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Rapid method development for chiral separation in drug discovery using sample pooling and supercritical fluid chromatography–mass spectrometry

Yining Zhao[a,*], Gregory Woo[b], Samuel Thomas[a], David Semin[a], Pat Sandra[c]

[a]*Discovery Analytical Science, Amgen Inc., One Amgen Center Drive, Mail Stop 29-M-B, Thousand Oaks, CA 91320, USA*
[b]*Research Informatics and Technology, Amgen Inc., One Amgen Center Drive, Mail Stop 29-M-B, Thousand Oaks, CA 91320, USA*
[c]*Research Institute for Chromatography, Kennedypark 20, B-8500 Kortrijk, Belgium*

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Abstract

A novel strategy for rapid chiral method development has been implemented using sample pooling and supercritical fluid chromatography–mass spectrometry (SFC–MS) on four chiral stationary phases, namely Chiralpak AD and AS, and Chiralcel OJ and OD, and eight different modifier concentrations (5 to 40% methanol–0.2% isopropylamine). The screening is performed under an outlet pressure of 110 bar at 35 °C, and at a flow-rate of 2.5 ml/min for the initial 20 min and then ramped up to 4 ml/min and held for 4.5 min to elute all solutes from the column. The entire process is fully automated from injection to data processing, and operates unattended for 15 h overnight to obtain optimal chiral separation for multiple compounds. A unique feature of using SFC–MS to monitor chiral synthesis is the negligible interferences from achiral impurities. In addition, with SFC–MS, enantiomeric excess can be determined with much lower detection limits than UV and much shorter analysis times compared to normal-phase/reversed-phase liquid chromatography.
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1. Introduction

As part of the continuing effort to select safe and effective drug candidates from thousands of lead compounds for clinical trials, attention has been focused on stereochemistry. Since the role of chirality has a large impact on drug profiles as a determinant of drug action, metabolism and toxicity [1,2], it has been considered one of the critical factors of drug developability.

As a result, the importance of enantioselective analysis has been addressed in the early lead optimization stage of drug discovery. The focal point has been how conventional method development strategies for chiral separation can meet the requirement of the highly demanding discovery analytical environment. In the discovery stage, the cycle time of developing methods for a large number of structurally diversified compounds is the key as opposed to the method's robustness in the development arena. To be specific, there are three major hurdles for rapid chiral

*Corresponding author. Tel.: +1-805-447-6742; fax: +1-805-480-3015.
E-mail address: yzhao@amgen.com (Y. Zhao).

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method development in drug discovery: (1) the number of compounds requested for chiral separation can be quite significant and one can expect multiple compounds from each project requested for chiral separation and/or purification on a daily basis due to the widely adopted parallel synthesis strategy; (2) the wide variety of compounds can be starting materials, intermediates or drug substances with only relatively small amounts (10 mg to 10 g) adequate to satisfy preclinical in vitro or in vivo testing, because, in most cases, the pure enantiomer is obtained by chiral separation instead of a time-consuming chiral synthesis; (3) the achiral purity of those compounds very often can be less than 90%, sometimes merely over 70%. Achiral impurities can severely interfere with the chiral method development.

To tackle these challenges, the first step is to increase the efficiency of method development so as to handle more chiral separation requests in a timely manner. The most effective and practical approach used so far turns out to be the rapid screening of an array of conditions, e.g., various chiral stationary phases (CSPs) and mobile phases, with separation techniques commonly used in the pharmaceutical industry, such as normal-phase/reversed-phase liquid chromatography (NP/RPLC) [3,4], capillary electrophoresis (CE) [5] and supercritical fluid chromatography (SFC) [6]. Because chiral discrimination is a very complex phenomenon and sometimes depends on little known properties, it is almost impossible to predict which CSP and modifier combination will provide the best separation [3,7]. Optimal conditions can vary greatly and are compound-specific. It is not unusual that a slight change in just one functional moiety on a molecule may require totally different CSPs and/or modifiers [6] in order to achieve the desired enantiomeric resolution. The aim of a screening strategy is not to achieve optimal separations, but to serve as a ''filter'' to rapidly determine which conditions can achieve an acceptable separation, and comprise a good starting point for further method optimization [3]. The screening also determines whether SFC–MS is a suitable technique for the set of compounds.

