile phase types. The more polar neutral molecules do well statistically in the polar organic mode which can be methanol or ethanol alone or in combinations or ACN with either of these alcohols (See Figure 11). The addition of MtBE in combination with acetonitrile/methanol has been an excellent method to enhance the influence of steric bulk and has been quite useful in these normal phase modes as seen in Figure 12.

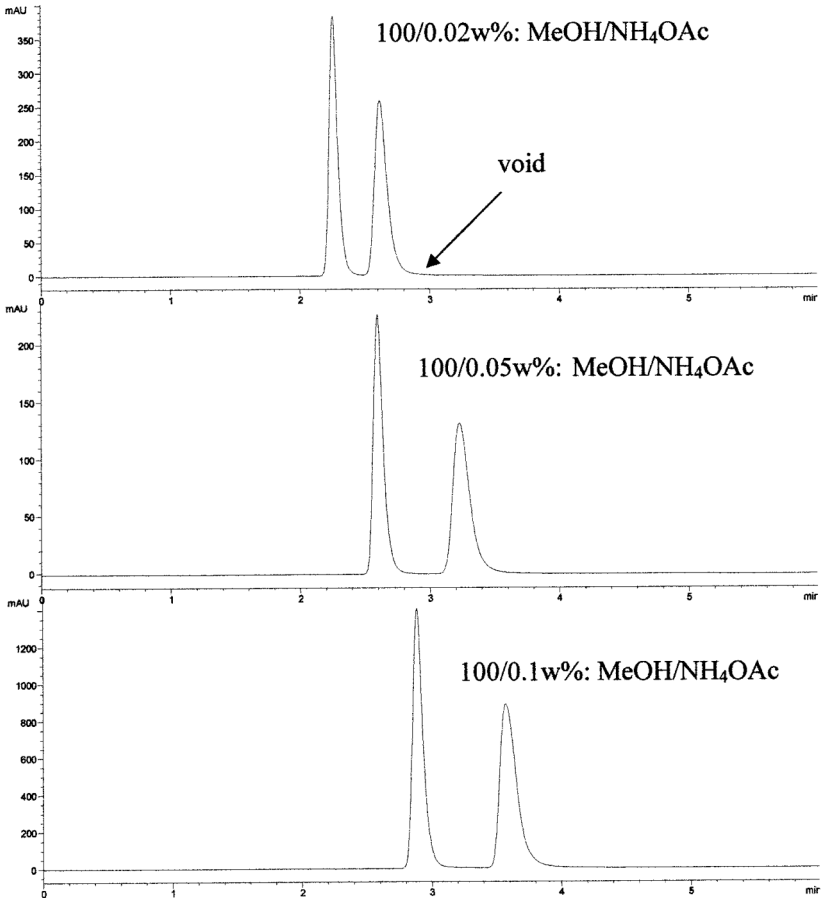

Figure 8. Another ionic repulsion phenomena between CHIROBIOTIC T (250 × 4.6 mm) and Dansyl methionine. Higher salt concentrations can reduce this response. The column void is 3.0 mL and the flow rate is 1 mL/min.

Resolution of All Types of Chiral Analytes: Reversed Phase Mode (RP)

Dominance of the ionic interaction for these ionizable compounds is also seen in the reversed phase mode (RP). Here, however, the inclusion mode offers unique opportunities for recognition of changes in the included hydrophobic portion of the molecule. Therefore, in addition to controlling the anion and cation concentration, the type of buffer used can be significant since many of these buffers can also affect the retention mechanism in the cavity. Several other operating parameters also offer

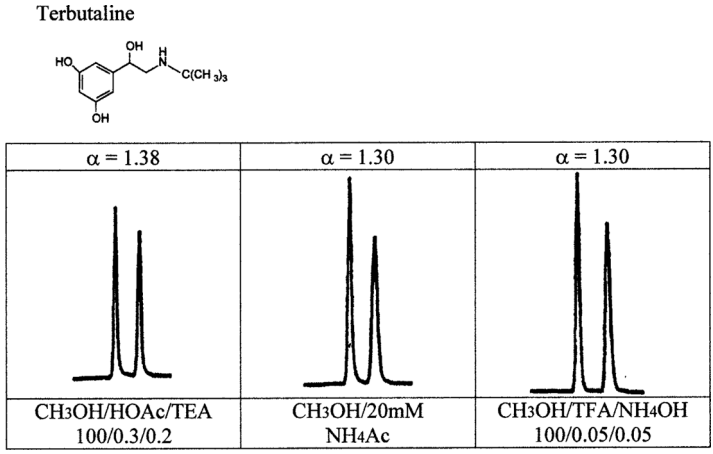

Figure 9. Effect of HOAc/TEA vs. NH_4TFA for terbutaline on CHIROBIOTIC T.

