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Preparative chromatographic resolution of racemates using HPLC and SFC in a pharmaceutical discovery environment☆

Larry Miller*, Matt Potter

Amgen, One Kendall Square, Bldg 1000, Cambridge, MA 02139, USA

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ABSTRACT

The preparative chromatographic resolution of racemates has become a standard approach for the generation of enantiomers in pharmaceutical discovery laboratories. This paper will discuss the use of preparative HPLC and SFC to generate individual enantiomers for discovery activities. Analytical HPLC and SFC method development to rapidly screen chiral stationary phases and solvent combinations will be presented. The usefulness of preparative chromatographic resolution of racemates will be demonstrated through the presentation of numerous non-routine case studies from the laboratories at Amgen.

1. Introduction

The necessity to generate individual enantiomers for testing is a growing priority in pharmaceutical research and discovery. There are two approaches to obtaining enantiomerically pure chemicals. These are (1) asymmetric synthesis of the desired enantiomer and (2) resolution of a racemic mixture into individual enantiomers. At a discovery stage where a very diverse set of molecules is made at small quantities, and where most compounds are only made once, asymmetric synthesis is not time or cost efficient. In addition, often both enantiomers are needed for biological testing, which would require two synthetic routes to be developed. Resolution of a racemic mixture has the advantage of producing both enantiomers, essential for testing. Resolution methods include recrystallization, enzymatic resolution, indirect chromatographic resolution and direct chromatographic resolution. The time required to develop a recrystallization method or an enzymatic resolution can also make this an inefficient approach for the generation of small quantities of enantiomers. Indirect chromatographic resolution, involving derivatization of the enantiomers to form a pair of diastereomers, followed by separation on an achiral stationary phase is useful for the generation of individual enantiomers [1]. While the separation of diastereomers is usually easier than the separation of enantiomers, and has the advantage of being easier to scale-up, there are limitations to this approach. First, high enantiomeric purity of the derivatizing agent is essential. Also, removal of the derivative after chromatographic separation should not cause racemization of the desired enantiomer. Finally the use of indirect chromatographic separation adds two steps to the synthesis, the reaction to form the derivatives prior to chromatography and the reaction to remove the derivatives after chromatography. This is especially time consuming in a discovery environment where the number of compounds being synthesized is large. The use of chromatographic resolution has become the quickest, most cost effective approach for enantiomer generation at the discovery stage [2–5].

Chromatographic resolution via HPLC has been utilized for the past 20 years [6–8]. Over the past 5 years the use of supercritical fluid chromatography (SFC) has gained greater acceptance as a fast, cost effective approach to resolution [9–15]. With SFC a majority of the solvent in the mobile phase, usually 60+% is supercritical CO_2. The low viscosity and high diffusivity of the SFC mobile phase allow higher flow rates relative to HPLC, resulting in shorter run times and increased efficiencies. A major advantage of preparative SFC vs. preparative HPLC is the lower solvent usage and the higher product concentrations post chromatography, reducing the time required for solvent removal and enantiomer isolation.

Polysaccharide based chiral stationary phases have been utilized for over 20 years for the analytical and preparative resolution of racemates. While these phases have proved extremely useful, they have severe solvent restrictions due to the derivatized cellulose or amylose being only adsorbed onto the silica gel. Contact with

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* Corresponding author.
E-mail address: millerl@amgen.com (L. Miller).

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certain solvents can result in dissolution of the cellulose/amylose and loss of resolution and/or column destruction. Over the past 5 years a series of immobilized cellulose/amylose chiral stationary phases (Chiralpak IA, IB, and IC) have been introduced. These phases have no solvent restrictions and have proven useful where solvents other than traditional solvents used for chiral separations (alkanes, alcohols, and acetonitrile) are required for resolution [16].

Typical detectors used for chromatographic resolution of enantiomers include UV and/or chiral detectors [17]. Recently the use of mass spectrometric (MS) with SFC has increased in the pharmaceutical industry [18–23]. MS detection has been utilized to accurately identify enantiomers in plasma [24–25] as well as during method development to allow sample pooling [26].

While multiple papers have been written on the use of preparative HPLC/SFC for chromatographic resolution, this paper will discuss some less straightforward and non-routine separations encountered in our laboratories. Several examples of chiral separations using HPLC and SFC will be discussed in this paper. Due to the small quantities of racemates to be resolved (usually less than 1 g) as well as the need to rapidly turn around these separations, these separations were not fully optimized.

