Journal of Chromatography A, 1218 (2011) 3080–3088

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Two-dimensional supercritical fluid chromatography/mass spectrometry for the enantiomeric analysis and purification of pharmaceutical samples

Lu Zeng*, Rongda Xu, Yinong Zhang, Daniel B. Kassel

Takeda San Diego, Inc., 10410 Science Center Drive, San Diego, CA 92121, USA

ARTICLE INFO

Article history:
Received 29 January 2011
Received in revised form 13 March 2011
Accepted 16 March 2011
Available online 12 April 2011

Keywords:
Two-dimensional SFC/SFC/MS
Two-dimensional achiral and chiral chromatography
Enantioselective method development and optimization

ABSTRACT

A new analytical two-dimensional supercritical fluid chromatography/mass spectrometry system (2D SFC/SFC/MS) has been designed and implemented to enhance the efficiency and quality of analytical support in drug discovery. The system consists of a Berger analytical SFC pump and a modifier pump, a Waters ZQ 2000 mass spectrometer, a set of switching valves, and a custom software program. The system integrates achiral and chiral separations into a single run to perform enantiomeric analysis and separation of a racemic compound from a complex mixture without prior clean up. The achiral chromatography in the first dimension separates the racemate from all other impurities, such as un-reacted starting materials and by-products. Mass-triggered fractionation is used to selectively fractionate the targeted racemic compound based on its molecular weight. The purified racemate from the achiral chromatography in the first dimension is then transferred to the chiral column in the second dimension to conduct the enantiomeric separation and analysis. A control software program, we coined SFC2D, was developed and integrated with MassLynx to retrieve acquisition status, current sample information, and real time mass spectrometric data as they are acquired. The SFC2D program also monitors the target ion signal to carry out mass-triggered fractionation by switching the valve to fractionate the desired peak. The 2D SFC/SFC/MS system uses one CO_2 pump and one modifier pump for both first and second dimension chromatographic separations using either gradient or isocratic elution. Similarly, a preparative 2D SFC/SFC/MS system has been constructed by modifying an existing Waters preparative LC/MS system. All components except the back pressure regulator are from the original LC/MS system. Applications of the 2D SFC/SFC/MS methods to the separation and the analysis of racemic pharmaceutical samples in complex mixtures demonstrated that an achiral separation (in first dimension) and a chiral separation (in second dimension) can be successfully combined into a single, streamlined process both in analytical and preparative scale.

1. Introduction

Structure based drug design is now commonplace in drug discovery laboratories. Access to protein crystal structure information allows for the design of highly selective and potent compounds based on 3-dimensional shape and pharmacophore features (i.e., key recognition features between functional groups on the designed compound and active site of the protein) [1,2]. One of the byproducts of early access and use of protein crystal structure information is that enantiomeric compounds are now routinely designed and selected for synthesis at earlier stages of the drug discovery. Most of these compounds are synthesized as crude racemic mixtures, since chiral synthesis of the desired enantiomer may not be feasible or cost effective at early stage of drug discovery. This presents a challenge for analytical discovery labs, as most of these labs are neither set up to rapidly establish a chiral method sufficient to support purification of single enantiomers from racemic mixtures nor are they set up to assess enantiopurity of the isolated product. Even for those few analytical discovery labs equipped with achiral and chiral capabilities, to obtain pure enantiomers from crude racemic mixtures generally requires that the samples be purified in a two-step process. The first step is to remove unreacted starting materials, reagents and reaction by-products by achiral reversed phase or normal phase liquid chromatography (LC). Following solvent removal, the purified racemate is then reconstituted and subjected to chiral chromatography, either in normal phase LC (rarely reversed phase) or supercritical fluid chromatography (SFC), to separate the two enantiomers, allowing for isolation of the desired product. A two-step purification process is generally required because the analysis and purification of an impure racemate without prior clean up is usually not feasible. Enantioseletive chromatographic method development using impure racemate samples is often unreliable because the impurity may co-elute with the targeted

* Corresponding author. Tel.: +1 858 731 3566; fax: +1 858 550 0526.
E-mail address: lu.zeng@takedasd.com (L. Zeng).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.03.041

enantiomer. Secondly, repetitive loading of crude samples onto the chiral column significantly reduces column lifetime. Because chiral columns are generally 3–4 times more costly than achiral columns, such an approach may become cost prohibitive. Such a two-step process is time-consuming, leading to unnecessary delays in the preparation of the desired enantiomer for biological screening.

Two-dimensional liquid chromatography (2D LC) has been used for the separation of complex mixtures [3–5], the primary benefit being that higher selectivity and resolving power can be achieved relative to one-dimensional chromatography. 2D LC has been applied in a few different ways. When used in an unbiased (or comprehensive 2D analysis) mode, all components from the first dimension separation are subjected to separation in the second dimension, generating a complete 2D map for all resolvable components. When applied to biased or target analysis, a heart-cutting 2D chromatography approach is applied [6,7]. Heart-cutting approaches have included achiral/achiral 2D LC and achiral/chiral 2D LC, the latter used for the analysis of enantiomers from complex samples (such as plasma, serum, urine, etc.) [8–13]. The achiral/chiral 2D LC systems usually incorporate ultraviolet (UV) [8–11], fluorescence [12], or mass spectrometric (MS) detection [13]. For achiral/chiral 2D LC, the first column is used for the extraction and clean-up of the interference materials and the second column for the analysis and separation of the enantiomers. However, one of the drawbacks of current 2D LC systems is that they are complex in design, consisting of multiple sets of pumps and multiple switching valves to support sample transfer from one column to the other. Furthermore, if different solvents are used in the first and second dimensions, the incompatibility of solvents may cause band dispersion or broadening and result in the failure of the chromatography [14]. In addition, column equilibration in two dimensions may be quite time consuming and challenging.