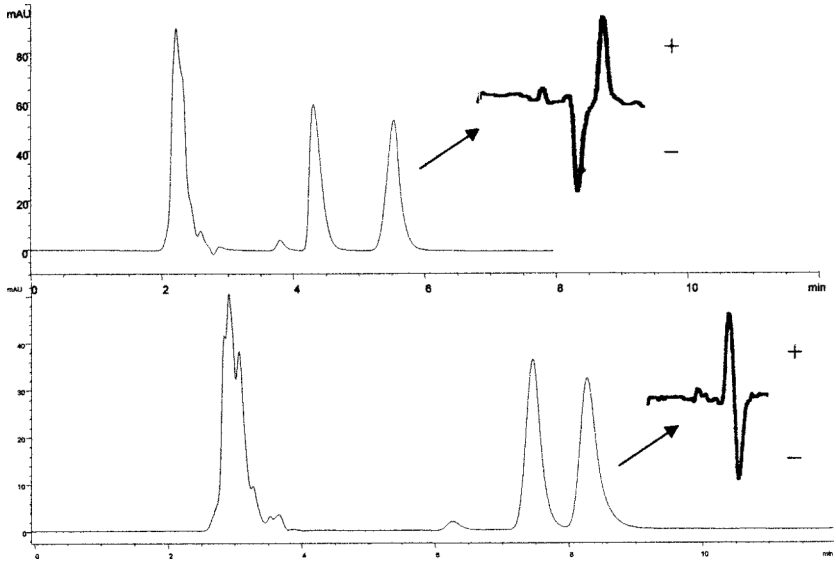

Figure 10. Reversal of elution order between CHIROBIOTIC V2 (top) and CHIROBIOTIC T2 (bottom) columns under the exact same condition. Mobile phase is 15 mM NH_4 formate in MeOH and the flow rate is 1 mL/min. The polarimeter showed the first peak is (−) on the CHIROBIOTIC V2 while the first peaks is (+) on the CHIROBIOTIC T2.

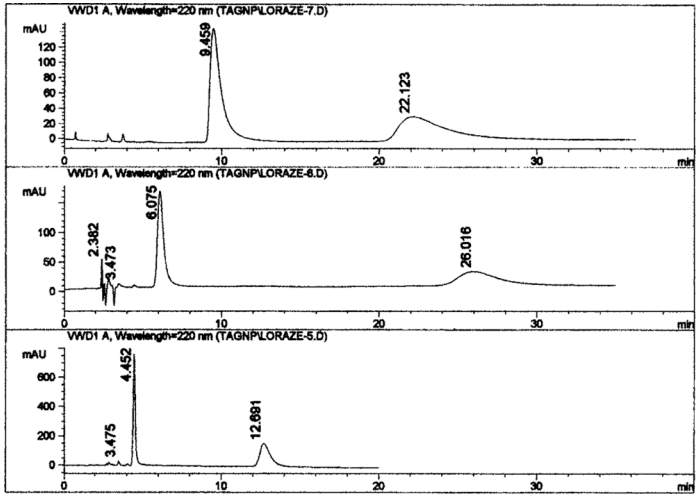

Figure 11. The separation of lorazepam using single solvent system by CHIROBIOTIC TAG (250 × 4.6 mm). The solvents are acetonitrile (top), ethanol (middle) and methanol (bottom), respectively. The flow rate is 1 mL/min.

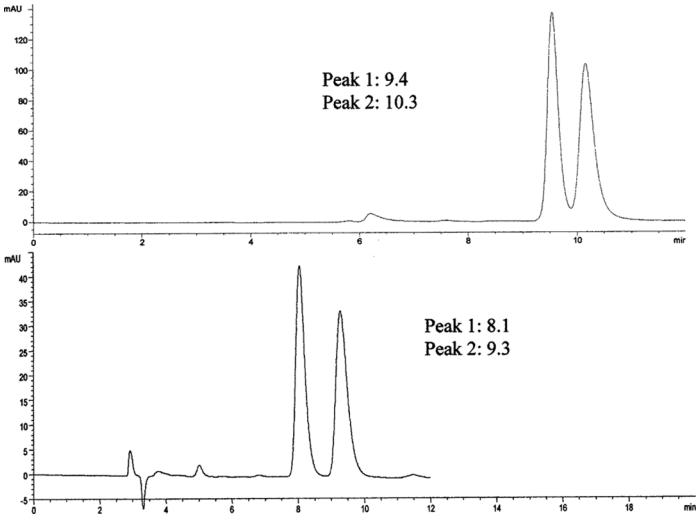

Figure 12. Methyl phenyl sulfoxide separation by CHIROBIOTIC V (250 × 4.6 mm). Top: 20/80, THF/20 mM NH_4NO_3. Bottom: 97/2/1/, MtBE/ACN/MeOH.