2. Experimental

2.1. Racemates

The racemates separated are proprietary pharmaceutical compounds belonging to and synthesized at Amgen (Cambridge, MA, USA). While no information regarding the structure of the compounds being separated is presented, the approach for separation discussed in this paper is generic and applicable for the separation of many small (<700 ama) molecular weight racemates.

2.2. Mobile phase and chiral stationary phase (CSP)

The mobile phases used in all case studies were varied, reagent grade or better and obtained from a variety of sources. The chiral stationary phases were obtained as 5 or 10 μm particle size prepacked columns from Chiral Technologies (West Chester, PA, USA).

2.3. HPLC equipment

The analytical chromatograph consisted of an Agilent 1100 system modified with multiple column switching and mobile phase switching valves to facilitate method development. The preparative HPLC chromatograph was a Varian SD-1 system (Wakefield, RI, USA).

2.4. Supercritical fluid chromatography (SFC) equipment

The analytical SFC chromatograph was a SFC method development station sold by Thar (Pittsburgh, PA, USA) equipped with a Waters ZQ mass spectrometer (Milford, MA, USA). The preparative SFC chromatograph was a SFC Prep 80 from Thar (Pittsburgh, PA, USA).

2.5. Analytical method development

During the discovery phase of pharmaceutical R&D, hundreds of compounds are being synthesized to find a molecule with the desired pharmacological properties. As the chemical requirements for initial discovery testing are small (often <50 mg) and the quantity of different molecules is large, an approach for analytical method development must be utilized that is both time and compound efficient. The initial CSP and mobile phase evaluation is not designed to give complete resolution, but to quickly differentiate between conditions which afford separation and those that do not. In addition, the evaluation must work for a range of compounds of varying polarities. The approach utilized at Amgen's Cambridge, MA site utilizes short columns (50 mm for HPLC, 100 mm for SFC) with fast flow rates and steep gradients for this initial evaluation. Conditions for HPLC and SFC method development are summarized in Table 1. For HPLC a total of eight experiments are performed in less than 80 min. For SFC a total of twelve experiments are completed in approximately 80 min. Evaluation of results from the gradient analyses is used to identify the best CSP/mobile phase. Isocratic conditions are then quickly developed at the analytical scale prior to preparative work. As preparative SFC has been shown to be more time and cost efficient than preparative HPLC, only SFC method development is explored for samples less than 1 g. Only if sample size is greater than 1 g, and/or resolution is insufficient HPLC analytical method development is also explored. For the preparative purifications longer (25 cm) columns are used relative to column lengths (5 or 10 cm) used for analytical method development. To avoid excessively long run times on the longer preparative columns we strive for low retention times when developing isocratic methods for scale-up. For SFC a retention time of less than 2 min is desired. This translates to a preparative retention time of less than

Table 1
HPLC and SFC method development conditions

	HPLC conditions	SFC conditions
Column dimensions (mm)	4.6 × 50	4.6 × 100
Chiral stationary phases	Chiralpak AD-H Chiralpak AS-H Chiralcel OD-H Chiralcel OJ-H	Chiralpak AD-H Chiralpak AS-H Chiralcel OD-H Chiralcel OJ-H
Flow rate (ml/min)	2.0	5.0
Co-solvents	Isopropanol* Ethanol*	Methanol w/0.2% diethylamine** Isopropanol w/0.2% diethylamine** Ethanol w/0.2% diethylamine**
Gradient conditions	5% for 0.5 min 5–55% over 1.5 min 55% for 0.75 min	5–55% over 3.5 min 55% for 1 min
Temperature	Ambient	40 C
Run time	3.5 min	4.5 min

* w/heptane.
** w/CO_2.

5 min. Due to the lower linear velocity in HPLC, a retention time of less than 5 min for the isocratic analytical method is desirable. This translates to a preparative run time of less than 12 min.