SFC offers unique advantages over LC for 2D enatioselective analysis and separation. Solvent compatibility between the first and the second column chromatography is generally much less of a concern since achiral and chiral SFC methods utilize the same solvent types. Column equilibration can be achieved much faster in comparison to LC because higher flow rates can be achieved due to the low viscosity of CO_2. Two-dimensional SFC/SFC (2D SFC) systems have been reported previously for analyses of complex samples using open tubular columns [15] and packed capillary columns [16] in both first and second dimensions, or packed capillary column in the first dimension and open tabular column chromatography in the second dimension [17]. These 2D SFC systems have incorporated flame ionization detection. Recently, 2D SFC systems using packed columns in both dimensions with UV detection have also been reported [18,19]. These packed 2D SFC systems were designed for comprehensive 2D chromatography with fractionation in the first dimension by stopping the flow [18] or fractionation based on expected elution times of the desired components [19]. Such systems are not ideally suited to targeted analyses due to their long run time. Serial-based-chromatography (also referred to as tandem column chromatography), which is achieved by direct coupling in series of either an achiral or chiral column to a chiral column, has been reported for the SFC analysis of enantiomeric β-blockers in complex samples [20] as well as for the SFC/MS analysis of diastereomers/enantiomers [21]. However, to realize the gains in selectivity and resolving power by coupling two types of columns in series, extensive screening experiments for the combination of columns and solvent conditions have to be conducted until satisfactory separations can be achieved [22]. To date, there have been no reports on targeted analyses using 2D SFC.

Here we report the design and implementation of a new analytical and preparative two-dimensional SFC/SFC/MS system that overcomes the limitations of existing approaches. With this method, it is possible to achieve rapid and efficient targeted enantioseletive analysis and purification in a single run. A single quadrupole mass spectrometer is used as the detector, allowing real-time mass monitoring of the two chromatographic processes in the first and second dimensions. Only one peak (containing the desired racemate) is targeted for fractionation from the first dimension (achiral column) and transferred to the second dimension (chiral column). Mass-triggered fractionation [23–25] is applied to facilitate the peak cutting as well as fraction transfer. The 2D SFC/SFC/MS systems use one CO_2 pump and one modifier pump for both first and second dimension chromatography with either gradient or isocratic elution through the well designed valve interface. The introduction of these new two-dimensional, orthogonal achiral–chiral separation methods for the pharmaceutical samples has improved the quality of enantioselective analysis and purification whilst reducing turn-around time.

2. Experimental

2.1. Chemicals

HPLC grade methanol (MeOH) and 2-propanol (IPA) were acquired from Fisher Scientific (Pittsburgh, PA, USA). Formic acid and ammonium acetate (NH_4OAc), cyclohexylamine (CHA) were purchased from Aldrich (Milwaukee, WI, USA). SFC grade liquid carbon dioxide for chromatographic separations was supplied by Airgas (Radnor, PA). The β-blockers, oxprenolol (OX), propranolol (PR), atenolol (AT), and pindolol (PI) as well as bupivacaine and warfarin were purchased from Sigma (St. Louis, MO, USA). Fmoc-Glu (N-α-Fmoc-L-glutamic acid γ-*t*-butyl ester) was purchased from EDM Chemicals (Gibbstown, NJ, USA). All other test compounds, including samples A, B, C and D were obtained from our synthetic laboratory.

2.2. Mass spectrometry

All analytical 2D SFC/MS studies were carried out on a Waters ZQ 2000 single quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ion source (ESI). Data was acquired in the positive selected ion monitoring (SIM) mode. Dwell time was 0.1 s with an inter-spray scan delay time of 0.1 s. The ion source parameters were used as following: sprayer voltage, 3.2 kV; cone voltage, 25 eV; desolvation temperature, 350 °C; and source temperature, 150 °C. The instrument resolution was 1000 (10% valley definition). MassLynx 4.1 software was used for data acquisition. To enhance ionization, a make-up flow of 0.02 mL/min of methanol–water (90/10 plus 0.05% formic acid) was added, by aid of a mixing tee, to the SFC column effluent prior to the ESI interface.

All preparative 2D SFC studies were carried out on a Waters SQD single quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ion source (ESI). Data was acquired in the positive selected ion monitoring (SIM) mode. Dwell time was 0.1 s with an inter-spray scan delay time of 0.1 s. The ion source parameters were used as following: sprayer voltage, 3.0 kV; cone voltage, 25 eV; desolvation temperature, 300 °C; source temperature, 120 °C; cone gas flow at 50 L/h; and desolvation gas flow at 500 L/h. The instrument resolution was 1000 (10% valley definition). MassLynx 4.1 software was used for data acquisition. To enhance ionization, a make-up flow of 0.1 mL/min of methanol–water (90/10 plus 0.05% formic acid) was added, by aid of a mixing tee, to the SFC column effluent prior to the ESI interface.

2.3. Supercritical fluid chromatography

For analytical SFC, a Berger Analytical SFC System (Thar Instrument/Waters Corp., Milford, MA, USA) equipped with a Berger

Table 1
Chromatographic conditions for analytical and preparative 2D SFC/SFC/MS.

Sample name	1st dimension SFC			2nd dimension SFC		
	Column[a] (mm)	Modifier[b] (gradient)	Flow rate (mL/min)	Column[c] (mm)	Modifier[b] (isocratic)	Flow rate (mL/min)
Sample A	2.1 × 200	MeOH–NH_4OAc (10–55%)	2	ChiralPak AD-H 2.1 × 150	MeOH–NH_4OAc (15%)	2
Sample B	4.6 × 300	MeOH–NH_4OAc (10–30%)	3	ChiralPak AD-H 4.6 × 250	IPA–cyclohexylamine (40%)	3
Sample C	4.6 × 150	MeOH–NH_4OAc (10–55%)	3	ChiralCel OJ-H 4.6 × 150	Acetronitrile (20%)	3
Sample D	19 × 150	MeOH (10–40%)	50	ChiralCel OJ-H 21 × 150	MeOH (40%)	40

[a] Pyridine columns (particle size 5 μm) were used in the 1st dimension.
[b] The concentrations of modifiers were 10 mM for NH_4OAc and 0.2% for cyclohexylamine, respectively.
[c] The particle size for all columns in the 2nd dimension was 5 μm.