Of the four screening techniques (NP/RPLC, CE and SFC), SFC is presently considered the first try for chiral separation because of its unique advantages over LC and CE in the drug discovery arena where high speed and high separation efficiency are greatly appreciated. Due to the low viscosity and high diffusivity of the eluent, SFC provides fast mass transfer and thus allows high flow-rates combined with fast column equilibration. Also, from an industrial perspective, SFC has proven to be cost-effective and can be considered a ''green'' technique for chiral purification by reducing organic solvent consumption and replacing flammable toxic solvents such as hexane, chloroform, etc.

Chiral SFC applications with packed columns [8–13] and a screening approach using automated column and modifier selection devices [6] have been extensively reported for over a decade, however most of the previous approaches have not taken advantage of the MS selectivity and sensitivity to help develop chiral methods or to determine the enantiomeric excess (EE%) in a complex mixture or matrix. Only a few scientists, e.g. Baker and Pinkston [14], have investigated the use of coupling SFC to mass spectrometry (MS) for chiral method development. In this contribution, a novel approach using SFC–MS and sample pooling for rapid chiral method development is presented. The extracted ion chromatogram (EIC) function of the MS is used to distinguish the enantiomers. Sample pooling substantially increases the throughput without fear of interferences from achiral impurities with the exception of structural isomers. Moreover, in the presented approach, selected conditions also take other aspects into consideration such as purification by semi-preparative operation and method transfer.

2. Experimental

2.1. Materials

Carbon dioxide (SFC grade) was obtained from BOC Gases (Murray Hill, NJ, USA). All chiral compounds were synthesized in-house. Methanol (MeOH) and isopropanol (IPOH) were HPLC-grade from Mallinckrodt Baker (Muskegon, MI, USA). Isopropylamine (IPA), diethylamine (DEA), triethylamine (TEA) and trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Chiral stationary phases

Columns packed with Chiralpak AD and Chiralpak AS, both amylose derivatives, and Chiralcel OD and Chiralcel OJ, both cellulose derivatives, were purchased from Chiral Technologies (Exton, PA, USA). Columns are referred with a two-letter designation, i.e. AD, AS, OD, or OJ, throughout the paper. Column dimensions were 25 cm×4.6 mm I.D., 10 μm particle size.

2.3. Sample preparation

Samples were dissolved to a concentration of approximately 0.1 mg/ml in methanol, and pooled into one mixture. Some samples with poor solubility in methanol were supplied in 10 μl 10 m*M* DMSO solutions, which were subsequently diluted 1000-fold in MeOH before injection.

2.4. Instrumentation

The heart of the chiral method development system is a Berger Supercritical Fluid Chromatograph (SFC) unit with a dual pump control module, an FCM1200 flow control module, a TCM2100 thermal column module (with temperature accurately controlled in the range 7–150 °C) and column and solvent selection valves that automatically attempts up to six different CSPs and four different modifiers all from Berger Instruments (Newark, DE, USA). The SFC was completed with a CTC LC Mini PAL autosampler (Leap Technologies, Carrboro, NC, USA), an Agilent 1100 photodiode array detector with a high-pressure flow cell (Agilent Technologies, Palo Alto, CA, USA), and a Waters ZQ benchtop single quadrupole mass spectrometer (Waters, Milford, MA, USA) with an APCI source. A post-column make-up fluid of MeOH can be delivered by an Agilent 1100 HPLC quaternary pump (Agilent Technologies) to improve the MS ionization, especially under ESI conditions. Instrument control, data acquisition and analysis for both SFC and MS are facilitated from one software platform by integrating the SFC ProNTo with the Waters MassLynx software. A schematic representation of the SFC–MS system is shown in Fig. 1.

An automatic data processing routine was written in Microsoft Visual Basic 6.0. This was accomplished by accessing the provided API libraries from Waters MassLynx v 3.4. Prior to data acquisition, the masses of each compound in the pool are entered into the Masslynx sample list. The macro was compiled into a windows executable file and con-