3. Results and discussion

3.1. Case I: SFC method development utilizing MS detection

The incorporation of a MS detector into analytical SFC equipment is a useful addition. A MS detector is routinely utilized for SFC method development at Amgen. 1.1 g of material (Racemate 1) was submitted for resolution. This compound, which contained two chiral centers was synthesized at a contract facility. The purity of one chiral center was set through purchase of enantiomerically pure starting material. The other starting material was racemic, generating a product containing two diastereomers. Reverse phase LC/MS showed two closely eluting peaks with the same mass. This sample was analyzed using the SFC method development screen described in Table 1. Evaluation of results on the Chiralpak AD-H using methanol/diethylamine co-solvent showed four peaks (Fig. 1a). The extracted ion trace for mass-to-charge 444 Da is shown in Fig. 1b. All four of these peaks had the same predominant ion signal. It appeared that racemization had occurred during the synthesis. Analytical SFC exhibited good separation of the first two peaks, while the last two peaks did not show adequate separation. The preparative separation is shown in Fig. 2. This separation resolved the first two peaks cleanly. An analytical method that separated the two late eluting peaks was developed (Fig. 3) and scaled to preparative loadings to afford the final two peaks. The two SFC purifications generated 195, 126, 174 and 113 mg, each with ee >98%.

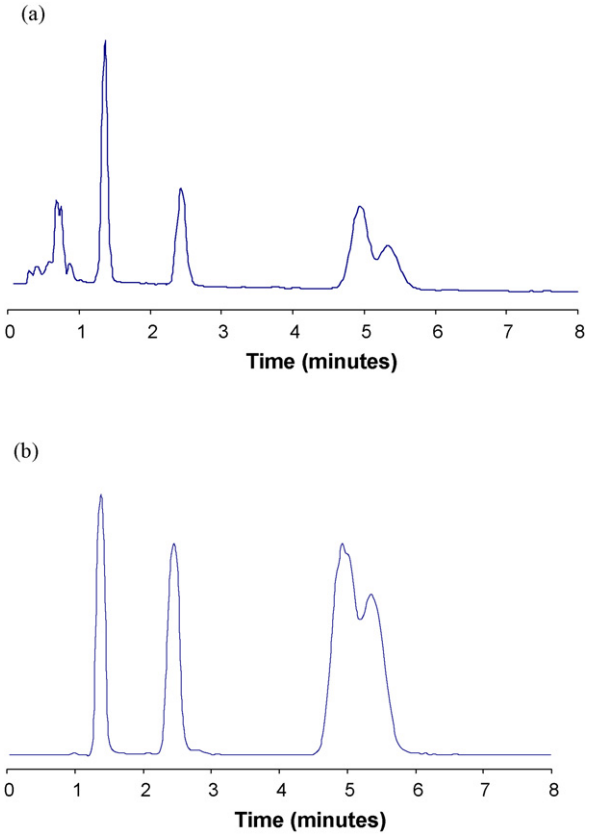

Fig. 1. Analytical SFC separation of Racemate 1. Analysis conducted on Chiralpak AD-H (100 mm × 4.6 mm I.D.), 90:10 CO_2–methanol (w/0.2% diethylamine), 40 C, 120 bar, 5 ml/min. (a) UV detection, (b) extracted mass of 444.

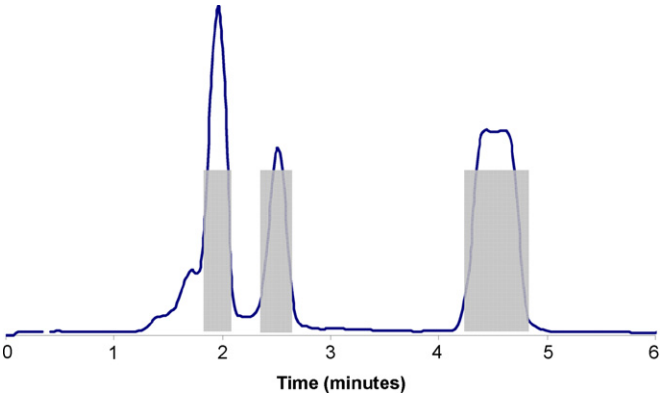

Fig. 2. Preparative SFC separation of Racemate 1. The purification was conducted on Chiralpak AD-H (250 mm × 20 mm I.D.) with a mobile phase of 85:15 CO_2–methanol (w/0.2% diethylamine), 40 C, 100 bar. A flow rate of 85 ml/min and a loading of 15 mg were used.