SFC Dual Pump Fluid Control Module (a FCM 1100/1200 CO_2 Pump, a FCM 1200 Modifier Fluid Pump), a Berger TCM 2000 Oven and an Agilent 1100 variable wavelength UV detector (Agilent Technologies, Santa Clara, CA, USA) was controlled by Berger SFC Massware version 4.03. A CTC PAL Autosampler (LEAP Technologies, Carrboro, NC, USA) was used to load samples onto the columns. For preparative SFC, a Waters 2525 pump was modified with chilled pump head to supply up to 70 mL/min flow of carbon dioxide. A back pressure regulator (Thar BPR 200, Waters Corp., Milford, MA, USA) and a column oven (Model C030, Torrey Pines Scientific, Inc., Carlsbad, USA) were added to convert an existing Waters preparative LC/MS system into a preparative SFC/MS system. Both analytical and preparative column ovens were operated at 40 °C and back pressure was controlled at 100 bar.

Method developments for achiral separations were performed routinely using the configuration of first dimension. The optimal method of separation was then directly transferred to achiral chromatography in the first dimension of 2D SFC/SFC/MS methods. For chiral method developments a parallel SFC/MS approach [26,27] was utilized to search for the optimal conditions, which were then transferred to chiral chromatography in the second dimension of 2D SFC/SFC/MS methods.

2.4. Two-dimensional SFC/SFC system configuration

For the analytical 2D SFC/SFC/MS, the achiral chromatographic separations in the first dimension were performed on a Pyridine column (Princeton Chromatography, NJ, USA) with MeOH–10 mM ammonium acetate (NH_4OAc) as the modifier in CO_2 and gradient elution or with MeOH as the modifier in CO_2 and gradient elution (Table 1). The enantiomeric separations in the second dimension were achieved on a ChiralPak AD-H column or a ChiralCel OD-H column (Chiral Technologies, PA, USA) with MeOH–10 mM NH_4OAc in CO_2, or with IPA–0.2% cyclohexylamine (CHA) in CO_2, or with acetronitrile in CO_2 and isocratic elution (Table 1). Sample concentrations varied from 0.1 to 0.5 mg/mL and a 20-μL aliquot was injected onto the column. To facilitate transfer from the first dimension achiral to second dimension chiral chromatography columns, a group of four Valco valves (two-position six-port valves, Valco Instruments, Houston, TX, USA) was configured to enable automated and intelligent mass-triggered peak cutting and fraction transferring controlled by a custom software, SFC2D (see details in Section 2.5).

For the preparative 2D SFC/SFC/MS, the achiral chromatographic separations in the first dimension were performed on a Pyridine column, Viridis 2-Ethylpyridine 5 (m OBD 19 × 150 mm, 5 μm (Waters Corp., Milford, MA, USA) with MeOH as the modifier in CO_2 and gradient elution (5–40% for a standard mixture of buipvacaine, warfarin, and Fmoc-Glu). The flow rate was from 30 to 70 mL/min. The enantiomeric separations in the second dimension were achieved on a ChiralPak AD-H column (for warfarin) or a ChiralCel OJ-H column (for sample D), both 21 × 150 mm, 5 μm (Chiral Technologies, PA) with MeOH as the modifiers in CO_2 and isocratic elution (30% for warfarin). Sample concentrations varied from 5.0 to 30.0 mg/mL and a 100 to 1000 μL aliquot was injected onto the column. To facilitate transfer from the first dimension achiral to second dimension chiral columns, a group of four Valco valves (two-position six-port valves, Valco Instruments, Houston, TX, USA) was configured to enable automated and intelligent mass-trigger peak cutting and fraction transferring controlled by a custom software, SFC2D Prep (see details in Section 2.5). To avoid aerosol generation during fraction collection that may cause cross contamination or losses of sample, a procedure consisting of sample trapping, pressure releasing, and compound eluting was utilized under an open-bed collection format. The fraction trapping system is comprised of two stream selection valves mounted on a single microelectronic actuator (Model C5 from Valco Instruments, Houston, TX, USA), which is controlled through SFC2D Prep software to facilitate the process. Two trap columns (Princeton diol, 21 × 150 mm, 5 μm, Princeton Chromatography, NJ, USA) were used to trap the first and second enantiomers respectively. The trapped fractions were then flushed out using methanol delivered from a Waters 2545 pump and collected through a LC fraction collector (Waters 2767 sampler manager).

2.5. Two-dimensional SFC/SFC control software (SFC2D and SFC2D Prep)

A Visual Basic program for controlling the analytical 2D SFC/SFC/MS system was developed to facilitate 2D achiral and chiral SFC analysis (see details in Section 3.2). This new control software program, we coined SFC2D, was integrated with MassLynx to retrieve acquisition status, sample information, and real time mass spectrometric data as they were acquired. The program monitored the target ion signal and carried out mass-triggered fraction trapping. As the ion signal intensity reached the user-defined thresholds, the software directs a switching valve (see also details in Section 3.2) to fractionate the desired racemate into a narrow band and then transfer the fraction to the second dimension chiral column for further separation and detection (via MS or UV).

A new control software program, SFC2D Prep, was developed for preparative 2D SFC/SFC/MS system (analogous to the analytical 2D SFC/SFC/MS software program). SFC2D Prep was designed to support two independent mass-triggering functions, one from the achiral chromatography in the first dimension and one from the chiral chromatography in the second dimension. The first mass-triggered fractionation, which occurs in the achiral chromatography step, isolates the racemate. The second mass-triggered fractionation allows for the collection of one or both of the separated enatiomers into the open bed fraction collector.

