# Chiral Method Development Screening Techniques: A practical guide and new approaches in LC-MS

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Retention mechanisms in chiral separations are complex and tend to be unpredictable. As a consequence, generic screening techniques utilizing many chiral stationary phases (CSPs) are typically used in method development practices. Normal phase chiral HPLC is commonly used in Drug Discovery and whilst such procedures are well established, there is a strong trend towards more sensitive detection by MS that now calls for a review of mobile phase selection. Additionally, the development of chiral pharmaceuticals has matured, generating larger and larger numbers of chiral molecules in late-stage product development. As a result, chiral LC-MS methods for DMPK and aqueous-compatible/LC-MS friendly methods for pharmaceutical (drug substance) analysis have become even more important.

The key to successful chiral method development is the availability of simple, rapid and reliable screening protocols that avoid the possibility of false positives/negatives. This article outlines a set of simple protocols that uses one of the well-known classes of chiral stationary phases (CSPs), the macrocyclic glycopeptides. CHIROBIOTIC phases are highly compatible with LC-MS and operate in a variety of different mobile phase types.

LC-MS can be used further in method development itself. HPLC combined with tandem MS is often used for the simultaneous quantification of parent drug and metabolites, but it also has the potential for even faster chiral method development. In this article, we also investigate simultaneous chiral method development, injecting a composite set of chiral molecules using LC-MS-MS. By comparing the results from the probe set with that from a single solute, the effect on the screening protocol of simultaneous screening is investigated.

## Macrocyclic Glycopeptide phases

Developed in 1994 as a result of research by Dr. Daniel Armstrong1, these innovative CSPs are based on the chemical bonding of macrocyclic glycopeptides on to silica. The glycopeptides used to date include vancomycin, teicoplanin and ristocetin A (commercialised as CHIROBIOTIC™ V and V2, T , T2 and TAG, and R, respectively). CHIROBIOTICS CSPs exhibit a large variety of potentially interacting functional groups such as the peptide backbone, amide and chiral sugar moieties for hydrogen bonding and dipole-dipole interactions, plus inclusion sites. Additionally, chloro-substituted aromatic groups provide the potential for π–π interactions. However, the unique aspect of these CSPs when compared to other phases is their ionic character arising from carboxylic acid and amine sites providing these phases with exceptional selectivity for ionisable molecules. The individual CSPs available are different from one another in the position, type and number of interactive sites and as a result offer complementary selectivity. Another result of this variety of interactive sites is that the phases operate in diverse mobile phase conditions, both aqueous and non-aqueous, without the need to reserve one column for each mobile phase. As the mobile phase is changed, the mechanism also changes, providing a wide range of enantiomeric applications for which these phases have become well established. A particular strength in reversed and polar mobile phases results in these phases being especially well-known for their effective interface with MS detection.

## Generic screening using glycopeptide chiral phases

Because the macrocyclic glycopeptides exhibit enantioselectivity in several mobile phase types, the screening protocol (Table 1) takes advantage of this and includes one run in each type, starting with polar mobile phases followed by reversed phase. Pure EtOH is used as an intermediate solvent in between reversed phase and normal phase and as a polar organic mode. Results from method development screening of fluoxetine for the first two mobile phases are shown in Figure 1.

The polar ionic mode – a polar organic solvent containing low concentrations of an ionic modifiers - seems unique to these phases and is used for all ionisable molecules. Both acid and base must be added but these can be replaced as part of the optimisation process with MS- and prep HPLC-friendly volatile salts. It is the ratio of acid to base that mainly controls selectivity with bases preferring higher acid and acids, higher base. Reversed phase is used for both ionisable and neutral solutes, while polar organic and normal phase are beneficial for more neutral molecules. The polar ionic mode has many advantages in terms of speed, MS compatibility and sample solubility, but is also beneficial for high throughput prep HPLC. The polar ionic mode is especially useful when solubility in more polar solvents means that normal phase prep is either not possible or inefficient.