3.2. Case II: processing of low solubility racemate, example 1

Development of an analytical chiral method requires identification of a suitable chiral stationary phase (CSP) and mobile phase. While chiral discrimination is required for purification, good racemate solubility (ideally greater than 20 mg/ml) is also necessary for a successful preparative resolution. Utilizing traditional amylose and cellulose CSP, solubility in methanol, ethanol, acetonitrile or isopropanol is required. The recent introduction of immobilized CSP (Chiralpak IA, IB and IC) has increased the range of solvents that can be utilized as mobile phases (and dissolution solvents) in analytical and preparative separations.

A total of 2.5 g of material (Racemate 2) was submitted for resolution. The racemate was impure, containing a 1:1:2 mixture of product:regioisomer:unknown impurity as well as other lower level impurities. As each of these compounds had a chiral center, six peaks may be seen when analyzed using a chiral stationary phase. As this would lead to a complicated chromatogram, it was decided to remove the regioisomer and the impurity prior to resolution of the product enantiomers. Initial attempts using normal phase chromatography were unsuccessful. Reverse phase chromatography was successful in removing the impurity, but did not remove the regioisomer. The product/regioisomer mixture (822 mg) was subjected to polar organic chiral HPLC method development. The results are shown in Table 2. Separation of the product and regioisomer enantiomers was observed with Chiralpak AD and a methanol mobile phase (Fig. 4). While this method gave adequate analytical

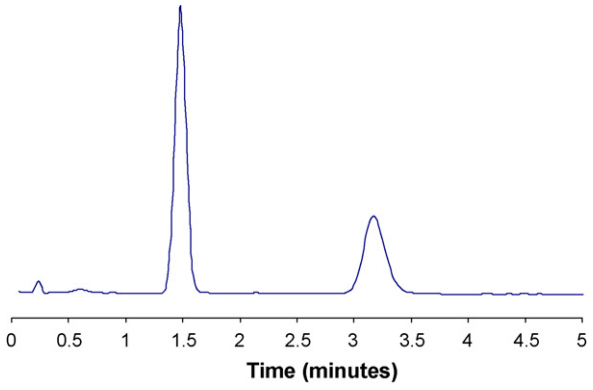

Fig. 3. Analytical SFC separation of peaks 3 and 4 of Racemate 1. Analysis conducted on Chiralcel OD-H (100 mm × 4.6 mm I.D.), 90:10 CO_2–methanol (w/0.2% diethylamine), 40 C, 120 bar, 5 ml/min.

Table 2
HPLC results for Racemate 2

CSP	Mobile phase	Regioisomer Peak 1 t_r	Regioisomer Peak 2 t_r	Product Peak 1 t_r	Product Peak 2 t_r
Chiralpak AD-H	Methanol	6.94	6.94	9.56	12.89
Chiralpak AS-H	Methanol	No separation, all peaks eluted in less than 5 min			
Chiralcel OD-H	Methanol	No separation, all peaks eluted in less than 5 min			
Chiralcel OJ-H	Methanol	No separation, all peaks eluted in less than 7 min			
Chiralpak IA	Methanol	8.11	8.11	10.32	12.32
Chiralpak AD-H	Ethanol	6.63	7.07	8.41	9.33
Chiralpak AS-H	Ethanol	No separation, all peaks eluted in less than 5 min			
Chiralcel OD-H	Ethanol	No separation, all peaks eluted in less than 5 min			
Chiralcel OJ-H	Ethanol	No separation, all peaks eluted in less than 5 min			
Chiralpak IA	Ethanol	7.84	7.84	9.23	9.93
Chiralpak AD-H	Acetonitrile	No separation, all peaks eluted in less than 5 min			
Chiralpak AS	Acetonitrile	No separation, all peaks eluted in less than 5 min			
Chiralpak IA	Acetonitrile	No separation, all peaks eluted in less than 5 min			

separation it was soon determined that this approach was not suitable for preparative work due to poor solubility in methanol (<1 mg/ml).

The sample was soluble in dichloromethane, ethyl acetate, methyl *t*-butyl ether or tetrahydrofuran (THF). These solvents were used as starting points for chiral method development using Chiralpak IA (immobilized version of Chiralpak AD). The method development results are summarized in Table 3. The best separation was observed with 30/70 (v/v) THF/heptane. The analytical separation shown in Fig. 5 was scaled to preparative loadings. The sample was dissolved in THF at a concentration of 134 mg/ml. Final purification conditions separated ~34 mg of sample/injection. The preparative chromatogram is shown in Fig. 6. 822 mg of sample was processed using these conditions to generate 170 mg of enantiomer 1 and 166 mg of enantiomer 2, both with ee >98.6%. The recoveries were low due to the presence of the regioisomer impurity in the original sample.