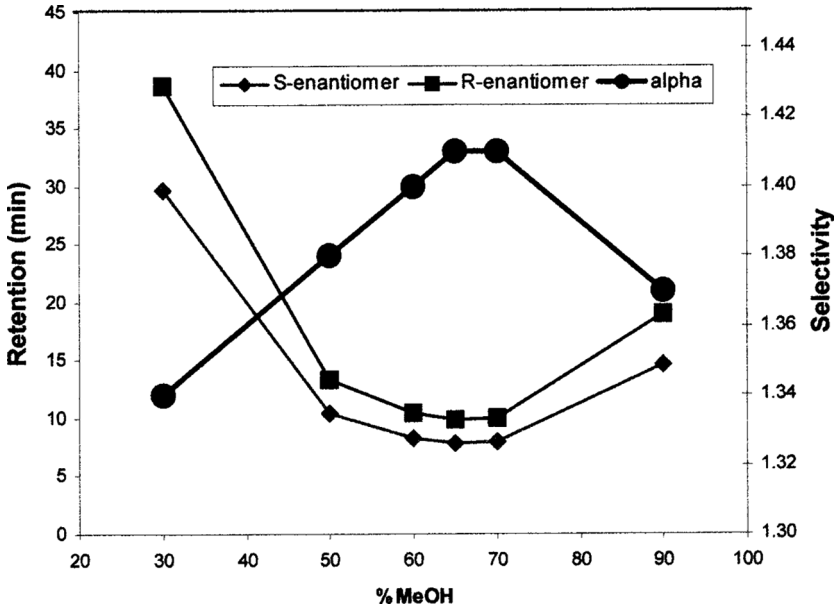

Figure 13. U-shape retention profile of fluoxetine on CHIROBIOTIC V2 (250 × 4.6 mm) using 20 mM NH_4OAc (pH 4) as a buffer in reversed phase mode. The highest selectivity is obtained at the lower retention that is at about 70% methanol.

unique opportunities. Changing from an reversed phase composition, high in aqueous buffer to one high in organic composition revealed a U shaped plot (Figure 13) with chiral selectivity at both extremes, useful for MS as well as preparative applications.[11] Another, very unusual, but extremely useful phenomenon in this mobile phase is the effect of flow rate. Decreased flow rate typically enhances chiral resolution with only a modest increase in retention. See additional information in the section dealing with Sample Injection.

SPECIAL OPERATING PARAMETERS AND CONDITIONS

Sample Injection

Solvent

The best choice for dissolution of a sample is the mobile phase in which the sample is to be run. It is possible for the majority of samples to be

dissolved in pure reagent grade methanol as the primary solvent but in those cases when weak interactions occur, the sample peaks may be distorted by this slight change in mobile phase composition, especially in normal phase conditions. Other reported sample solvents have included acetonitrile and ethanol. When using ethanol it must be free of acid and polar additives that can influence the separation. Once mobile phase conditions are established the sample dissolution solvent could be tested separately from the above choices.

Concentration and Volume

Typical sample concentrations are in the range of 0.1–1.0 mg/mL. It is best to start with the minimum amount of sample until the response to overload and peak shape with the chosen detector are determined. An "on column" sample concentration of 1–5 micrograms is a good starting point, again, depending on the requirements of the chosen detector. For the CHIROBIOTIC phases it is better to inject a large volume of a dilute sample rather then a small volume of a concentrated sample. A 5 μL injection of a 1.0 mg/mL sample is a good starting point. In other cases, the load volume and concentration may be 5 to 10 times higher without effecting resolution.

Flow Rate

This operating parameter offers some advantages not seen in standard liquid chromatography. For standard chromatography the optimal linear velocity in terms of optimal efficiency tends to be 1 mm/sec or 1.0 mL/min for a 4.6 mm id column. In the reversed phase mode, regardless of which CHIROBIOTIC phase you use, selectivity/resolution has been seen to increase with decreasing flow rate as low as 0.2 mL/min, dependent on the compound being analyzed. The phenomenon appears to be a function of the strength of the inclusion complex in the hydrophobic cavity of these CSPs and the general polarity of the analyte under study. The greater the polarity of the analyte surrounding the hydrophobic portion that is to be included in the cavity the more beneficial is a slower flow rate.

While decreased flow rate has a diminished effect on resolution for the polar ionic mode or polar organic mode, it does offer a simple solution to modestly increasing enantioresolution by increasing efficiency. This increase in resolution with decreased flow rate does not exist at all with typical normal phase modes. See Figure 14.

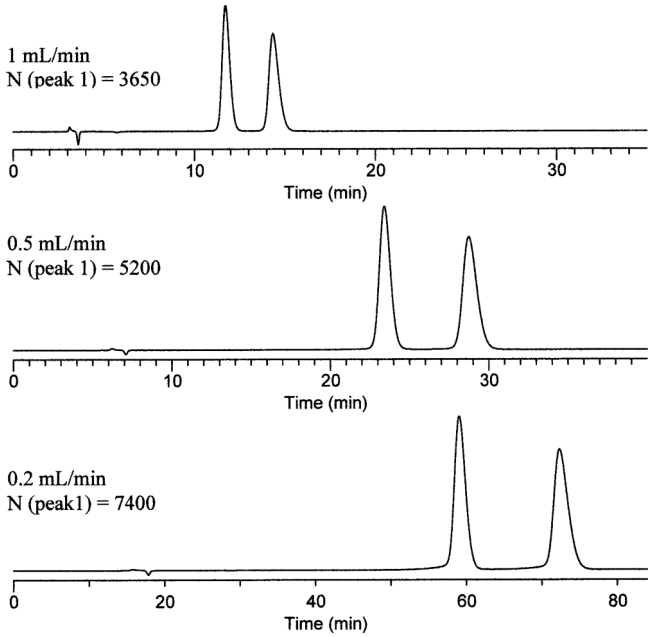

Figure 14. Flow rate effects on the separation of fluoxetine in polar ionic mode (100/0.1/0.1, MeOH/HOAc/TEA) by CHIROBIOTIC V2 (250 × 4.6 mm). The peak efficiency is still increasing even at 0.2 mL/min.