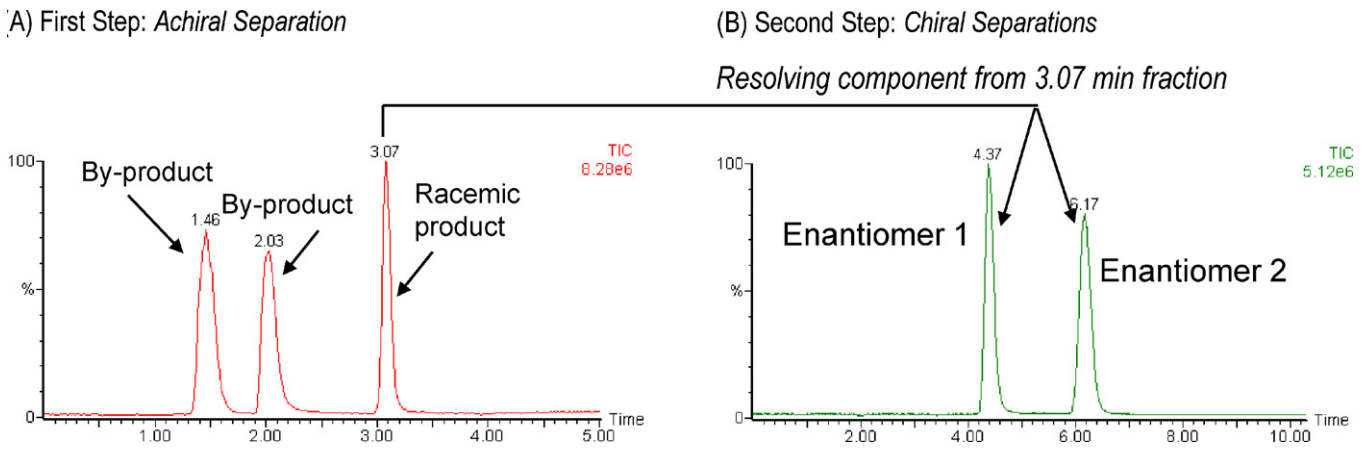

Fig. 1. Conventional two-step process of achiral and chiral analysis and separation of a three-component mixture (sample A) by SFC. (A) The first step of achiral SFC chromatogram showed the targeted racemic product (peak at 3.07 min) was separated from two by-products. (B) The second step of chiral SFC chromatogram showed the separation of two enantiomers following the achiral purification.

3. Results and discussion

3.1. Concept of analysis and purification of racemic mixture in a single step process

The purification of a pair of enantiomers from a crude reaction mixture has generally required a two-step chromatographic process. An achiral separation is performed first to remove unwanted by-products, un-reacted starting materials, and other impurities and then a chiral separation is performed to separate the racemic mixture into two enantiomers. An example in Fig. 1A shows the achiral separation of a crude racemic mixture (sample A) containing three components in which a desired racemate with the retention time of 3.07 min was readily separated from two by-products with the retention times of 1.46 and 2.03 min, respectively. After the achiral purification, which removes these two by-products as well as other impurities, the clean racemic material (over 95% purity) is then subjected to the chiral separation to resolve the two enantiomers (Fig. 1B). The entire process involves two chromatographic separations performed using two different chromatographic methods. There are several additional procedures conducted in between the two chromatographic steps, including collection of the fraction, evaporation of the solvents, reconstitution of the racemic sample for the next step of chiral analysis and separation. Each of these steps is both resource and time consuming. Conceptually, two chromatographic separations can be combined into an automatic process, either in one single run or two consecutive runs, thereby eliminating these time-consuming and resource-intensive steps. Therefore, we designed and implemented the 2D SFC/SFC/MS systems to combine two chromatographic separations into a single run.

3.2. Analytical 2D- SFC/SFC/MS system design and implementation

The analytical 2D SFC/SFC/MS system was designed to combine the two-step analysis and separation of racemic samples into a single step process (Fig. 2). This system is configured with one set of SFC pumps (a CO_2 pump and a modifier pump), and is capable of running gradient and isocratic elution across a wide range of solvents. The first dimension chromatography separations are performed on an achiral column. Mass-triggered fractionation facilitates accurate peak-cutting and fraction-transfer to the chiral column in the second dimension. Four two-way, six-position

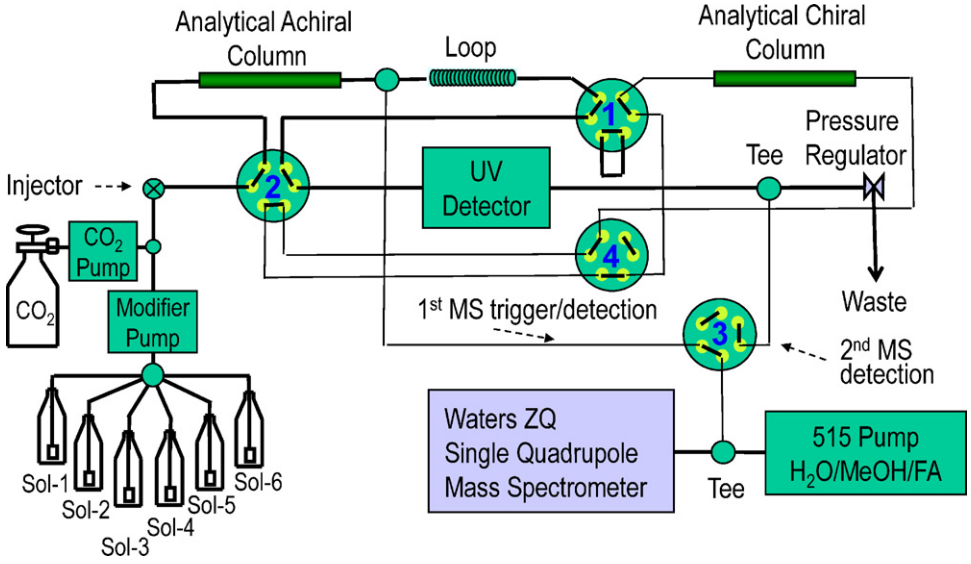

Fig. 2. System configuration of analytical 2D SFC/SFC/MS including four-valve interface positions of 1A, 2A, 3A, and 4A to carry out the achiral chromatography in the first dimension.