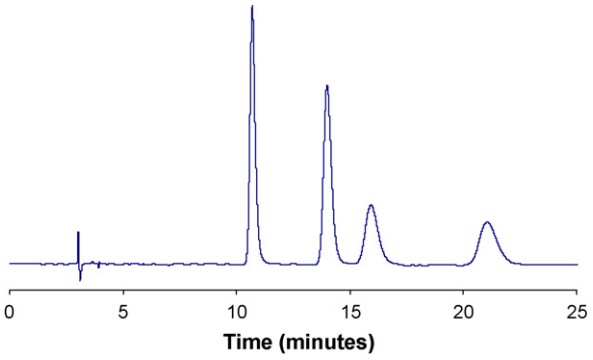

Fig. 5. Analytical HPLC separation of Racemate 2. Analysis conducted on Chiralpak IA (150 mm × 4.6 mm), mobile phase of 30/70 (v/v) THF/heptane and a flow rate of 1 ml/min. Peaks at 10.7 and 14 min are product enantiomers, Peaks at 15.9 and 21 min are regioisomer enantiomers.

3.3. Case III: processing of low solubility racemate, example 2

A total of 450 mg of material (Racemate 3) was submitted for resolution. Chiral screening using HPLC and SFC was performed. The results are summarized in Table 4. The best separation was obtained in 40/60 (v/v) ethanol/heptane on Chiralcel OD-H. The analytical separation is shown in Fig. 7. For the preparative separation dissolution in ethanol was attempted, but solubility was poor (<1 mg/ml). After discussion with the submitter, it was determined that the racemate was soluble only in tetrahydrofuran (THF). Chiralpak IB is the immobilized version of Chiralcel OD and thus was utilized for analytical method development. A method was developed using Chiralpak IB and a mobile phase of 25/75 (v/v) ethanol/heptane. Often satisfactory purification results are obtained when the solvent used for sample dissolution and injection is different than the mobile phase. This approach was attempted using THF as the dissolution solvent and ethanol/heptane as the mobile phase. Poor chromatographic performance was obtained and this approach was abandoned. Additional method development using non-traditional solvents was performed with a Chiralpak IB column. Acceptable conditions were developed using a mobile phase of 5/95 (v/v)

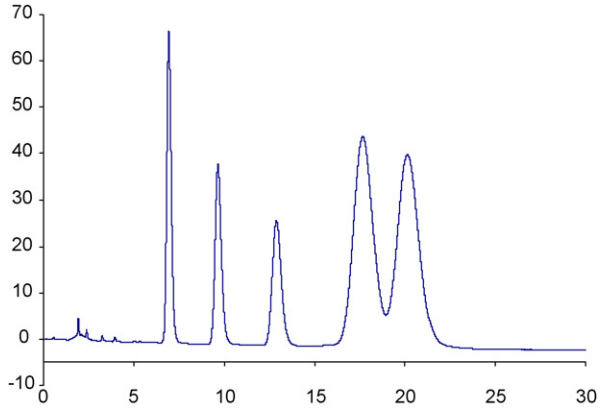

Fig. 4. Analytical HPLC separation of Racemate 2. Analysis conducted on Chiralpak AD-H (150 mm × 4.6 mm I.D.), mobile phase of methanol and a flow rate of 1 ml/min. Peak at ~7 min contains both regioisomer enantiomers, peaks at 9.7 and 12.9 min are the product enantiomers and peaks at 17.7 and 20.14 min are the impurity enantiomers.

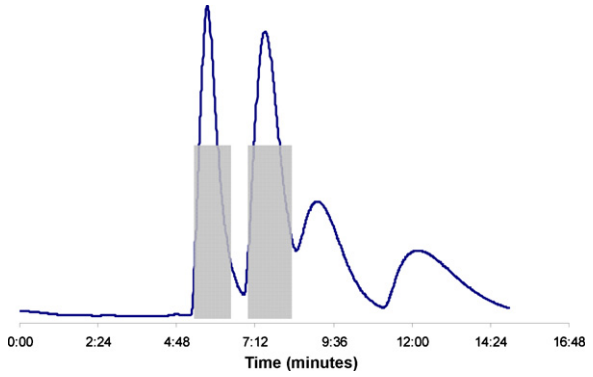

Fig. 6. Preparative HPLC separation of Racemate 2. The purification was conducted on Chiralpak IA (250 mm × 20 mm I.D.) with a mobile phase of 30/70 THF/heptane and a flow rate of 40 ml/min. 34 mg of material was injected.