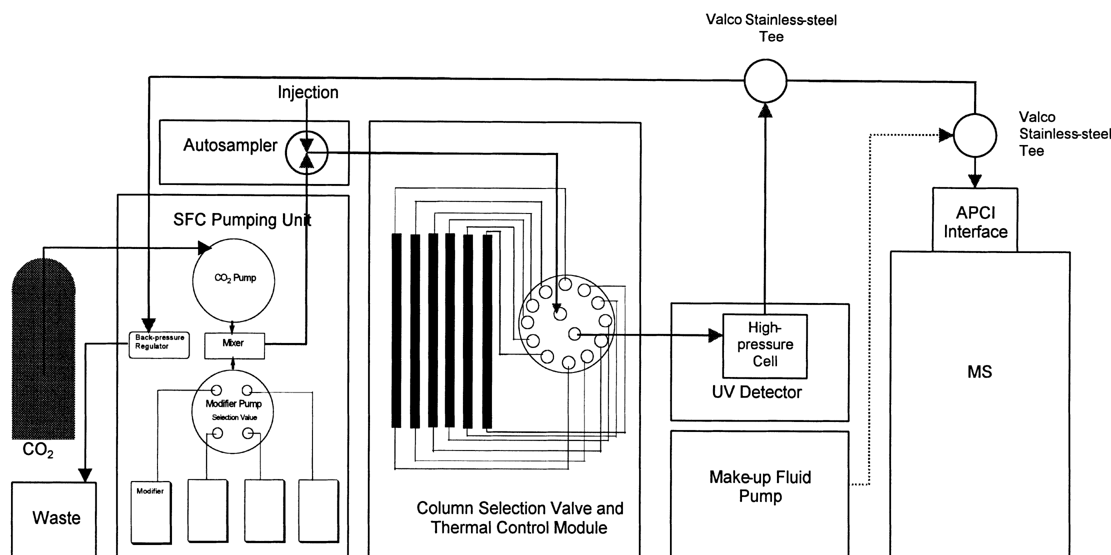

Fig. 1. Schematic diagram of the SFC–MS system with mobile phase flow path.

Table 1
Experimental conditions for the chiral SFC–MS screening strategy

Column	AD, AS, OJ, OD installed in an oven with automatic column switcher and screened in this order
Mobile phase	A: CO_2, B: MeOH with 0.2% IPA or 0.1% TFA
Temperature	35 °C (isothermal)
Outlet pressure	110 bar
Gradient	Screening performed under eight gradients (eight starting points), starting with B (%): 40, 35, 30, 25, 20, 15, 10 and 5%, hold for 20 min and ramp up to 45% within 0.25 min, and hold for 4.5 min to elute all the compounds, and then return to 40% within 0.25 min
Analysis time	Each run time is 25 min plus 1.5 min column equilibration time and 0.5 min autosampler injection time. The total screening sequence runs through four columns and eight gradients which takes about 15 h (often set as an overnight run in our laboratory)
Flow-rate	2.5 ml/min isocratic for 20 min and then ramp up to 4.5 ml/min within 0.25 min and hold for 4.5 min, then return to 2.3 ml/min within 0.25 min
Detection	UV at 215 and 254 nm. MS with APCI source in positive mode
Injection volume	10 μl
MS operating conditions	Corona current, 4.6 μA; cone voltage, 25 V; extractor voltage, 6 V; RF lens voltage, 0.3 V; source temperature, 146 °C; APCI probe temperature, 350 °C; desolvation gas flow, 362 l/h; cone gas flow, 50 l/h; scan range, 100–900 amu

figured to execute once after each injection. The program prints out the total ion chromatogram (TIC) along with the individual extracted ion chromatograms (EICs) for each compound of the pool.

2.5. Analysis conditions

The experimental conditions for the SFC–MS screening strategy are described in Table 1. Conditions for further method optimization or eventually preparative purification are mentioned in the context of Results and discussion.

3. Results and discussion

3.1. Experimental design

SFC has been successfully applied with simple mobile phase compositions to nearly all commercially available CSPs [15–18]. Four types of polysaccharide-based CSPs (cellulose and amylose derivatives), i.e., AD, AS, OJ and OD, were selected because they have proved to be able to resolve more than 80% of the chiral drugs currently on the market [19]. Based on our and other groups' observations [6,20], the rate for successful chiral separation seems to be in the order AD>AS>OJ>OD. Therefore, the columns were also screened in that order in an attempt to achieve as fast a separation as possible. MeOH, unlike in NPLC, is preferred over EtOH and IPOH as the starting modifier in SFC–MS for several reasons. MeOH combines low viscosity and high polarity with a low boiling point, which is favorable when the method is transferred to preparative purification. Moreover, because of its lower surface tension it gives better ionization efficiency and sensitivity in MS compared with EtOH and IPOH. 0.2% IPA was chosen as additive instead of the widely accepted DEA [11] or TEA [12] to improve the peak shapes of basic solutes by masking residual silanol groups on the CSPs. With IPA, no impact on bifunctional compounds or degradation of the peak shapes of most of the acids was noted. Unlike in LC where additive effectiveness follows basicity, in SFC the additive effectiveness might also be affected by steric considerations. It was observed that sterically hindered amines, e.g. TEA, are much less effective than primary amines, even though they are generally stronger bases, because they are much easier to elute