Temperature

A great deal has been written on the effect of temperature in chiral separations. The most comprehensive paper written specifically for the CHIROBIOTIC phases occurred in 2004.[13] Conclusions were drawn that reduced temperature can increase enantioresolution without a large increase in retention. If sufficient selectivity is obtained, increased temperature can reduce tailing of the most retained enantiomer, further increasing enantioresolution and finally low temperature can permit the effective separation of rotomers (0°C) or stabilize induced temperature interconversion (15°C) for compounds like the diazepins as seen in Figure 15. For the separation of seven aryl-substituted β-lactams, Berkecz et al.[14] stated that optimization to baseline on the CHIROBIOTIC TAG was more quickly obtained for these compounds by adjusting temperature than making mobile phase adjustments.

In another study, four macrocyclic glycopeptides were evaluated for the separation of phenylcarbamic acid derivatives.[15] The enantiomers were separated isothermally in the range of 0–50°C with 10°C

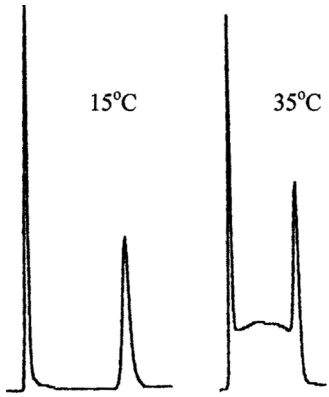

Figure 15. Temperature effect of oxazepam separation by CHIROBIOTIC T. Mobile phase is pure methanol at 0.9 mL/min. On column racemization occurs at elevated temperatures for some compounds.

increments. Thermodynamic properties were calculated and evaluated for; enthalpies (ΔH), entropies (ΔS) and Gibbs energies (ΔG) of transfer. The study shows that the polar ionic mode can produce enthalpy as well as entropy driven separations. A methylated version of the CHIROBIOTIC TAG demonstrated that enthalpy was the driving force for the separation of these compounds while entropy is more important for the CHIROBIOTIC V CSP. The response of temperature to all classes of molecules is not consistent. For the baseline resolution of 10 chiral sulfoxides in the polar organic mode (100% MeOH) on the CHIROBIOTIC TAG, the ln k versus1/T and ln α versus 1/T were linear for all enantiomers.[16] All chiral sulfoxides showed the (S) enantiomer eluting first at all temperatures and the attractive and steric interactions play very important roles during enantioseparation of sulfoxides.

Detection Methods

The benefits of the CHIROBIOTIC CSPs manifest themselves also in the potential choice of detector options due in large part to the flexible composition of mobile phase components and the various options for volatile salt components. In the clinical application section of this text we have extracted from the literature a variety of detector choices. One of the most frequently cited applications is the LC/MS platform with the polar ionic mode. The ease of method validation by the addition, by weight,

of a volatile salt like ammonium formate, ammonium trifluoroacetate or ammonium acetate to methanol makes this an attractive methodology along with the increase detection sensitivity and speed of method development.

For amino acid analysis, the CHIROBIOTIC T or TAG utilizing simple alcohol/water or acetonitrile/water mixtures depending on the polarity of the amino acid, allows for the detection of these types of analytes with very low UV or ELSD type detectors. See Supelco amino acid handbook for further details.[9]

METHOD DEVELOPMENT PROTOCOL

Proposed Chiral Screening Protocol

During the early part of this decade, a great deal of work went into the development of chiral screening protocols in an effort to increase proficiency in obtaining chiral separation methods. Four columns screens to as many as 12 were evaluated experimentally to find the optimum protocol to produce hit rates in the high 90 percentile. From all the published work, a configuration of eight columns seems to be the best methodology but the combination of columns depended on the drug development platform that was being used for drug discovery in the particular company. It has been widely stated, however, that the combination of cellulose and amylose phases in combination with the CHIROBIOTICS and CYCLOBOND (cyclodextrin derivatives) has produced hit rates of $>96\%$ in a number of companies.[17] The CHIROBIOTIC and CYCLOBOND phases are considered complementary to each other providing a minimum of overlap in stereoselectivity.

Supelco, Bellefonte, PA has produced a Methods Development Chart revised from that originally developed at Astec for the screening of racemates with four CHIROBIOTIC phases and four CYCLOBOND phases in 8 sequential mobile phase conditions to produce high hit rates (see Figure 17).

Evaluation Statistics of Chiral Screening Protocol

In the evaluation process, as a final test, 40 racemic switches were chosen to cover a broad range of racemate types. The following table lists the compounds and the CSPs showing best resolution from the results of the screen.

