

A Novel Approach in HPLC Chiral Method Development: Dealing with Multiple Chiral Centers

David S. Bell¹, Anders Fridstrom², and Gaurang Parmar¹

¹Supelco, Div. of Sigma-Aldrich
Bellefonte, PA 16823 USA

²Sigma-Aldrich GmbH
Industriestrasse 25
CH-9471 Buchs, Switzerland



T413140

sigma-aldrich.com/analytical

Abstract

Development of methods capable of robust and reproducible separation of enantiomers is significantly challenging. Add additional chiral centers and the complexity exponentially increases. Many traditional pharmaceutical compounds exhibit multiple chiral centers, requiring methods that can at least separate the potential enantiomers and diastereomers from the API. Even more desirable is a method that can separate each of the potential isomeric impurities for accurate quantitation; however, this is rarely accomplished. Chiral stationary phases are capable of separating enantiomeric pairs, but often lack in the selectivity required to concomitantly separate the potential diastereomeric impurities. This study describes a systematic process that combines chiral and achiral chromatography in series that greatly increases the probability for generating full separation of enantiomeric and diastereomeric impurities in multiple chiral center molecules.

Abstract (contd.)

The process involves the initial establishment of conditions suitable for all enantiomeric pairs that may exist, disregarding diastereomeric selectivity. The next step is to pair an achiral stationary phase to the conditions set forth in the first step to enable separation of the diastereomers. The chiral and achiral columns may then be coupled in series and geometries of each optimized to tailor the separation to specific requirements. Examples of the process as applied to multiple chiral center molecules highlight the benefits of the approach.

Introduction

Due to the highly complex and analyte specific retention mechanisms involved in chiral separations, attaining resolution of 8 stereoisomers from an analyte racemic at 3 chiral centers is always arduous and usually impossible. Where diastereomers may often be separated achirally by HPLC, separation of enantiomers by HPLC may only be achieved with the use of chiral stationary phases without the use of chiral mobile phase modifiers and/or derivatization. Often, in the separation of multiple stereoisomers, more than one chromatographic system is needed to achieve selectivity.

Unlike a number of other CSP's, CHIROBIOTIC™ stationary phases operate well in the traditionally used reversed-phase mode typically run on achiral columns. This study highlights the use of the chiral CHIROBIOTIC V2 in sequence with the achiral Ascentis® Phenyl in reversed-phase mode to resolve 8 stereoisomers.

Structural Information of the Analytes

- Full structure is proprietary, however:
 - 3 Chiral Centers, 8 Stereoisomers
 - Functional Groups Include:
 - Carboxylic acid
 - 3 Aromatic rings
 - Secondary Amine

Chiral Screen of 8 Stereoisomers

HPLC chiral column screening

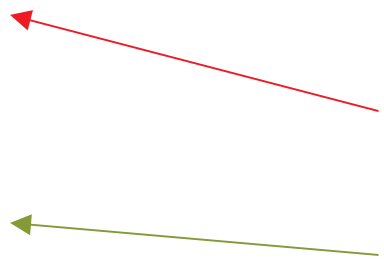
- Established protocol includes a 3 mobile phase set of conditions run using 6 different stationary phase chemistries as a front line screening.

Columns:

- CHIROBIOTIC
 - V2
 - T
 - TAG
- CYCLOBOND™
 - Native β CD
 - DMP
 - HP-RSP

Mobile Phases:

- Polar-Ionic Mode (PIM)
 - 100:0.1:0.1, methanol:acetic acid:triethylamine
- Reversed-Phase (RP)
 - 70:30, 20 mM ammonium acetate (pH 4.0):acetonitrile
- Polar-Organic Mode (POM)
 - 95:5:0.3:0.2, acetonitrile:methanol:acetic acid:triethylamine.



Screen of Main Compound with Enantiomer

To simplify the analysis of the results, two of the analytes were combined and screened to determine if enantiomeric selectivity could be achieved.

The main compound, Analyte 1, was combined with its enantiomer, Analyte 5, and subjected to the aforementioned 3 mobile phase, 6 column screen.