than primary amines [21,22]. In addition, IPA has a lower boiling point (34 °C) than DEA (55 °C) and TEA (89 °C), which is considered important when transferring the method to preparative purification where all traces of additives and solvents must be removed under mild temperatures to avoid compound decomposition. Although a number of studies [11,14] have shown that simultaneously adding a basic additive and an acidic additive such as TFA to the eluent can lead to an improvement in enantioselectivity for both basic and acidic compounds, this is not generally applicable and very much compound-dependent. It may also complicate the interpretation of the separation profile, misleading analysts to come to the wrong conclusion, particularly when sample pooling is used. On the other hand, most of the pharmaceutical compounds and in-house potential drug candidates are basic and/or neutral [23] (>75%) and IPA is most appropriate. If needed, before pooling the samples, the basics/neutrals and acids are segregated. The mobile phases are MeOH–0.2% IPA and MeOH–0.1% TFA for basic/neutral and acidic compounds, respectively.

The temperature and outlet pressure are set at 35 °C and 110 bar during screening to avoid modifying too many variables and also due to their relatively smaller impact on resolution than selection of CSPs and modifiers. However, they can be varied for further optimization if needed as will be illustrated further. The flow-rate is set at 2.5 ml/min for the analytical columns (4.6 mm I.D.) because it can later be proportionally scaled up to 50 ml/min for 20 mm I.D. columns for semi-preparative purification without much change in the peaks' retention times or capacity factors and resolution.

Based on our previous investigations of SFC–MS [24] and RPLC–MS with sample pooling for high-throughput analysis [25], the SFC-based chiral method screening approach proposed by Villeneuve and Anderegg [6] was strongly improved by coupling SFC to MS, and by using extracted ion chromatograms (EIC) to distinguish the different molecular ions of pooled compounds and monitoring each compound's separation profile during method development. In principle, one can pool as many compounds as one wants as long as the compounds have different molecular masses. However, in practice, we only pool up to 12 samples into one mixture in order to avoid ion suppression and obtain better signals in the MS. The data processing program was written to automatically print out each compound's chiral separation profile after each single run by compiling their EICs onto one page. This allows one to visually select the best separation conditions and then decide whether further optimization is required.

Since the implementation of this screening strategy, all of our chiral separation requests, representing a wide variety of chemical classes and broad mass ranges (75–900 amu), have been performed by the described strategy in approximately one-fourth of the time it used to take.

Examples have been selected to demonstrate how the overall screening process works and how the best screening result is then transferred to perform further method optimization, preparative purification, or EE% determination in various matrices.

Six racemic compounds from three different projects with different pharmacophores and all with basic/neutral characteristics were pooled into one mixture and injected onto the SFC–MS system for chiral method development. After screening 32 conditions (four CSPs times eight different modifier concentrations), 32 printouts, each containing a MS TIC and six EICs for all compounds, were generated automatically. As an example, Fig. 2 shows the chiral separation profiles of each compound on the AD column at 20% (A) and 25% MeOH (B), respectively. All other conditions are the same.

Under these two conditions, compounds **2**, **3** and **5** are baseline separated, **1** and **6** are almost separated while **4** shows no separation at all. At 15% MeOH concentration, **1** was baseline separated, but **6** was not. For that particular compound, lower MeOH concentrations resulted in baseline separation, but the dataset for the OJ column was applied for optimization because of the shorter analysis times on OJ compared to AD (see further). For compound **5** the modifier at 30% provided baseline separation with better peakshape for the first enantiomer compared to 20% MeOH and a shorter analysis time compared to 25% MeOH (see further). In the fine-tuning process for compound **6** the MeOH modifier was replaced by IPOH, giving baseline separation on OJ with 15 or 10% modifier in very short analysis times (Fig. 3).