CHIROBIOTIC Phases

Compound	Column:resolution	Compound	Column:resolution
Albuterol	T2:2.8	Naproxen	V:1.5
Amlodipine	V2:1.5	Nefopam	V:1.5
Bupivacaine	V2:2.5	Nicardipine	V:4.5
Carnitine	TAG:1.8	Omeprazole	R:1.6
Citalopram	V:1.5	Oxamniquine	V2:2.2
Clenbuterol	T2:2.5	Cis-permethrin	TAG:2.5
Fluoxetine	V2:2.5	Propranolol	T:1.9
Ibuprofen	V:1.5	Pseudo-ephedrine	T2:1.7
Isoproterenol	T2:3.3	Sotalol	T:1.5
Ketoprofen	R:3.0	Terbutaline	T2:7.0
Lercanidipine	V:1.5	Thalidomide	V:7.0
Lorazepam	T:11	Tolperisone	V2:2.7
Methylphenidate	V2:4.0	Trimipramine	V2:2.3
Metoprolol	T:2.0	Warfarin	V:3.0
Miaserin	V2:2.8		
Mosapride	V:3.3		

Of forty racemic switch drugs tested in this study:
The success rate for CHIROBIOTIC phases is $\sim$ 80%.
The success rate for CYCLOBOND phases is $\sim$ 40%.
Eight compounds can be separated by both types $\sim$ (20%).
Resolution range:
CYCLOBOND: 1.5 to 2.8
CHIROBIOTICS: 1.5 to 11.0

APPLICATIONS: REFERENCED PERIOD 2004–2008

Chiral Assays Listed by Compound Class

Methods for assaying chiral compounds are continually being developed on CHIROBIOTIC phases and the reasons cited are typically, recognition of metabolites, degradation products, speed of analysis and resolution. The following examples from the literature are listed alphabetically by compound type.

Primary Amino Acids. A direct enantioselective HPLC method for the determination of pure L-arginine was developed on the CHIROBIOTIC T (18). The reversed phase system consisted of methanol/50 mM sodium dihydrogen phosphate, pH 4.6; 2/8;v/v. Analysis at 214 nm allowed for the determination of 0.0025% (w/w) of D- arginine. Threonine that

has demonstrated a wide range of physiological activity has been assayed from different sources on the CHIROBIOTIC T using APCI and ESI detection methods yielding excellent sensitivity down to 10 ng/mL.[19] The adsorption behavior of L,D-threonine and L,D-methonine were investigated under non-linear isotherm conditions on CHIROBIOTIC T[20] Methanol, ethanol, 2-propanol and acetonitrile were investigated in the reversed phase mode indicating a heterogeneous adsorption mechanism of the amino acids strongly affected by the nature of the organic modifier. The values of the isotherm coefficients decreased with increase of the water content and after reaching minimum increased again, exhibiting a U-shaped dependency on the modifier content. Adsorption of water increased with the increasing carbon chain length of the alcohol. This study suggested a two-site mechanism of adsorption of amino acids; low energy sites involving weak polar and apolar interactions and high energy sites involving strong ionic interactions.

Secondary Amino Acids. A method for the direct separation of 18 unnatural amino acids was reported including several β-3-homo-amino acids.[21] All 6 CHIROBIOTIC phases were tested: V2/V, T/ T2, TAG and R. The effects of organic modifier, mobile phase composition and pH were investigated. The CHIROBIOTIC T2 was cited as the best CSP for the β-3-homo-amino acids. The elution sequence was determined for some samples indicating the CHIROBIOTIC R demonstrated a reversal of elution order from the other CSPs. A comparison of the CHIROBIOTIC T and TAG for the separation of secondary amino acid analogs found that the TAG yielded better results, the retention being $S < R$.[22] This work was also verified by a study of 15 unnatural conformationally constrained amino acids and 12 β-amino acids having cycloalkane or cycloalkene skeletons.[23] A method for the analysis of pharmaceutical grade propionyl carnitine was developed on the CHIROBIOTIC TAG in the reversed phase mode.[24] This method coupled an SCX column to the CSP to remove related impurities to obtain an ee of 98.9% for the drug substance.

Derivatized Amino Acids. A study on the effect of derivatization for amino acid enantiomer resolution using the CHIROBIOTIC T was reported.[25] It was found that the size of the analyte or tagging reagent had a great influence on enantioselectivity. Specifically, the π-π interaction was enhanced by derivatizing the aromatic of the tagging reagent with electron withdrawing groups. The enantioresolution of a series of unsaturated N-methyoxycarbonyl-α-H-α-amino acids was demonstrated on the CHIROBIOTIC T, V and R in both reversed phase and polar

ionic modes.[26] The best results in terms of selectivity and resolution were obtained on the CHIROBIOTIC R phase with the polar ionic mode giving the highest resolution per minute. The assay was applied to monitoring the enantioselectivity of alcalase reactions using N-FMOC-α-H-α amino acid esters as substrate. In another report, direct enantioseparations of seven N-fluorenylmethoxycarbonyl-amino acids was demonstrated on the CHIROBIOTIC T[27] This latter paper also compared the retention behavior and enantionseparation of N-FMOC-Val and its non blocked analog. The polar ionic mode was studied for these separations noting the effects of acetic acid and triethylamine and its concentration on enantioseparation for these derivatized amino acids as well as temperature and flow rate effects. The developed method has been successfully applied to the determination of optical purity of some derivatized amino acids.