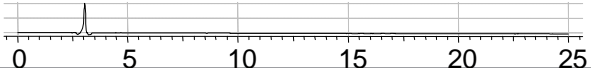

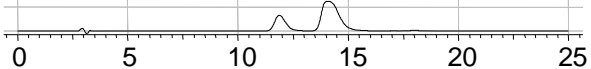
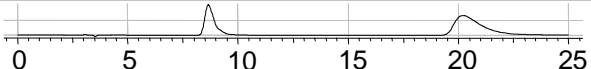
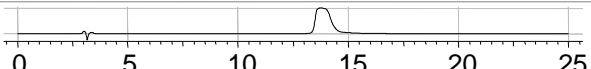
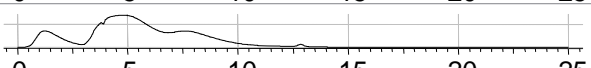
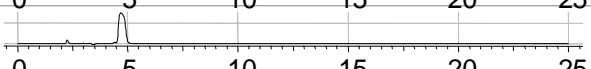
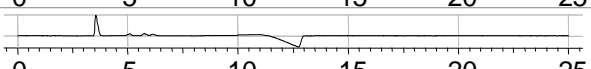
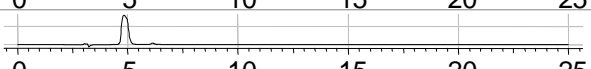
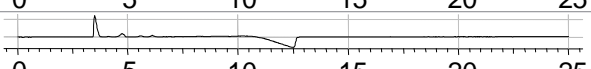
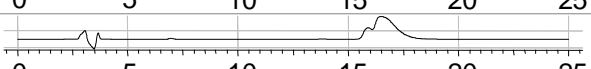
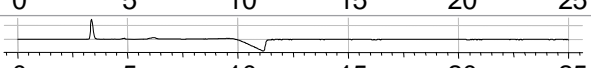
Screening Results

The following combinations of stationary phase and chromatographic mode provided evidence of enantiomeric selectivity: (See Figure 1).

Mixture of Main Compound (Analyte 1) and its enantiomer, Analyte 5

- CHIROBIOTIC V2: RP mode
- CHIROBIOTIC V2: PI mode
- CYCLOBOND DMP: RP mode (partial separation)

Figure 1. Summary of Primary Screen, Mixture of Main Compound and Enantiomer

Chromatogram	Column	Mode	Elution
	CHIROBIOTIC TAG	RP	No Retention
	CHIROBIOTIC TAG	PIM	Unknown
	CHIROBIOTIC V2	RP	Separation
	CHIROBIOTIC V2	PIM	Separation
	CHIROBIOTIC T	RP	No Separation
	CHIROBIOTIC T	PIM	Unknown
	CYCLOBOND I 2000	RP	No Separation
	CYCLOBOND I 2000	POM	No Separation
	CYCLOBOND 2000 HP-RSP	RP	No Separation
	CYCLOBOND 2000 HP-RSP	POM	No Retention
	CYCLOBOND 2000 DMP	RP	Partial Separation
	CYCLOBOND 2000 DMP	POM	No Retention