| Mobile phase type | CHIROBIOTIC V2, T, R, TAG |
|---|---|
| Polar Ionic Mode | 100/0.1/0.1 (v/v/v), MeOH/HOAc/TEA |
| Reversed Phase | 30/70, ACN/20mM NH4Ac, pH 4.0 |
| Polar Organic Mode | 100% EtOH |
| Normal Phase | 30/70, EtOH/Heptane |

*Table 1. Suggested method development screening mobile phases*

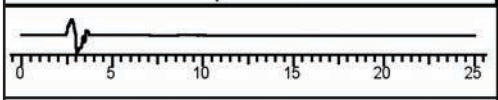
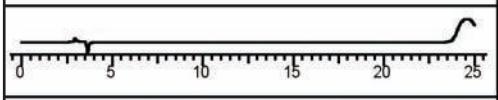
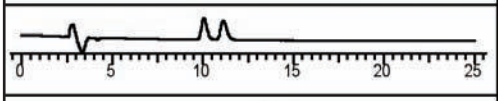
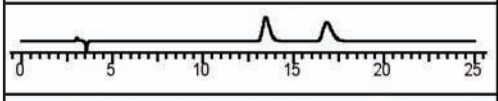
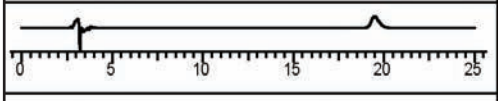
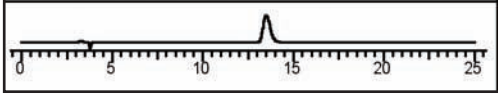
| Spectrum | Column | mode | elution |
|---|---|---|---|
|  | CHIROBIOTIC TAG | RP | No Elution |
|  | CHIROBIOTIC TAG | PIM | No Separation |
|  | CHIROBIOTIC V2 | RP | Separation |
|  | CHIROBIOTIC V2 | PIM | Separation |
|  | CHIROBIOTIC T | RP | No separation |
|  | CHIROBIOTIC T | PIM | No Separation |

*Figure 1. Method development screening for Fluoxetine: CHIROBIOTIC V2 in both RP and Polar Ionic Mode show selectivity.*

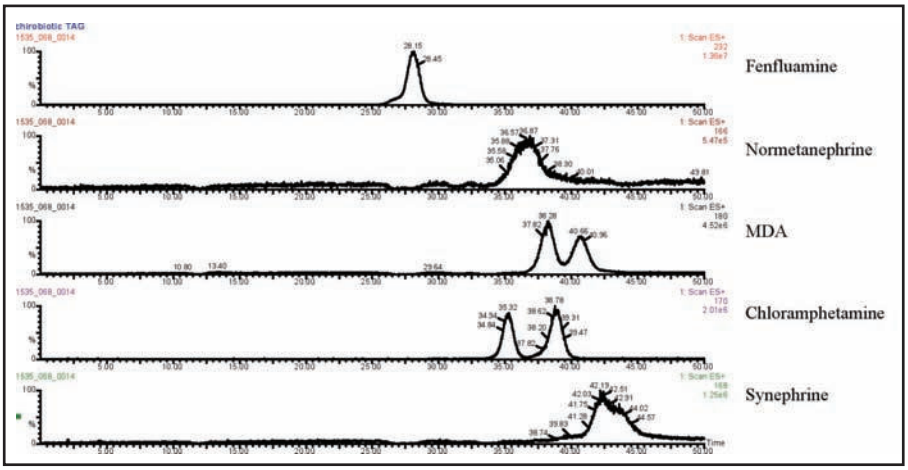



*Figure 2. Partial results of batch screening on CHIROBIOTIC TAG in the Polar Ionic Mode (Extracted Ion Current)*