β-Agonists (Beta Blockers)

A number of publications have cited the use of the CHIROBIOTIC phases for the enantioseparation of this class of drugs. Bambuterol and albuterol, a long acting and a short acting β-agonist drug, were tested on several cellulose and amylose CSPs and the CHIROBIOTIC V, Whelk-01 and Ultron ES-OVM.[28] For bambuterol, the ChiralPak AD was chosen in a typical normal phase mode while the CHIROBIOTIC V gave optimal results for the albuterol in the polar ionic mode. Validation of the methods after an investigation of the mobile phase components, demonstrated a selective and linear detector response for both pairs of enantiomers. In a subsequent paper in 2007, six β-agonists were evaluated on three CHIROBIOTIC phase, V, T, and R in three different mobile phase conditions; RP, NP and PIM.[29] In this study, all six beta blockers; bambuterol, clenbuterol, clenproperol, furnoterol, mabuterol and terbutaline were resolved in the polar ionic mode requiring both CHIROBIOTIC T & V to resolve all six racemates. A validated method for the screening, confirmation, determination and quantitation of salbutamol was reported[30] utilizing a 250×4.6 mm CHIROBIOTIC T in the polar ionic mode: 60/40/0.3/0.2; ACN/MeOH/HOAc/TEA at a flow rate of 1.2 mL/min. Detection was 276 nm UV to obtain a linear range for quantitation of 0.125–1.5 mg/mL.

Fused Polycycles

For the enantioseparation of 13 new chiral polycycles, both cyclodextrin based and macrocyclic glycopeptides chiral stationary phases were

evaluated in normal phase, reversed phase and polar organic modes.[31] The most selective CSPs were found to be the CYCLOBOND 1 2000 DM and CYCLOBOND I 2000 RSP on which 11 were baseline resolved. The macrocyclic glycopeptides showed selectivity for only two of these racemic polycycles with very high enantioselectivity on both the CHIROBIOTIC T and TAG, separated in both reversed phase and normal phase modes. The reversed phase mode was the best mobile phase conditions for separation consistent with the concept of utilizing steric bulk as a mechanism when using the cavity of these phases. This was further substantiated by the fact that the resolved enantiomers were more highly substituted. For the separation of pterocarpans, another class of fused rings, a similar situation was reported[32] but, in this case, the CHIROBIOTIC R demonstrated the highest resolution ($\sim$7.1) along with the CHIROBIOTIC V again in a methanol/water, reversed phase mode.

Peptides

Peptides with one or more amino acid differences, or the chirality of a single amino acid have been reported separated on CHIROBIOTIC CSPs (33). Forty-two peptides from 11 families were resolved in the reversed phase mode using ESI-MS compatible mobile phases. A review of the separation of amino acids and small peptides on macrocyclic glycopeptides indicated that the CHIROBIOTIC phases are complementary to one another for the separation of unusual amino acids, native as well as derivatized amino acids and small peptides.[34] HPLC coupled to APCI was used for the separation of amino acids and peptide enantiomers.[35] APCI was and order of magnitude greater sensitivity for 25 amino acids down to 250 pg and for low mass peptides. As the chain length of the peptide increased >300Da, ESI proved to be more ideal. A mobile phase of 1% ammonium trifluoroacetate in methanol and 0.1% formic acid in water increased the sensitivity of the APCI method significantly.

Other

The first reported enantiomeric separation of hypericin, a naturally occurring perylene quinone with recently discovered light-induced biological activity was reported separated on the CHIRBIOTIC TAG in 1% triethylamineacetate in methanol.[36] This separation method was also used to prepare the first recovered pure enantiomers of hypericin.

Clinical Chiral Assays Listed by Detection Method

LC/MS

A comprehensive review on the use of chiral liquid chromatography-tandem mass spectrometry was published for the characterization of the drug metabolism and pharmacokinetic profiles of enantiomers.[37] The utility of the CHIROBIOTIC phases especially in the polar ionic mode and reversed phase mode was analyzed from a number of cited studies. Various ionization techniques including electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric photoionization (APPI) coupled to chiral chromatography are described in terms of their ionization efficiency, matrix effects and limitations. Examples were chosen to demonstrate the applicability of these methods for enantionselective bioanalysis. In addition, a report on transforming chiral HPLC methodologies into more sensitive LC/MS methodologies without losing enantioselectivity was published for 19 pharmaceutical compounds of interest on CHIROBIOTIC T, V or R.[38] A rather unique and environmentally friendly solvent for normal phase applications on the CHIROBIOTIC phases in conjunction with APCI and ESI MS detection modes was reported.[39]

a. Benidipine in human plasma.[40]
   CHIROBIOTIC V (150 × 4.6 mm, 5μ)
   Mobile phase: 100/0.01/0.0001; v/v/v MeOH/HOAc/TEA
   Flow rate: 1.0 mL/min
   LC/MS: positive ion mode
   Coefficient of variation for the assay was cited as<8% and the detection limit was 0.05 ng/mL for 1 mL plasma.
b. Propranolol and hyoscyamine in human plasma.[41]
   CHIROBIOTC V (250 × 4.6 mm, 5μ)
   Mobile phase: 100/0.05/0.04 to 0.1 (25 min gradient); MeOH/HOAc/TEA
   Flow rate: 1.0 mL/min
   LC/MS: APCI mode
   Temp: 30°C
c. Lactic acid (urine).[42]
   CHIROBIOTIC TAG (150 × 2.1 mm, 5μ)
   Mobile phase: 83/17/0.12/0.30; EtOH/H_2O/HOAc/TEA
   Flow rate: 0.2 mL/min
   LC/MS/MS
   Temp: 25°C
d. Ifosamide/metabolites (3) in human plasma.[43]
   CHIROBIOTIC T2 (250 × 4.6 mm , 10 μ)

Mobile phase: 2-propanol/methanol; 60/40, v/v
Flow rate: 0.5 mL/min
LC/ESI, SIM mode.