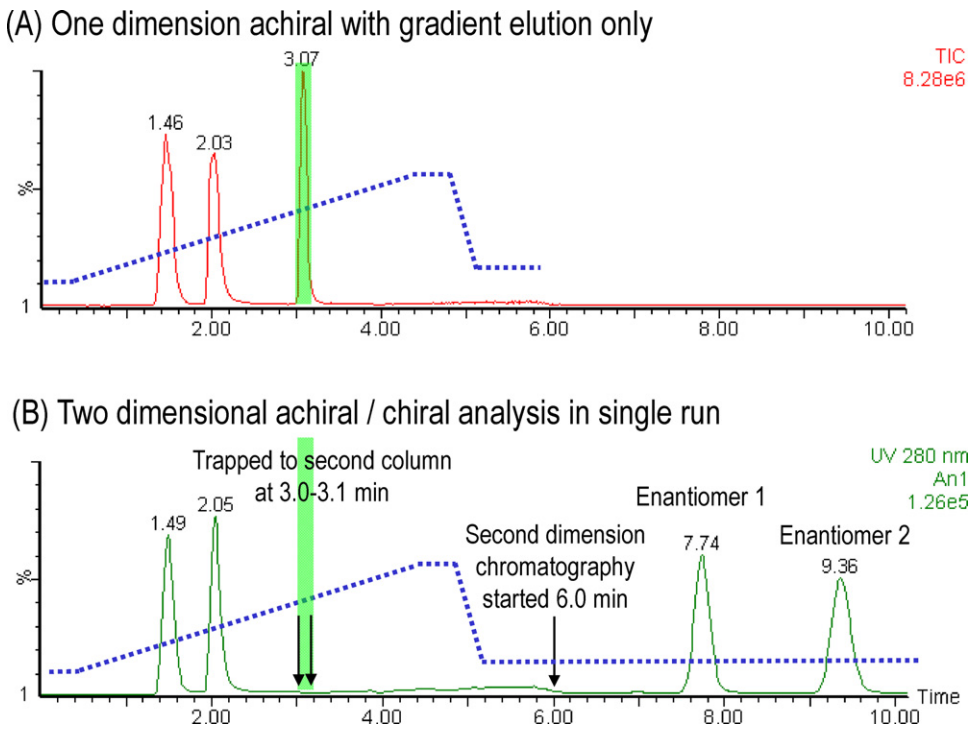

Fig. 3. Analytical achiral/chiral 2D SFC/SFC/MS analysis and separation of a three-component mixture (sample A). (A) The first dimension achiral SFC/MS total ion current (TIC) chromatogram. (B) 2D SFC/SFC/UV chromatogram (UV 280 nm) with peak-cutting and fraction transfer to the second dimension (dotted line indicating the gradient profile).

valves (valves 1–4) are incorporated into the system. The in-house Visual Basic program, SFC2D, controls the system for the mass triggered function, MS detection for either first or second dimension chromatography, as well as fast equilibration of the system, if the solvent is changed. The four switching valves are positioned in the following ways: (1) when the valves are in the 1A, 2A, 3A, and 4A position (the valve position shown in Fig. 2 all in A position), the system is running the first dimension of achiral chromatography separation; (2) when the valves are in the 1A, 2B, 3B, and 4A position, the system is running the second dimension of enantiomeric separation; (3) when the valves are in the 1B, 2A, 3A, and 4A position, the system is performing peak-cutting and fraction-transferring; (4) when the valves are in the 1A, 2B, 3B, and 4B position, the system is in the flush mode, which by-passes the column to allow quick system flush when the solvent has changed. A flow splitter at the outlet of the first column enables the mass-trigger fractionation in the first dimension. The second flow splitter, positioned just before the pressure regulator, enables MS detection in the second dimension. Both split flows pass through valve 3 and are then combined with the make-up flow to deliver the appropriate flow to the MS detector. Since SFC is a pressure-regulated chromatographic system (usually at 100 bar), these four valves have been designed to synchronize for switching simultaneously, allowing the system pressure to be maintained and regulated properly.

3.3. Analytical 2D-SFC/SFC/MS using single-solvent approach

The same three-component mixture (sample A), in the example of conventional two-step process shown in Fig. 1, was used to illustrate the 2D SFC/SFC/MS process (Fig. 3). The racemate is shown to be well separated from the two impurities on the achiral column (Fig. 3A). The racemate is separated into the individual enantiomers in the second dimension separation (Fig. 3B). The SFC2D software monitored the racemate peak (m/z 323.2). When the ion signal reached the defined intensity threshold, valve 1 was switched from position A to position B and the peak was cut and transferred directly to the chiral column in the second dimension. As soon as the signal returned to the defined threshold, valve 1 switched back from position B to position A for the system to continue running the gradient elution to complete the first dimension achiral separation. Arrows in Fig. 3B showed the absence of the targeted peak from the down-stream UV detector, indicating that the targeted peak was completely transferred to the chiral column. After the achiral separation was completed, the SFC2D software simultaneously switched valves 2 and 3 from position A to position B, allowing the system to run the second dimension chiral analysis and separation. The achiral and chiral separations were achieved in a total of 10 min without any intermediate steps. The enantioseparations achieved using the 2D SFC/SFC approach (Fig. 3B) compared favorably with the conventional, two-step approach (Fig. 1A and B). More importantly, this process required no manual steps between the achiral and chiral separations and satisfactory results were achieved in significantly less time.