In order to transfer the method to a purification process, the highest resolution is desired to allow

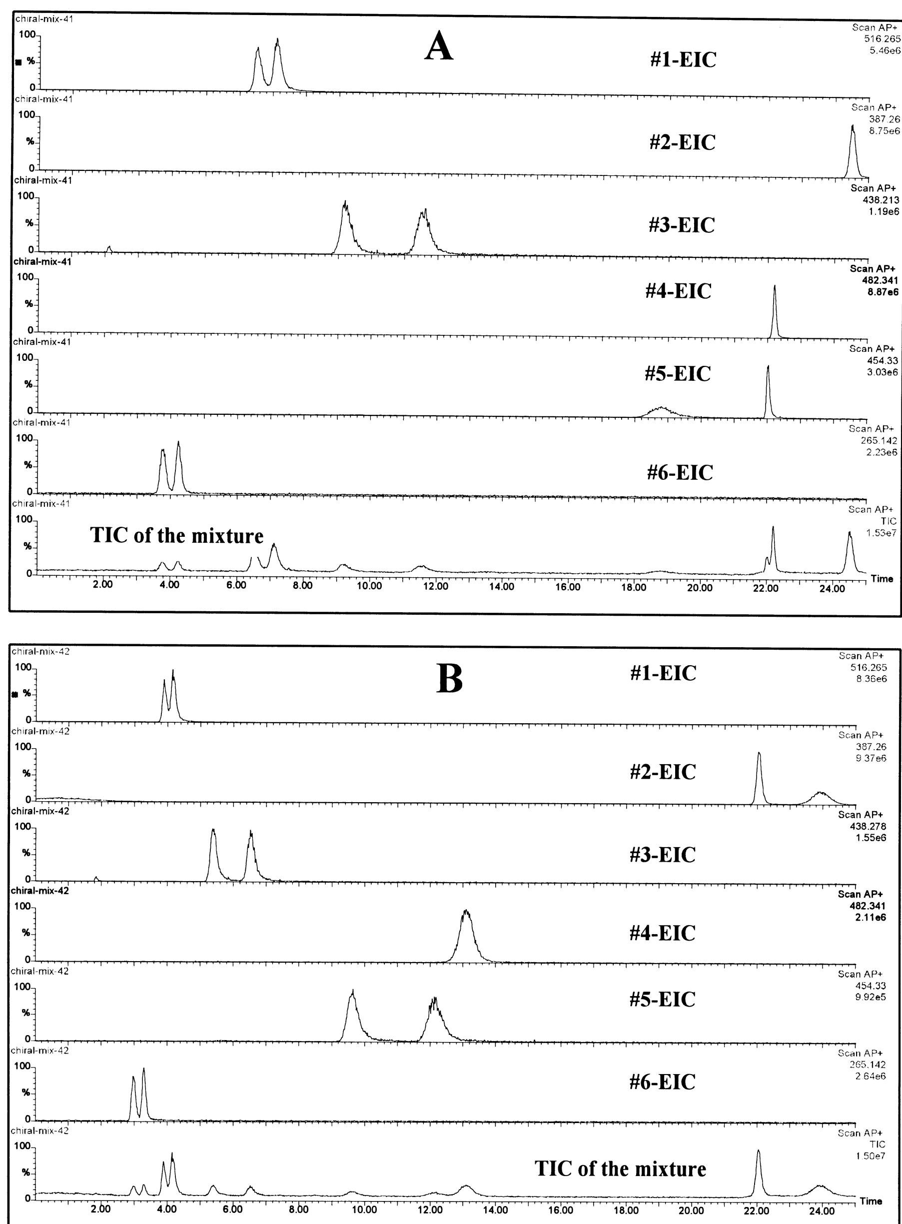

Fig. 2. TIC of six pooled compounds and each compound's EIC under the conditions of 20% MeOH–0.2% IPA (A) and 25% MeOH–0.2% IPA (B) on AD, 2.5 ml/min, 35 °C and 110 bar outlet pressure. The printout is generated automatically at the end of the run.

large sample loading onto a preparative column and therefore 10% modifier was chosen as an optimum for compound **6**.

Concerning compound **2**, although a good resolution is achieved on AD using 35% modifier (Fig. 4), in order to apply this method in a high-throughput fashion for the determination of the EE% in biological matrices, we tried to improve the peak shape (especially for the second peak) by increasing the flow-rate to 4 ml/min, the temperature to 40 °C, and the outlet pressure to 120 bar. As a result, the enhanced separation efficiency significantly reduces