e. Previous to 2004, a series of papers were published utilizing CHIROBIOTIC phases in MS. A summary of these LC/MS applications is available form Supelco.[44]

UV

CHIROBIOTIC phases have had excellent success in developing useful assays with UV detection. The ability to resolve drug metabolites, degradation products and excepients due the wide variety of hydrogen binding sites in these phases has led to their success.

a. Antipsychotic: butaclamol[45]
CHIROBIOTIC V (250 × 4.6 mm, 5μ)
Mobile Phase: MeOH/HOAc/TEA; 100:0.2/0.05
Flow rate: 0.5 mL/mim.
UV: 262 nm
Temperature: ambient
b. Mabuterol (Rat plasma).[46]
CHIROBIOTC V (150 × 4.6 mm, 5μ)
Mobile phase: 100/0.1/0.1, MeOH/HOAc/TEA, v/v/v
Flow rate: 1.0 mL/min
UV: 254 nm
Temp: 25°C
c. Methotrexate in human plasma.[47]
CHIROBIOTC T (250 × 4.6 mm, 5μ)
Mobile phase: 100/0.2/0.1, MeOH/HOAc/TEA
Flow rate: 0.8 mL/min
UV: 303 nm
Temp: 20°C
d. Bufuralol in human plasma[48]
CHIROBIOTC V (150 × 4.6 mm, 5μ)
Mobile phase: 100/0.015/0.01, MeOH/HOAc/TEA
Flow rate: 0.5 mL/min
UV: 254 nm
Temp: 25°C
e. Trimebutine, lafutidine and ondansetron.[49]
CHIROBIOTIC V2 (250 × 4.6 mm, 5μ)
Mobile phase: 100/0.075/0.025, MeOH/HOAc/TEA
Flow rate: 1.0 mL/min
UV: 254 nm
Temp: 20°C

f. Duloxetine.[50]
CHIROBIOTIC V (150 × 4.6 mm, 5μ)
Mobile phase: 100/0.04/0.01; MeOH/HOAc/TEA
Flow rate: 1.0 mL/min
UV: 214 nm
Temperature: 17°C
LOD: 0.06μ/mL

Fluorescence

a. Bisoprolol in human plasma.[51,52]
CHIROBIOTIC T (150 × 4.6 mm, 5μ)
Mobile phase: 100/0.02/0.025; MeOH/HOAc/TEA
Flow rate: 1.5 mL/min
Fluorescence: 275/305 nm
Temp: 25°C
b. Selenomethionine in breast and formula milk.[53]
CHIROBIOTIC T (250 × 4.6 mm,10μ)
Mobile phase: water
Flow rate: 1.0 mL/min
Atomic fluorescence spectrometry
Detection limits: 3.1 ng/mL Se

ELSD

a. Eflornithine in human plasma.[54]
CHIROBIOTIC TAG (250 × 4.6 mm, 5μ)
Mobile phase: 25/75, EtOH/0.01 M HOAc + TEA
Flow rate: 1.0 mL/min
Temp: 25°C

Preparative Applications

CHIROBIOTIC phases have been employed in all phases of medium to large scale chiral separations including direct elution, stacked injections, simulated moving bed (SMB) and SFC. Since these phases have no solvent limitations, separations can be optimized for sample solubility.

Direct Elution: Batch Chromatography

Applications have been largely of two mobile phase types; polar ionic mode and reversed phase mode. The PIM mode lends itself nicely to

stacked injections based on typical speed of analysis and peak efficiency. Recovery is a matter of evaporation. If acetic acid along with the triethylamine are used as additives, then final extraction with an immiscible solvent will help to remove the acetic acid. An alternative method is to pass the sample through a small column of silica in a minimum amount of mobile phase or methanol then ethyl ether can be used to elute off the acetic acid. The preparative purification of dihydrocoumarins was carried out on CHIROBIOTIC TAG in heptane/ethanol[55] while some related compounds were purified on a CHIROBIOTIC T in a reversed phase mode. The RP mode has many advantages for preparative applications; economics, green chemistry and high capacity for polar molecules. In the RP mode, the mobile phase can be made very high in water content to maximize selectivity and sample solubility. Recovery is easy, utilizing a standard C_{18} column as an affinity support with pH adjustment to get maximum capacity. The separation can be optimized also for selectivity with proper choice of buffer that is easily washed off the C_{18} column in the recovery step. For more details on this methodology see Chirobiotic Handbook, 5th edition, pages 32–36 or Supelco Application Report LC008: Experimental protocols for preparative purifications using macrocyclic glycopeptide chiral stationary phases. A reversible change in adsorption behavior was reported for the preparative separation of amino acids on the CHIROBIOTIC TAG.[56] Prolonged retention times were observed after harsh overload conditions attributable to the contribution of both a selective and nonselective interaction. The influence of experimental conditions; type and concentration of pH modifier, organic modifier and temperature could be regulated to negate this retention shift completely. In a study of peak shape for the separation of β-blockers on CHIROBIOTIC T it was demonstrated that it was possible to tune the peak shapes of the two enantiomers by varying the organic solvent composition.[57] This allowed for the first eluted peak to be transformed into an anti-Langmuir shape while keeping the second enantiomer in a normal Langmuir shape. It is then possible to obtain baseline resolution at higher load than when both enantiomers tail in the same direction. The authors state this manipulation makes it possible to develop very efficient preparative separations in terms of throughput and productivity.