Separation of Enantiomers on the CHIROBIOTIC V2

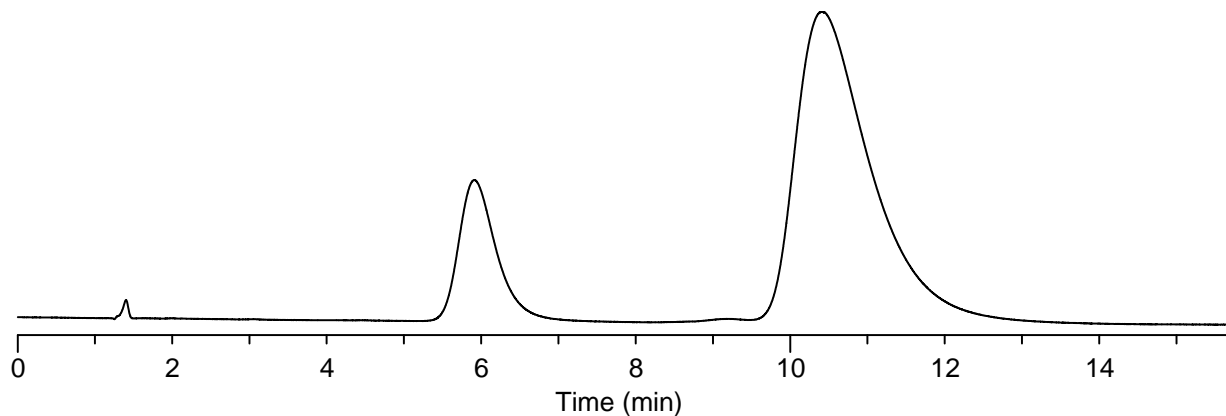
The screening protocol revealed the best selectivity on the CHIROBIOTIC V2. In hopes of ultimately utilizing a non-chiral phase to separate diastereomers in conjunction with a chiral phase to separate the enantiomers, the reversed-phase mobile phase was further optimized.

The organic component was switched from acetonitrile to methanol to see if better selectivity would be observed.

Injection of Analyte 1 and Analyte 5 produced two well-resolved peaks with the use of methanol as the organic component. See Figure 2.

Figure 2. Separation of Enantiomers on the CHIROBIOTIC V2

instrument: Agilent® 1100
column: CHIROBIOTIC V2, 10 cm x 4.6 mm, 5 μ m
mobile phase: 50:50, 20 mM ammonium acetate (pH 4.0): methanol
flow rate: 1 mL/min
temp.: 25 °C
det.: UV at 287 nm
injection: 10 μ L
sample: 2-component mix, 100 μ g/mL in 70:30, 20 mM ammonium acetate (pH 4.0):acetonitrile



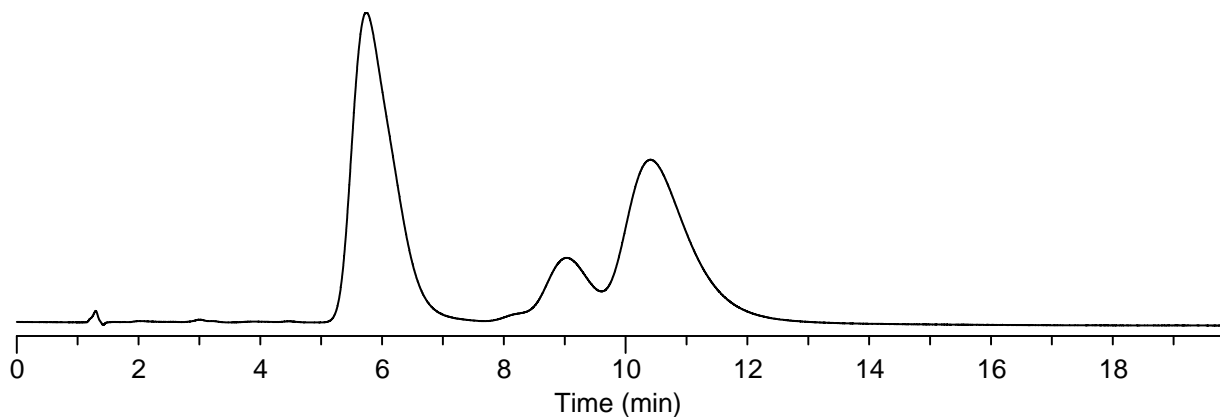
Injection of 8 Stereoisomers on CHIROBIOTIC V2 in RP

An injection of all 8 stereoisomers under the same conditions produced 3 major peaks showing that the diastereomers are not likely to show separation under these conditions. See Figure 3.

Therefore, supplementation with an achiral phase may be necessary.