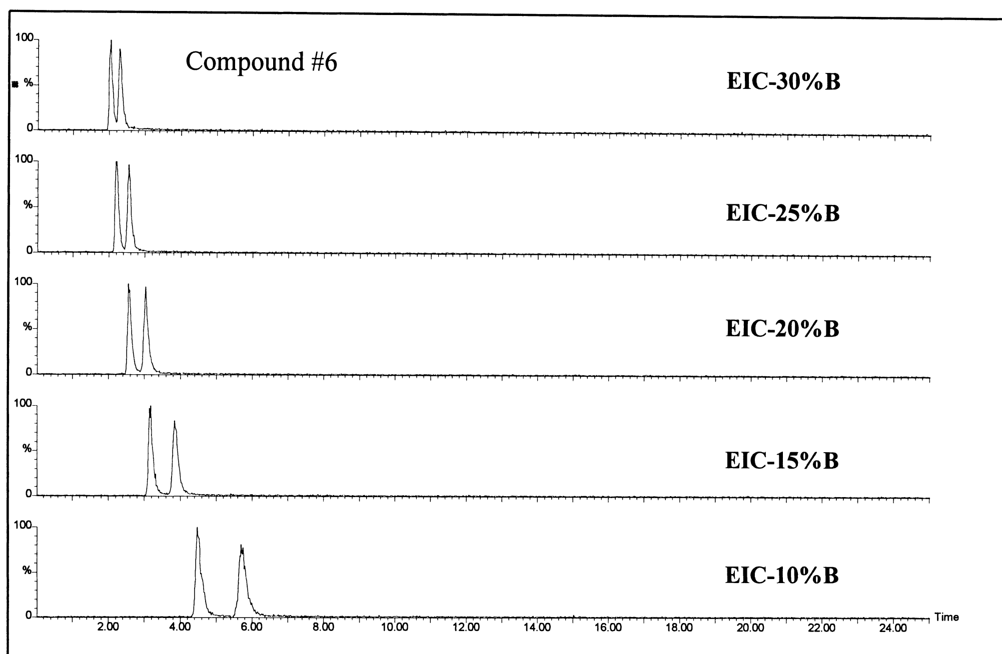

Fig. 3. Chiral method screening of compound **6** on OJ with a different modifier and content (IPOH–0.2% IPA) at 2.5 ml/min, 35 °C and 110 bar outlet pressure.

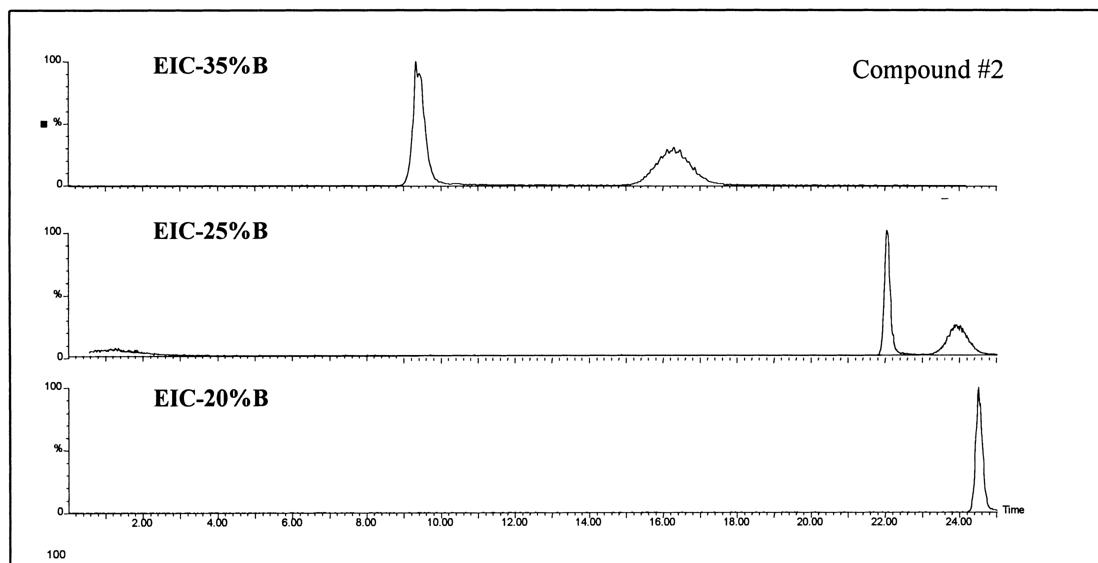

Fig. 4. Chiral method screening of compound **2** on AD with different modifier contents (MeOH–0.2% IPA) at 2.5 ml/min, 35 °C and 110 bar outlet pressure.