Table 3
HPLC Results for Racemate 2

CSP	Mobile phase	Regioisomer Peak 1 t_r	Regioisomer Peak 2 t_r	Product Peak 1 t_r	Product Peak 2 t_r
Chiralpak IA	50/50 (v/v) dichloromethane/heptane	15.07	16.40	15.64	17.04
Chiralpak IA	30/70 (v/v) tetrahydrofuran/heptane	15.93	21.05	10.75	14.12
Chiralpak IA	50/50 (v/v) methyl *tert*-butyl ether	4.64	4.82	5.10	5.28
Chiralpak IA	50/50 (v/v) ethyl acetate/heptane	6.40	6.40	6.29	6.92

Table 4
SFC results for Racemate 3

CSP[a]	Co-solvent[b]	t_r^1	t_r^2
Chiralpak AD-H	Isopropanol	6.05	6.28
Chiralpak AS-H	Isopropanol	5.16	5.16
Chiralcel OD-H	Isopropanol	7.13	7.13
Chiralcel OJ-H	Isopropanol	4.13	4.13
Chiralpak IA	Isopropanol	5.82	6.04
Chiralpak AS-H	Ethanol	3.68	3.68
Chiralcel OD-H	Ethanol	4.87	5.82
Chiralcel OJ-H	Ethanol	6.99	6.99
Chiralpak IA	Ethanol	5.40	5.40

[a] Column dimensions: 4.6 mm × 150 mm.
[b] Flow rate: 1 ml/min, gradient conditions: hold at 5% for 1 min, 5–40% over 14 min.

THF/methyl *t*-butyl ether (Fig. 8). The sample was processed using 10 mg injections on a 10 mm × 250 mm column (Fig. 9). These purification conditions generated material with ee >96%. Additional achiral impurities were present in the resolved enantiomers and were removed using reverse phase preparative HPLC.

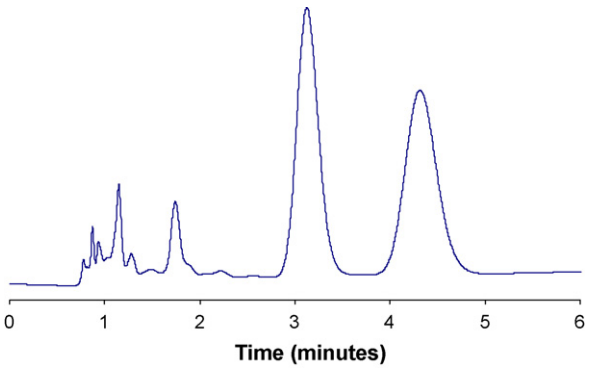

Fig. 7. Analytical HPLC separation of Racemate 3. Analysis conducted on Chiralcel OD (150 mm × 4.6 mm I.D.), mobile phase of 40/60 (v/v) ethanol/heptane and a flow rate of 1 ml/min.

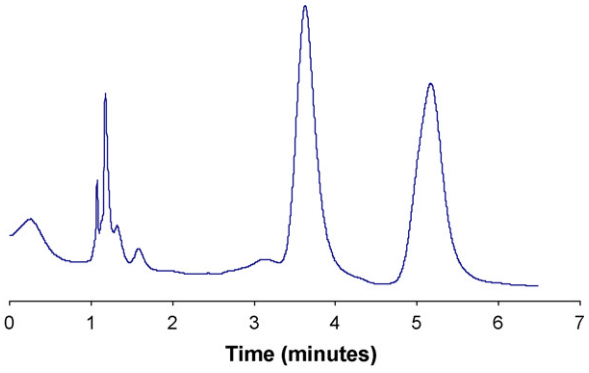

Fig. 8. Analytical HPLC separation of Racemate 3. Analysis conducted on Chiralpak IB (250 mm × 4.6 mm I.D.), mobile phase of 5/95 (v/v) THF/heptane and a flow rate of 2 ml/min. Peak at 0.2 min is from previous injection.