Simulated Moving Bed (SMB)

The utility of the Chirobiotics for the routine purification of racemic compounds by simulated moving bed has been highlighted in a recent publication for the purification of amino acids in high yield.[58] Integration of SMB and biotransformation has been reported for amino acid production utilizing the CHIROBIOTIC TAG.[59] Coupling a continuous mild

enzymatic racemization to the distomer and recycling of the reactor effluent to the SMB feed makes it possible to transform the racemate into a single desired enantiomer in theoredically 100% yield. The organic component for this system was in the range of 10–20% to provide efficient catalysis and SMB operation.

COLUMN TESTING AND STORAGE

CHIROBIOTIC columns showing decreased resolution or an unstable baseline, can be regenerated by passing 10–20 columns volumes of 50/50; acetonitrile/50 mM ammonium acetate at 0.5 mL/min followed by an equivalent volume of pure methanol. A test mix of 5-methyl-5-phenylhydantoin should be run following the washing treatment to insure the integrity of the column before storage. Never store a CHIROBIOTIC column in buffer even for short periods of time. Other solvents that can be used for long term storage include ethanol and isopropanol.

TROUBLE SHOOTING TIPS

Noisy Baseline. New columns require longer equilibration times (downward drift) and should be washed with the 50/50; acetonitrile/50 mm ammonium acetate, followed by methanol before conditioning to the mobile phase of choice. Often ethanol, stabilized with IPA only, is a better last wash then methanol for difficult situations. Most often unstable baselines can be traced to contaminated organic solvent or the UV transparency of the solvent or additive. Triethyamine is often used and it does oxidize slowly at room temperature, creating a colored contaminant. This additive is best stored in sealed vials in small quantities in the refrigerator.

Bad Tailing Peaks. For basic analytes, lower the pH of the buffer, conversely, for acids, raise the pH. The safe range of pH is 3.0 to 7.0. Changing the organic solvent either from methanol to acetonitrile or often from acetonitrile to methanol will have a positive effect on tailing. Methanol is often a better solvent since it can help reduce non chiral hydrogen bonding for polar molecules. See also Ref. [42]. If sufficient resolution is present increasing column temperature typically from ambient to 35–40°C can alleviate tailing for all analytes. The maximum allowable temperature for any CHIROBIOTIC phase is 50°C for aqueous mobile phases. Anhydrous mobile phases have a maximum temperature of 70°C.

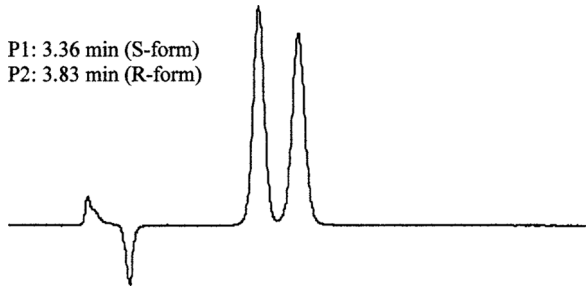

Figure 16. LC/MS applications of fluoxetine by CHIROBIOTIC V2 in polar ionic mode. The mobile phase is 15 mM ammonium formate in CH_3OH, 1 mL/min. Supelco Application Library@sial.com

Improving UV Sensitivity. First step is to use more UV transparent components like substituting ammonium phosphate for ammonium acetate or acetic acid/triethylamine, low pH only or substituting acetonitrile for methanol in reversed phase mode. It is also useful for low UV detection to clean the HPLC system with 0.5% nitric acid, followed with water until neutral and finally acetonitrile as a pure solvent. Nitric acid does not attach stainless steel but will clean degradation products from the cell windows as well as valve seats.

Avoid Mobile Phase Switching Problems. When switching from normal phase to reversed phase and vice versa, be sure to flush the entire HPLC

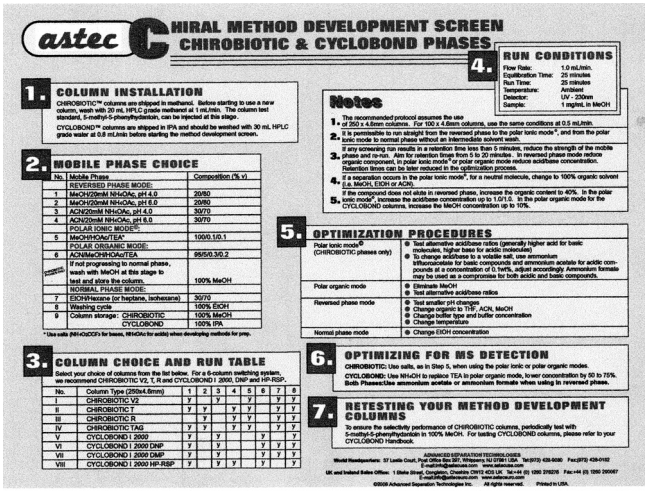

Figure 17. Chiral methods development wall chart.

system including tubing with either ethanol (stabilized with IPA) or isopropanol. These latter two solvents are infinitely miscible with both hydrocarbons like heptane and aqueous components in reversed phase systems.

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