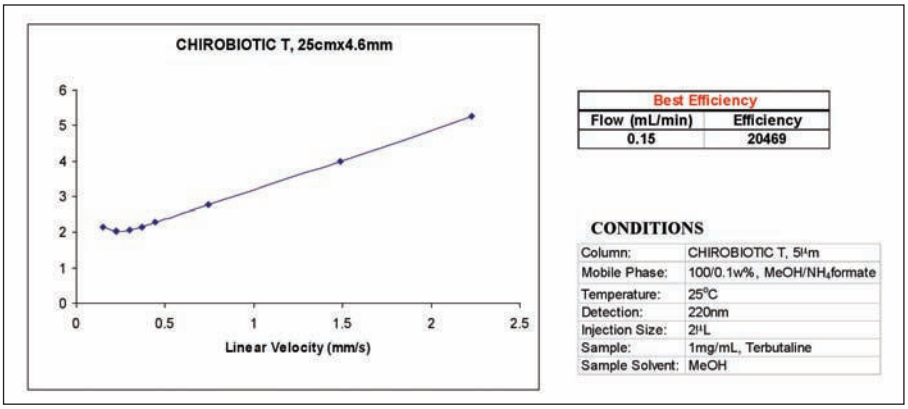



*Figure 3. Van Deemter curve for Chirobiotic T in Polar Ionic Mobile Phase*

### Chiral LC-MS-MS

Since LC-MS has the ability to separate in the mass/charge dimension, it should be possible to screen for a chiral method for many analytes simultaneously. CHIROBIOTIC stationary phases operate best in both reversed-phase and polar ionic modes, both of which are highly amenable to LC-MS. In a recent LC-MS study in our laboratory the polar ionic mode was used to test the efficacy of batch screening to identify unique selectivity, using a set of 14 basic probes differing over a wide range of pKa values, hydrophobicity and molecular weight. The extracted ion current of the β-blocker metoprolol was used to monitor impact of mobile phase variables on selectivity and retention, comparing single injection results against the probe mix in order to investigate the potential for batch injections in method development. In one such comparison, a slight variation in enantiomer response due to ion-suppression by co-eluting peaks was observed; however, retention and selectivity were not compromised when using the probe test mix approach (Table 2). Since this approach is being developed for qualitative method development purposes only, this indicates that batch injections would not result in a false negative result. The results from batch screens on the Chirobiotic TAG showed high selectivity and retention for many bases. Figure 2 shows the results from the amphetamines in the probe mix, and these columns also showed good selectivity towards the beta-blockers and overall greater retention for bases compared to the other Chirobiotic phases.

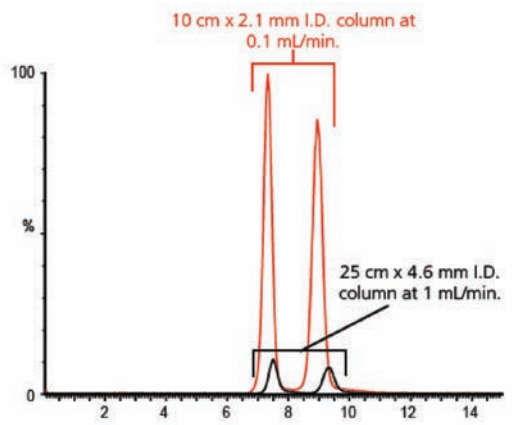
| | |
|---|---|
| Instrument: | Waters/Micromass ZQ, Single Quadrupole, Waters Alliance 2690 |
| Column: | Chirobiotic T, 150 cm x 4.6 mm, 5 µm |
| Temperature: | 35° C |
| Mobile Phase: | 0.1%, w/v ammonium acetate in methanol (Polar Ionic Mode) |
| Flow Rate: | 1 mL/min |
| Detection: | ESI, Positive Ion Mode, scan range m/z 150–500 |
| Inj. Volume: | 5 µL |