3.4. Case IV: utilization of immobilized chiral stationary phase (CSP)

While the screening protocol described in Table 1 is often successful (~95% of in house requests) in identifying acceptable conditions for purification of discovery scale quantities, there are instances where the standard approach does not yield satisfactory results. 1.2 g of material (Racemate 4) was submitted for resolution. The submitter provided HPLC conditions that were used at another laboratory to resolve this compound (Chiralcel OJ with 15/85 ethanol (0.1% DEA)/heptane). When these analytical conditions were explored in our laboratory, broad peaks were obtained. Using these conditions with small injection quantities with peak shaving and/or recycle conditions, material of sufficient purity could have been obtained. As greater than 1 g of material needed to be resolved, additional method development was performed. The SFC screen described in Table 1 gave no hits. Acetonitrile was also investigated as a co-solvent, but was not successful. A new CSP (Chiralpak IC) had recently been introduced to the marketplace and was evaluated for this racemate. An SFC method utilizing methanol w/0.2% diethylamine as a co-solvent gave good separation of the enantiomers while also resolving numerous achiral impurities (Fig. 10). This method was scaled up to preparative loadings (Fig. 11). Using these conditions approximately 600 mg of enantiomer 1 with an ee of >98% and 575 mg of enantiomer 2 with an ee of >99% were recovered.

3.5. Case V: sample precipitation during injection

A total of 4.3 g of material (Racemate 5) was submitted for resolution. The structure contained two chiral centers and existed as two trans isomers. HPLC method development using conditions in Table 1 showed late elution, necessitating the evaluation of polar organic mode. The results of this work are summarized in Table 5. The best separation was obtained on Chiralpak AD-H with an ethanol mobile phase. These conditions were used for purification.

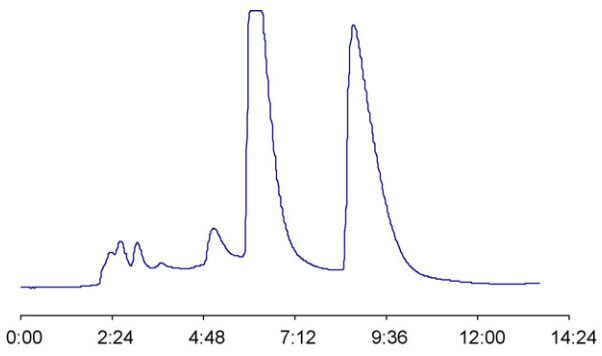

Fig. 9. Preparative HPLC separation of Racemate 3. The purification was conducted on Chiralpak IB (250 mm × 10 mm I.D.) with a mobile phase of 5/95 (v/v) THF/heptane and a flow rate of 10 ml/min.

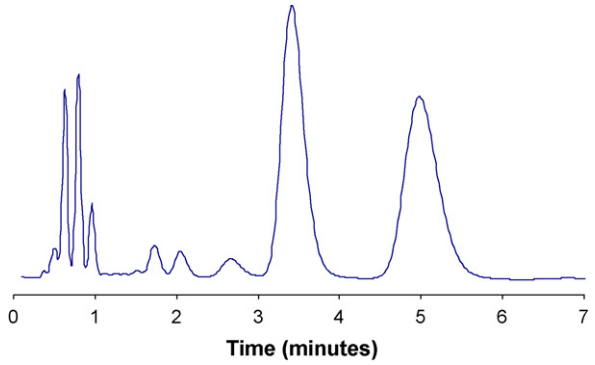

Fig. 10. Analytical SFC separation of Racemate 4. Analysis conducted on Chiralpak IC (100 mm × 4.6 mm I.D.), 65:35 CO_2–methanol (w/0.2% diethylamine), 40 C, 120 bar, 5 ml/min.

The enantiomer separation for this compound was large ($\alpha = 2.83$) which should result in a large loading capacity. Initial loading studies were performed with 5 ml injection volumes (~220 mg sample) via injection pump. The separation at this load was satisfactory and still larger injection volumes were explored. While resolution was sufficient at 7 ml injection volumes (~300 mg), larger amounts of racemate could be applied to the column before touching bands were observed. At the end of the injection cycle high column back pressure (>1500 psi) occurred, resulting in system shutdown. While the racemate had high solubility in ethanol (>40 mg/ml), upon introduction to the column there was an unknown interaction between the compound and the CSP/mobile phase that resulted in high pressure. Interestingly,