Figure 3. Injection of 8 Stereoisomers on CHIROBIOTIC V2 in RP

instrument: Agilent 1100
column: CHIROBIOTIC V2, 10 cm x 4.6 mm, 5 μ m
mobile phase: 50:50, 20 mM ammonium acetate (pH 4.0):methanol
flow rate: 1 mL/min
temp.: 25 °C
det.: UV at 287 nm
injection: 10 μ L
sample: 8-component mix, 100 μ g/mL in methanol



Injection of Individual Enantiomers on CHIROBIOTIC V2

To ensure that the individual enantiomer pairs were all selective on the V2, 1 mL stock solutions were made of each analyte, and the following mixtures were made and injected under the aforementioned conditions:

- Mix 1, 2:1: 200 μ L Analyte 1 + 100 μ L Analyte 5 + 700 μ L, 20 mM ammonium acetate (pH 4.0)
- Mix 2, 2:1: 200 μ L Analyte 2 + 100 μ L Analyte 8 + 700 μ L, 20 mM ammonium acetate (pH 4.0)
- Mix 3, 2:1: 200 μ L Analyte 3 + 100 μ L Analyte 7 + 700 μ L, 20 mM ammonium acetate (pH 4.0)
- Mix 4, 2:1: 200 μ L Analyte 4 + 100 μ L Analyte 6 + 700 μ L, 20 mM ammonium acetate (pH 4.0)

As shown in Figure 4, All enantiomer pairs showed well-resolved peaks with very little difference in retention observed between the diastereomers.

Figure 4. Injection of Individual Enantiomers on CHIROBIOTIC V2

Chromatogram	Column	Elution	Sample
	CHIROBIOTIC V2	Separation	09-28 Mixture 1
	CHIROBIOTIC V2	Separation	09-28 Mixture 2
	CHIROBIOTIC V2	Separation	09-28 Mixture 3
	CHIROBIOTIC V2	Separation	09-28 Mixture 4

Achiral Separation of Diastereomers

Among the achiral columns investigated were the Ascentis[®] C18, Ascentis RP-Amide, Discovery[®] HS F5, and the Ascentis Phenyl.

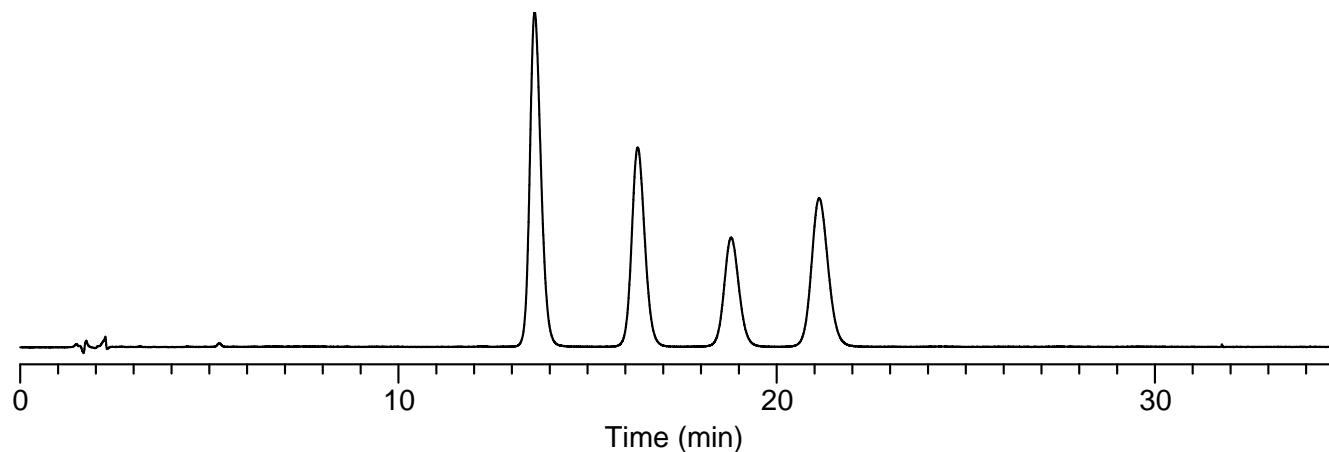
The columns were run using reversed-phase mobile phases that utilized methanol or acetonitrile as the organic component.

Both ammonium acetate and ammonium formate were investigated as mobile phase buffers.