*Table 2: Conditions for simultaneous LC-MS chiral screen*

The probe mix was also used to study the impact of buffer (salt) type, buffer concentration, and acid/base ratio on retention and selectivity in the polar ionic mode2 Frequently, when optimising the method for MS detection or prep HPLC, a volatile salt such as ammonium acetate or ammonium formate replaces the traditional

| | |
|---|---|
| salt type | some impact on retention and peak shape, little impact on selectivity |
| salt concentration | major impact on retention and peak shape, little impact on selectivity |
| acid/base ratio | some impact on retention, moderate impact on selectivity |
| stationary phase | major impact on selectivity and retention (need for screening still exists) |

*Table 3: Summary of results from the LC-MS study of metaprolol*




**CONDITIONS**

| | |
|---|---|
| column: | Astec CHIROBIOTIC V2, 25 cm x 4.6 mm (black) or 10 cm x 2.1 mm I.D. (red), 5 µm particles |
| mobile phase: | 10 mM ammonium formate in 10:90 v/v water:methanol |
| flow rate: | 1.0 (black), 0.1 (red) mL/min. |
| temp.: | ambient |
| detector.: | +ESI, m/z 310 |
| injection: | 2 µL |
| sample: | 5 µg/mL in methanol |

*Figure 4. Relative LC-MS response of fluoxetine enantiomers on CHIROBIOTIC V2 in reversed Phase*

acetic acid/triethylamine in this mobile phase. In the investigation of differences between salt types, no selectivity was observed using one salt that was not observed when using the other two salts in any of the probe mix separations, so choosing one of these as a generic screen would also be acceptable. A comparison of the results for various concentrations of ammonium formate in methanol for metoprolol confirmed that retention of this base increases with decreasing concentration, indicating a dominating ionic interaction. Lowering salt concentration appears to assist selectivity. The results are summarized in Table 3. Figure 3 shows the results from one such batch screen using a Chirobiotic TAG column in the polar ionic mode.

## Flow rate studies

An interesting characteristic of the Chirobiotic CSPs is that optimum flow rates for maximum efficiency are quite low. Using van Deemter curves (Figure 3), the optimum flow rates for analytical (25cm x 4.6 mm) columns are observed to be as low as 0.2 mL/min, with an almost 100% increase in efficiency over that at 1.0 ml/min. Results from method development screening run at 1.0 mL/min can then optimised by a simple reduction in flow rate; this may also require some slight changes to the mobile phase or temperature in order to maintain fast separation methods.

An example of the improvement in speed, cost and sensitivity gained by using short columns with narrow internal diameter are shown in Figure 4. This compares the separation of fluoxetine enantiomers on a 25 cm x 4.6 mm I.D. column to a 10 cm x 2.1 mm I.D. column, both packed with 5 µm Chirobiotic V2. The flow rate was adjusted to obtain the same linear velocity on both columns. By decreasing the flow rate on the short column, compared to the equivalent linear velocity on the larger column, equivalent resolution with dramatically improved sensitivity was obtained. The injection volume was maintained at 2µl for each column. The lower flow rates also permit direct connection to the MS without flow splitting.

## Conclusions

Screening protocols provide a speedy answer to the question – which column, which mobile phase – in preparation for optimisation studies. The generic screens shown offer fast screening to achieve this. The effect on the screening protocol of injecting a composite set of chiral molecules using LC-MS may be minimal, affecting only sensitivity such that batch screening shows excellent potential for speeding up the column selection process further, without the need for use of parallel HPLC systems and producing methods that are immediately useful for MS detection. Further studies currently under way are hoped to lead to an MS protocol.

*References*

*1. D.W.Armstrong, Y Tang, S Chen, Y Zhou, C Bagwell, J-R Chen; Anal Chem, 66 (1994) 1473-1484.*

*2. D S Bell, J E Claus, J Jones; Chirality 2009, Breckenridge, US , Poster P102 . Significant Improvements in Chiral Method Development Using an LC-MS-Based Screening Approach, July 12 - 15, 2009.*