A second example of analytical 2D achiral and chiral SFC/SFC/MS separations is shown for a mixture of four β-adrenergic blockers (β-blockers), oxprenolol (OX), propranolol (PR), atenolol (AT), and pindolol (PI). In this case, we were interested in isolating the two enantiomers of oxprenolol in a single run, without the need for extensive chiral method development and optimization. The achiral SFC separation of these four β-blockers was achieved in 5 min and the enantioseparations of oxprenolol enantiomers in the second dimension was achieved in another 5 min (Fig. 4). The SFC2D software monitored oxprenolol at m/z 266.2, and controlled the two-dimensional separations (arrows in Fig. 4A indicating the targeted peak being cut and completely transferred to chiral column). Gradient elution was applied to the first dimension separation and isocratic elution for the second dimension

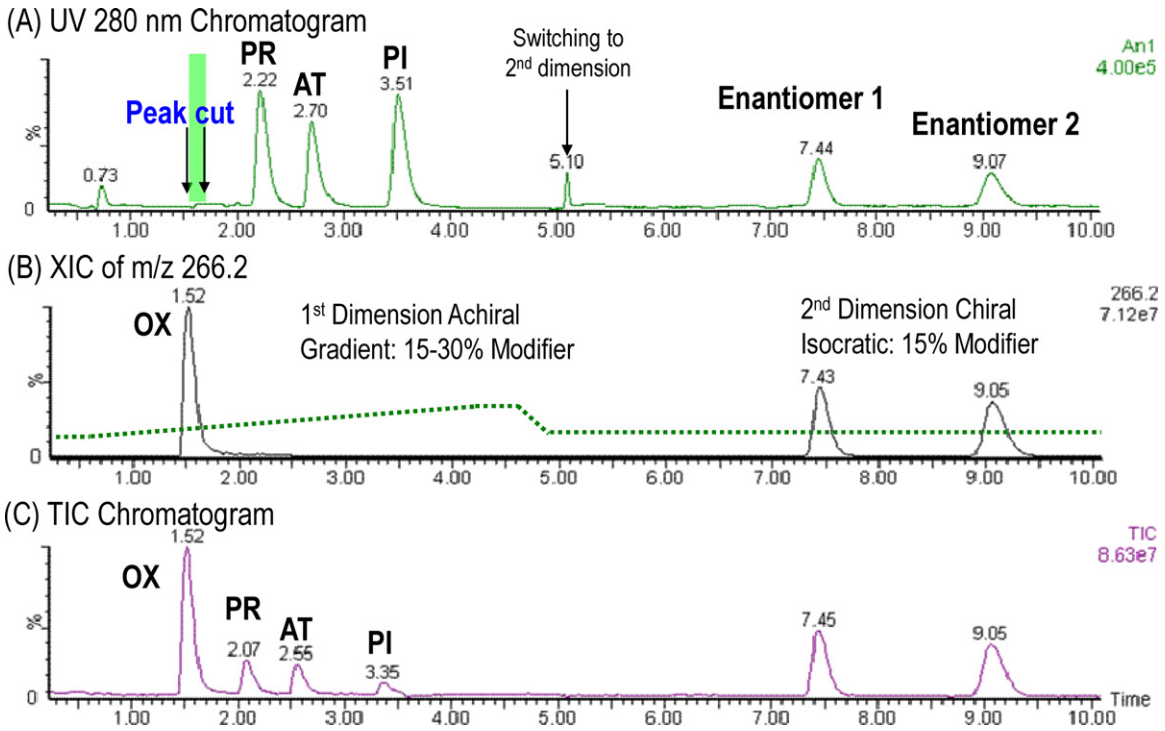

Fig. 4. Single-solvent approach for the 2D SFC/SFC/MS analysis and separation of a four-component mixture of β-blockers. (A) UV chromatogram (280 nm) with arrows indicating the peak-cutting of oxprenolol (OX). (B) Extracted ion chromatogram (XIC) of oxprenolol at *m*/*z* 266.2 with dotted line indicating the gradient profile of modifier. (C) TIC of four β-blockers in the first dimension of achiral chromatography (0–5 min).

separation (gradient profile indicated in Fig. 4B with a dotted line). The total ion current chromatogram (Fig. 4C) of all four components can be seen in the first dimension of achiral separation (from retention times of 0–5 min) and two enantiomers of oxprenolol (one of the four racemates) were baseline resolved in the second dimension of chiral separation (from retention times of 5–10 min). The 2D achiral–chiral analysis and separation using a single-solvent approach was completed with a total run time of 10 min. A similar approach of 2D SFC/SFC/MS can also apply to heart-cutting and chromatographically resolving the enantiomeric pairs for propranolol, atenolol, and pindolol respectively.