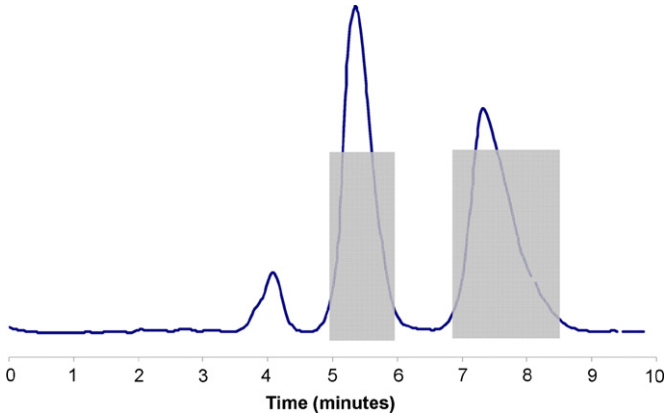

Fig. 11. Preparative SFC separation of Racemate 4. The purification was conducted on Chiralpak IC-H (250 mm × 20 mm I.D.) with a mobile phase of 55:45 CO_2–methanol (w/0.2% diethylamine), 40 C, 100 bar. A flow rate of 70 ml/min and a loading of 18 mg were used.

Table 5
SFC results for Racemate 5

CSP[a]	Mobile phase	k'_1[a]	k'_2[b]	α
Chiralpak AD-H	Ethanol	1.06	3.0	2.83
Chiralpak AS-H	Ethanol	1.07	2.05	1.92
Chiralcel OD-H	Ethanol	1.01	2.23	2.21
Chiralcel OJ-H	Ethanol	3.08	3.08	1.00
Chiralpak AD-H	Acetonitrile	0.88	0.88	1.00
Chiralpak AS-H	Acetonitrile	1.22	1.69	1.39
Chiralpak AD-H	Methanol	1.02	2.26	2.22
Chiralcel OD-H	Methanol	1.25	2.41	1.93

[a] Capacity factor for first eluting enantiomer.
[b] Capacity factor for second eluting enantiomer.

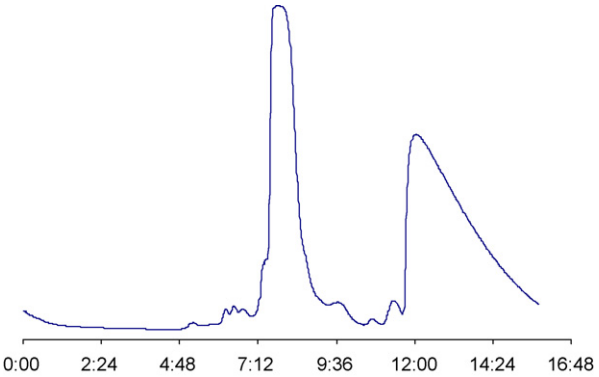

Fig. 12. Preparative HPLC separation of Racemate 5. The purification was conducted on Chiralpak AD (250 mm × 30 mm I.D.) with a mobile phase of ethanol and a flow rate of 25 ml/min.

after a few seconds the pressure dropped to normal (~1000 psi), allowing the purification to proceed. If larger quantities of racemate had to be resolved, alternate purification conditions would have been explored. As the quantity of racemate to be processed was small (<5 g) it was not necessary to explore an alternate purification process. In addition, the small quantity of racemate and the need to process the material quickly did not allow investigation of the cause of the pressure spike. To process the racemate, a 15 s hold with no flow was programmed immediately after the injection. The product was then eluted at normal flow rates. The remainder of the sample was processed using these conditions. The preparative chromatogram is shown in Fig. 12. From this purification 1.77 g of enantiomer 1 (ee = 100%) and 1.72 g of enantiomer 2 (ee = 99.2%) were generated.

4. Conclusions

Preparative resolution of enantiomers using HPLC and SFC is a powerful technique for rapid generation of enantiomers in pharmaceutical discovery. The use of SFC for analytical (SFC/MS) and preparative resolution of racemates has been shown to be particularly useful, reducing time requirements nearly two-fold relative to HPLC. While the broad applicability of CSP allows the majority of racemates to be purified routinely, there are some racemates that do not scale-up as expected due to low solubility, poor loading or other unknown reasons. The introduction of immobilized chiral stationary phases enables separation of racemates that would be very time consuming and/or impossible due to poor compound solubility.

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