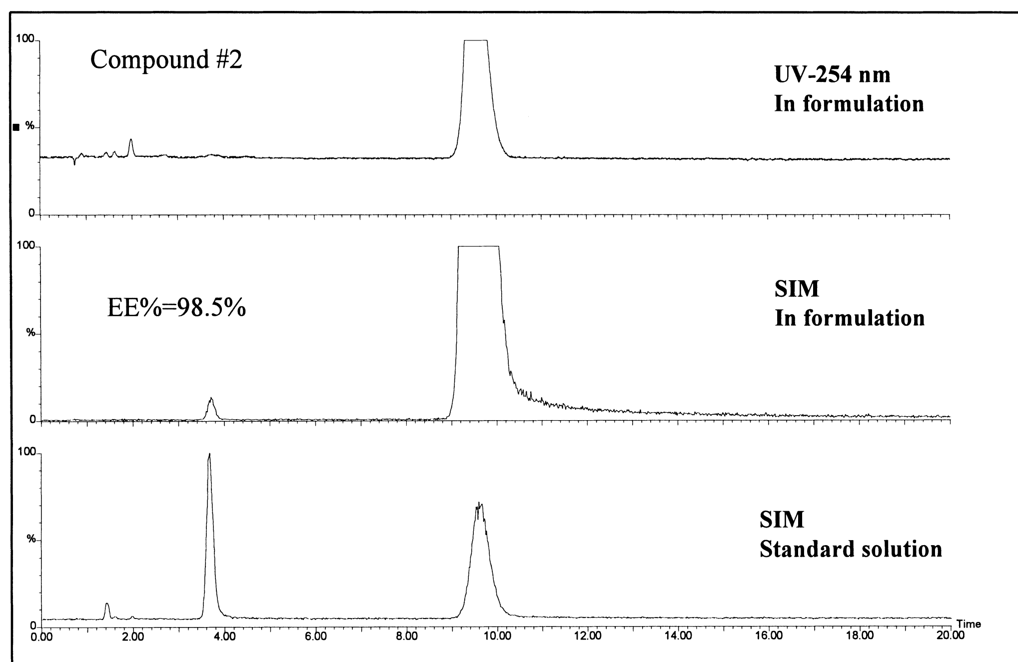

Fig. 5. Enantiomeric excess determination (EE%) of compound **2** in a formulation using UV and SIM under the conditions of 35% MeOH–0.2% IPA, 4 ml/min, 40 °C and 120 bar outlet pressure.

the MS detection limit owing to a higher signal-to-noise ratio (Fig. 5). Because of its high sensitivity and selectivity, single ion monitoring (SIM) offers at least 10 times the sensitivity of UV as compound **2** has a relatively weak UV absorption in the formulation.

Finally, compound **4** was found to be separated on the OJ column with 15% MeOH–0.2% IPA. The optimal conditions for each compound in this pool are shown in Fig. 6.

For real-world analysis in drug discovery, a compound's achiral purity is not always controlled when it is submitted for chiral separation. Particularly when exploring a new synthetic route, medicinal chemists only want to first monitor the chirality of the compound, and leave all the achiral impurities for later purification. In these cases, SFC–MS plays an indispensable role in providing chemists with a quick answer to their chiral synthesis without requiring pure racemates. Fig. 7 shows a typical example of a SFC–MS chiral separation of an impure intermediate with two chiral centers. Based on the MS EICs, one can distinguish the impurities from the enantiomers, which definitely cannot be achieved by UV.

4. Conclusion

A novel SFC–MS-based screening strategy for rapid chiral method development in drug discovery is proposed. Implementing this strategy together with sample pooling allows us to screen an array of conditions for multiple samples at the same time by taking advantage of the selectivity and sensitivity of MS. Moreover, SFC–MS provides much lower detection limits than UV and shorter analysis times than NP/RPLC for the determination of EE%. A similar approach of efficiently developing chiral methods could also be applied to NP/RPLC–MS. Since much effort has recently been expended in trying to extend SFCs unique capabilities into a highly regulated environment [26], we believe that, sooner or later, it will be accepted to a greater extent under cGMP conditions because of the high-throughput features of SFC.