The Ascentis Phenyl provided the best results by showing separation of the 4 sets of stereoisomers using a reversed-phase mobile phase with acetonitrile as the organic component. The results are shown in Figure 5.

Figure 5. Separation of Diastereomers on the Ascentis Phenyl in RP Mode

instrument: Agilent 1100
column: Ascentis Phenyl, 15 cm x 4.6 mm I.D., 5 μ m particles
mobile phase: 75:25, 20 mM ammonium acetate (pH 4.0):acetonitrile
flow rate: 1 mL/min
temp.: 25 $^{\circ}$ C
det.: UV at 287 nm
injection: 10 μ L
sample: 8-component mix, 100 μ g/mL in methanol



Injection of 8 Stereoisomers on the CHIROBIOTIC V2 in Sequence with the Achiral Ascentis Phenyl

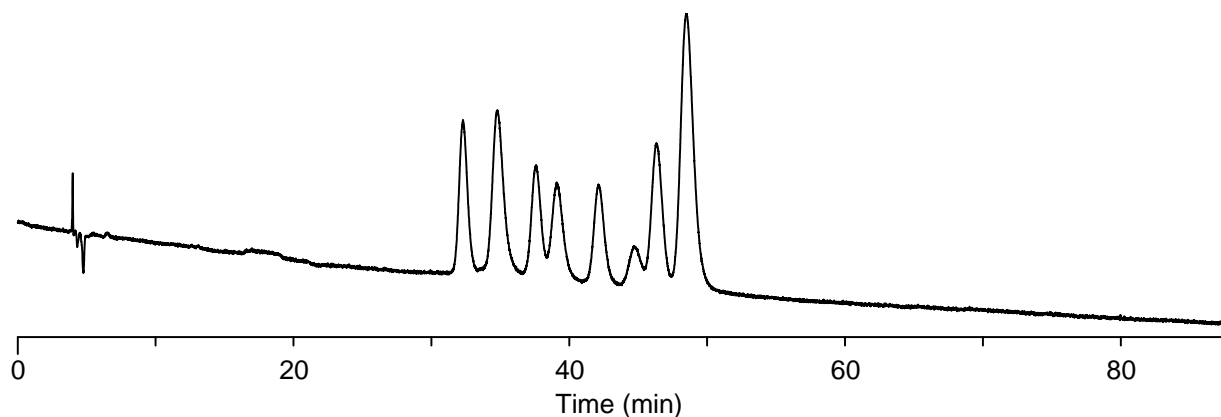
When combining a V2 (5 cm x 4.6 mm I.D., 5 μ m particles) with an Ascentis Phenyl (15 cm x 4.6 mm I.D., 5 μ m particles) and injecting the mixture of all 8 analytes, enantiomeric selectivity of 7 out of the 8 peaks was observed.

Increasing the length of the V2 column to 10 cm and decreasing the acetonitrile to 20% shows improved resolution of the 7 peaks observed previously.

As shown in Figure 6, increasing the length of the Ascentis Phenyl to a 25 cm column produced resolution of all 8 peaks.

Figure 6. Resolution of 8 Stereoisomers on the CHIROBIOTIC V2 in Series with the Achiral Ascentis Phenyl

instrument: Agilent 1100
column: CHIROBIOTIC V2, 10 cm x 4.6 mm I.D., 5 μ m particles,
Ascentis Phenyl, 15 cm x 4.6 mm I.D., 5 μ m particles
mobile phase: 80:20, 20 mM ammonium acetate (pH 4.0):acetonitrile
flow rate: 1 mL/min
temp.: 25 $^{\circ}$ C
det.: UV at 287 nm
injection: 10 μ L
sample: 8-component mix, 100 μ g/mL in methanol