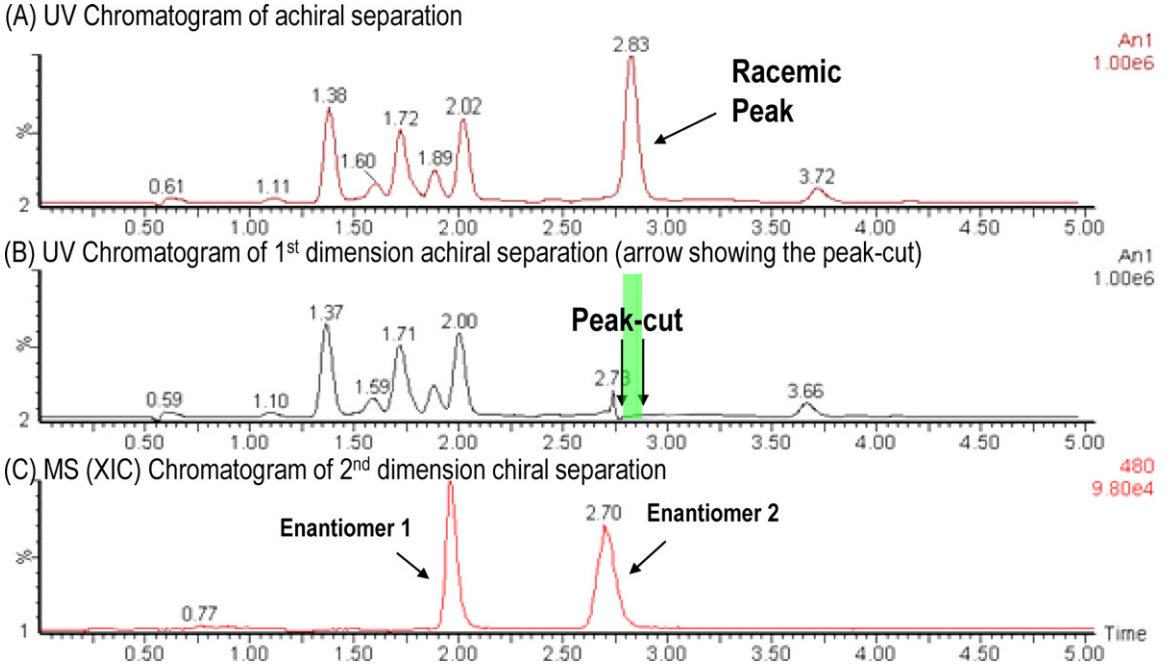

Fig. 5. Two-solvent approach for the 2D SFC/SFC/MS analysis and separation of a complex mixture (sample B). (A) The first dimension chromatogram (UV 280 nm). (B) The first dimension chromatogram (UV 280 nm) using MeOH–NH_4OAc as modifier (arrows showing the targeted peak-cutting). (C) The second dimension extracted ion chromatogram (XIC, *m*/*z* 480.0) using IPA–0.2% CHA as modifier.

3.4. Analytical two-dimensional SFC/SFC/MS using two-solvent approach

To separate a racemate from non-racemate impurities by achiral SFC chromatography and to achieve acceptable enantioseparation by chiral SFC chromatography often requires the use of different solvents as the modifiers. An example in Fig. 5 illustrates a 2D-chromatographic analysis incorporating two unique solvents. A crude synthetic mixture (sample B) was separated on a Pyridine column using a gradient elution of 10–30% methanol (with 10 mM NH_4OAc) in CO_2 as the modifier. The targeted racemate eluted at 2.83 min (Fig. 5A). The racemate peak was monitored by SFC2D software. When its signal intensity reached the pre-set threshold, the peak was cut and the fraction was transferred to the second dimension chiral column (Fig. 5B with the arrows indicating the peak cut) through switching the valve 1 from A to B and back to A as the signal rose above and dropped back below the threshold. After the first dimension achiral separation was completed, the second dimension chiral analysis and enantioseparation was initiated. The SFC2D program controlled the valves to switch to a new solvent, 40% 2-propanol with cyclohexylamine (CHA), required for the second dimension chiral enantioseparation. The first and second dimension chromatograms were recorded into two separate chromatograms (Fig. 5B and C). At the completion of the chiral step, the SFC2D switched valves and solvent back to equilibrate the system for the next sample to be separated in the first dimension.

3.5. Two-dimensional analytical SFC/SFC/MS separation of a mixture of regioisomers/enantiomers

An intermediate of a desired product was synthesized as a mixture of regioisomers/enantiomers (Fig. 6 indicating the structures of sample C as C1 and C2, i.e., 6- and 4-chloro-indole with 2-substituent derivatives). An LC/MS method failed to provide sufficient resolution whereas an SFC separated the two regioisomers (Fig. 7A). A 2D SFC/SFC/MS method was further developed for the separation of the regioisomers (sample C1 and C2) and their enantiomers. Baseline separation was achieved in the first dimension achiral chromatography (Pyridine column) and the C1 peak was transferred to the second dimension chiral chromatography (ChiralCel OJ-H column) by mass-triggered peak-cutting, yielding a successful baseline resolution for the two enantiomers (C1E1 and C1E2). The regioisomer C2 was also resolved in the same way in a separate injection (data not shown). The total 2D chromatographic time was only 10 min for each of the regioisomers (Fig. 7B).

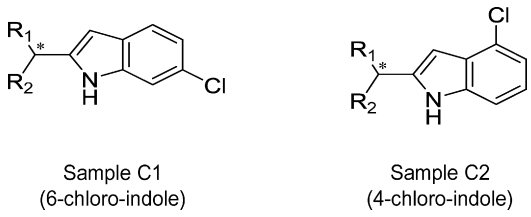

Fig. 6. The structures of sample C, a mixture of a pair of regioisomers (6-chloro-indole, sample C1 and 4-chloro-indole, sample C2 with the star indicating the chiral center on the side chain) and a pair of enantiomers (R1 and R2 = aromatic or aliphatic substitution).

3.6. Preparative two-dimensional SFC/SFC/MS system design and implementation

A preparative 2D SFC/SFC/MS system was constructed by modifying an existing preparative LC/MS system. As illustrated in Fig. 8 a preparative LC pump (Waters 2525) was modified to allow the delivery of modifier (MeOH) and CO_2. A custom chiller was added to one set of pump heads along with the chilled tubing to maintain the supercritical state and accommodate the higher flow rate of the CO_2 effluent. Tubing connection scheme on the autosampler (Waters 2767 Sample Manager) was also modified so that the samples were loaded from the modifier flow path before mixing with supercritical CO_2 effluent to avoid sample precipitation in the injection loop. The custom software, SFC2D Prep, monitored the MS signal and controlled valve 1 and valve 5 to facilitate the peak-