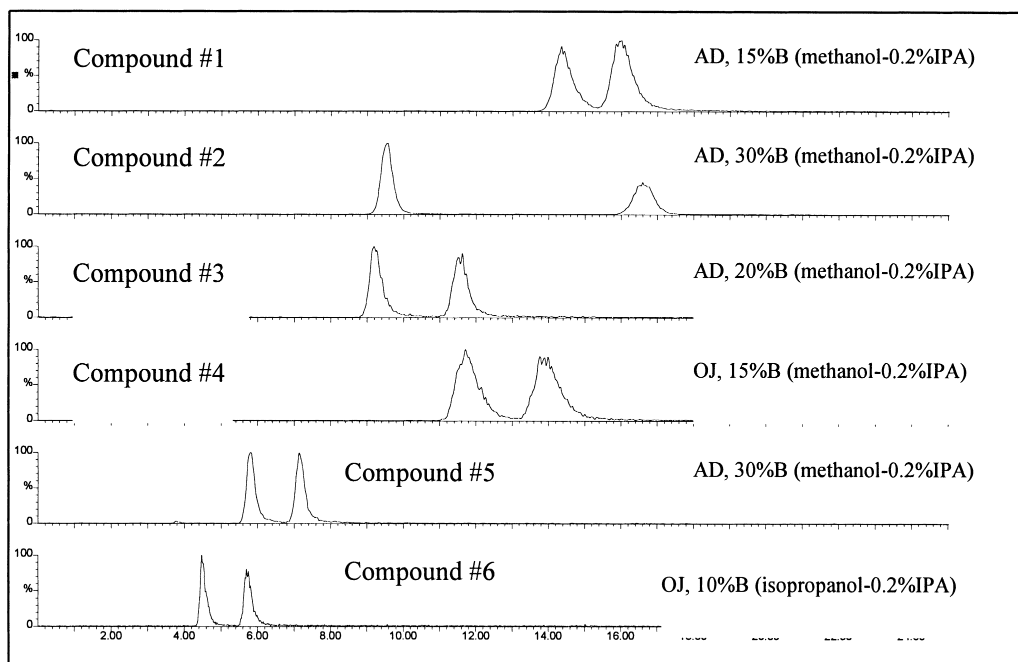

Fig. 6. Optimal conditions obtained from the screening at 2.5 ml/min, 35 °C and 110 bar outlet pressure.

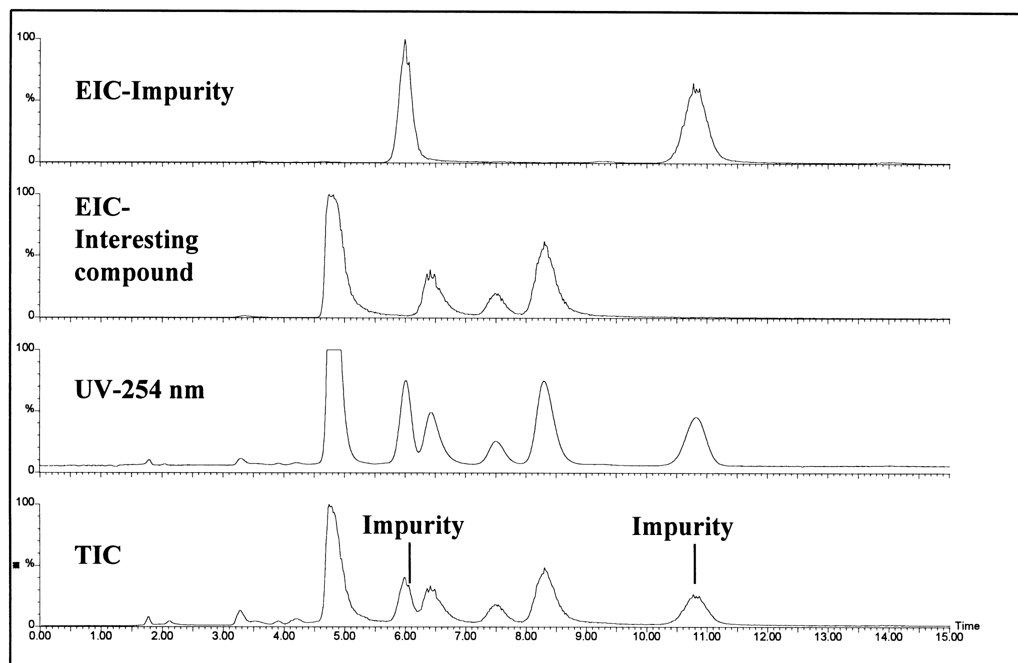

Fig. 7. SFC/UV–MS chiral separation of an interesting compound with two chiral centers under the conditions of 20% IPOH–0.2% IPA at 2.5 ml/min, 40 °C and 120 bar outlet pressure.

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