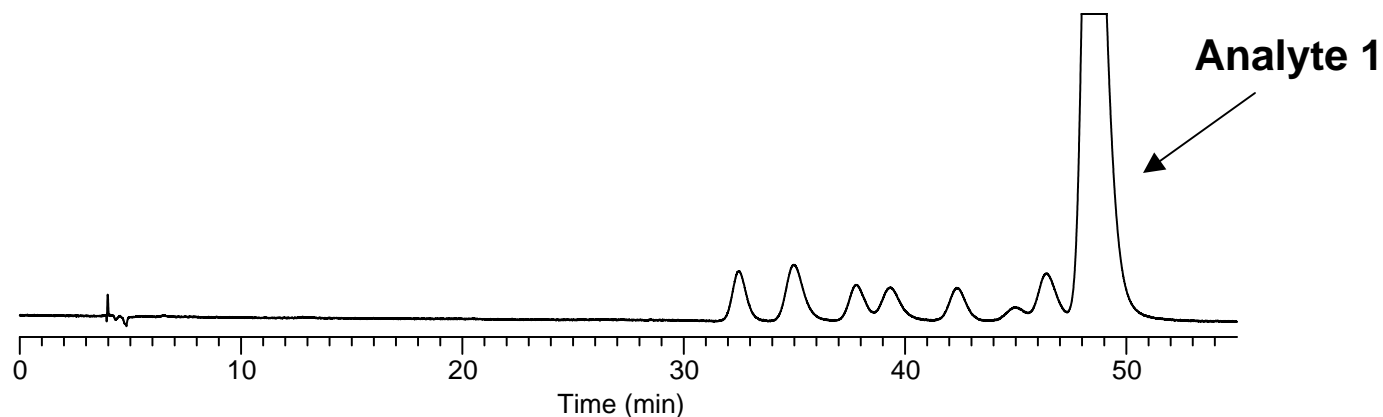
Elution of Main Compound Within the Mix of 8 Stereoisomers on the V2 and the Ascentis Phenyl

The mix was then spiked with main compound, Analyte 1, to determine when the main compound elutes within the mixture of stereoisomers.

Injection of the spiked sample under the developed conditions revealed that Analyte 1 is the final compound to elute within the mix as shown in Figure 7.

Figure 7. Elution of Main Compound Within the Mix of 8 Stereoisomers

instrument: Agilent 1100
column: CHIROBIOTIC V2, 10 cm x 4.6 mm I.D., 5 μ m particles,
Ascentis Phenyl, 15 cm x 4.6 mm I.D., 5 μ m particles
mobile phase: 80:20, 20 mM ammonium acetate (pH 4.0):acetonitrile
flow rate: 1 mL/min
temp.: 25 °C
det.: UV at 287 nm
injection: 10 μ L
sample: 8-component mix spiked with Analyte 1, 100 μ g/mL in methanol



Summary

- A primary screen of 8 stereoisomers from an analyte racemic at 3 chiral centers showed partial separation on the CHIROBIOTIC V2 and T, as well as the CYCLOBOND I 2000 and the DMP. Screening of the main compound, Analyte 1, and its enantiomer, Analyte 5, showed excellent selectivity on the CHIROBIOTIC V2 in both modes of chromatography.
- To ensure that the individual enantiomer pairs were all selective on the V2, mixtures of each enantiomeric pair were injected on the V2 under RP conditions. All enantiomer pairs showed well resolved peaks; however, very little difference in retention was observed between the diastereomers, making the incorporation of an achiral phase a necessity.
- Various achiral columns, including the Ascentis Express C18, RP-Amide, Discovery HS F5, and the Ascentis Phenyl, were investigated using reversed-phase mobile phases. Of these phases Ascentis Phenyl provided the best results.

Summary (contd.)

- Ultimately, the combination of chiral CHIROBIOTIC V2 (10 cm x 4.6 mm I.D., 5 μ m particles) in line with the achiral Ascentis Phenyl (25 cm x 4.6 mm I.D., 5 μ m particles) in reversed-phase mode showed resolution of all 8 stereoisomers in less than one hour.
- By spiking the mixture of 8 stereoisomers with main compound Analyte 1, it was determined that Analyte 1 is the final compound to elute within the mix.
- Since the CHIROBIOTIC CSPs are amenable to multiple mobile phases including the traditional reversed-phase mode, it is possible, and very useful to employ them in combination with achiral columns.
- The use of CHIROBIOTIC CSPs in combination with achiral columns is a valuable means to achieve chiral separation of multiple chiral center molecules.

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