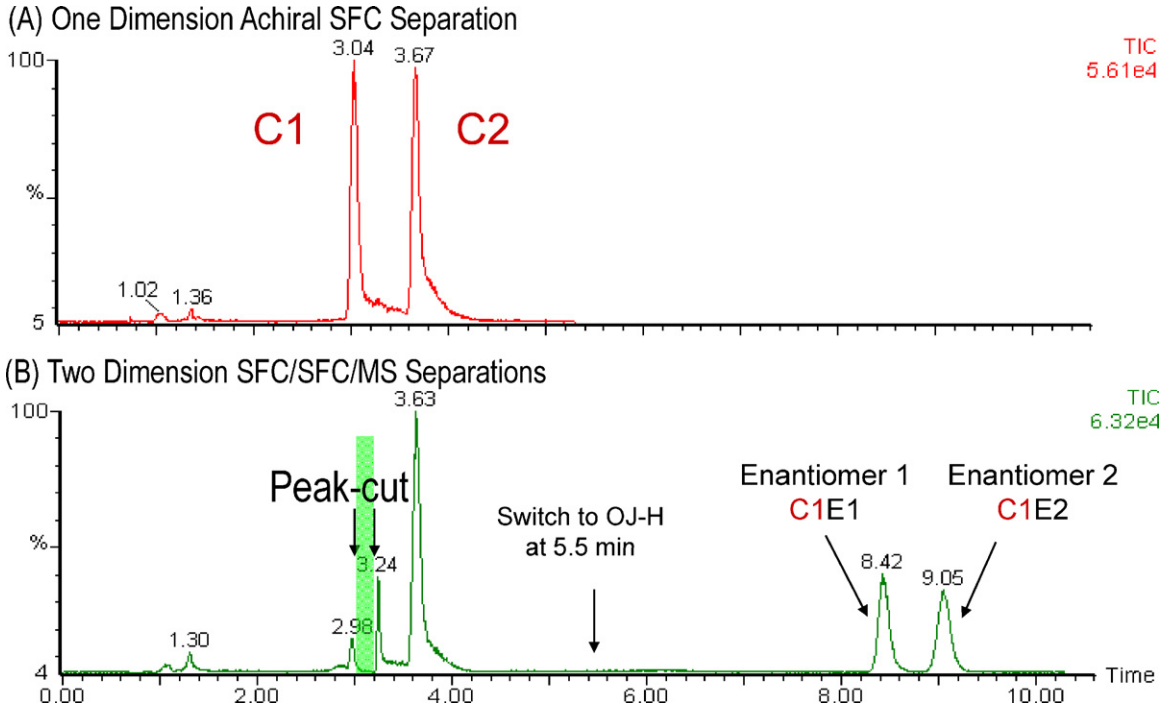

Fig. 7. Two dimensional achiral–chiral SFC/SFC/MS analysis and separation of a mixture of regioisomers/enantiomers (sample C). (A) TIC chromatogram in the first dimension achiral separation. (B) The 2D SFC/SFC/MS chromatogram (TIC) showing the peak-cutting and transfer to the second dimension.

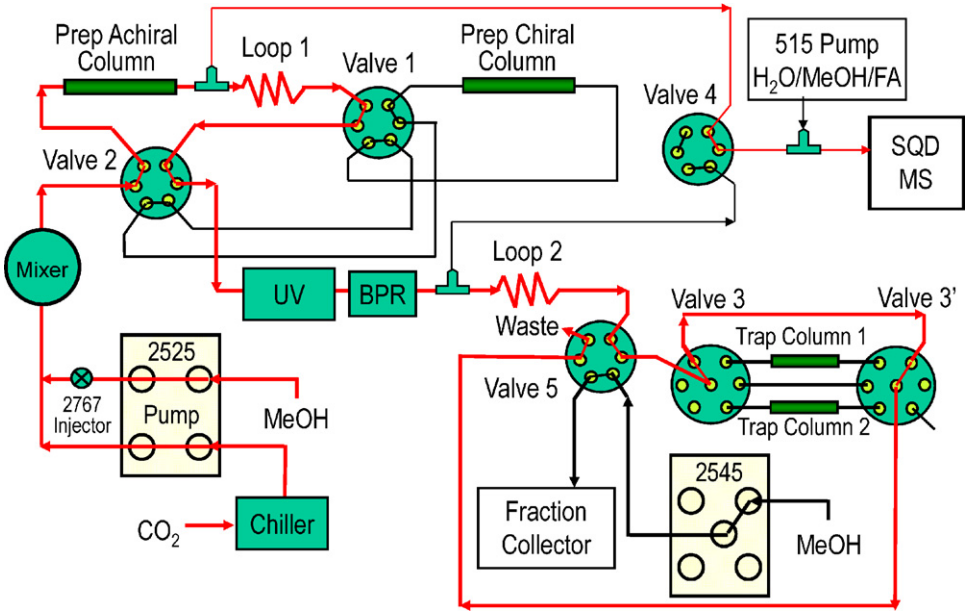

Fig. 8. System configuration for preparative 2D SFC/SFC/MS including four-valve interface (valves 1, 2, 4, and 5) with the positions of 1A, 2A, 4A, and 5A to carry out the preparative achiral chromatography in the first dimension whilst the fraction collection valve (valve 3 and 3′) is in the by-pass position.

cutting from the achiral chromatography in first dimension and the fraction trapping for chiral chromatography in the second dimension, respectively. The capability to perform two-mass triggered functions is one of the important new features for the preparative 2D SFC/SFC/MS system. Open-bed fraction collection was achieved without the issue of aerosol generation by trapping fractions after the second chiral chromatographic separation through the incorporation of a set of switching valves (valve 3 and valve 3′) and pressure release steps. Following pressure release, isolated enantiomers were eluted (or washed) from the fraction collection trap columns into a standard HPLC fraction collector (Waters 2767 sample manager), which was controlled by FractionLynx (either MS or UV trigger). The performance of the preparative 2D SFC/SFC/MS system was assessed using a standard mixture of three compounds (bupivacaine, warfarin, and Fmoc-Glu) for achiral chromatography in the first dimension and using a racemate standard compound

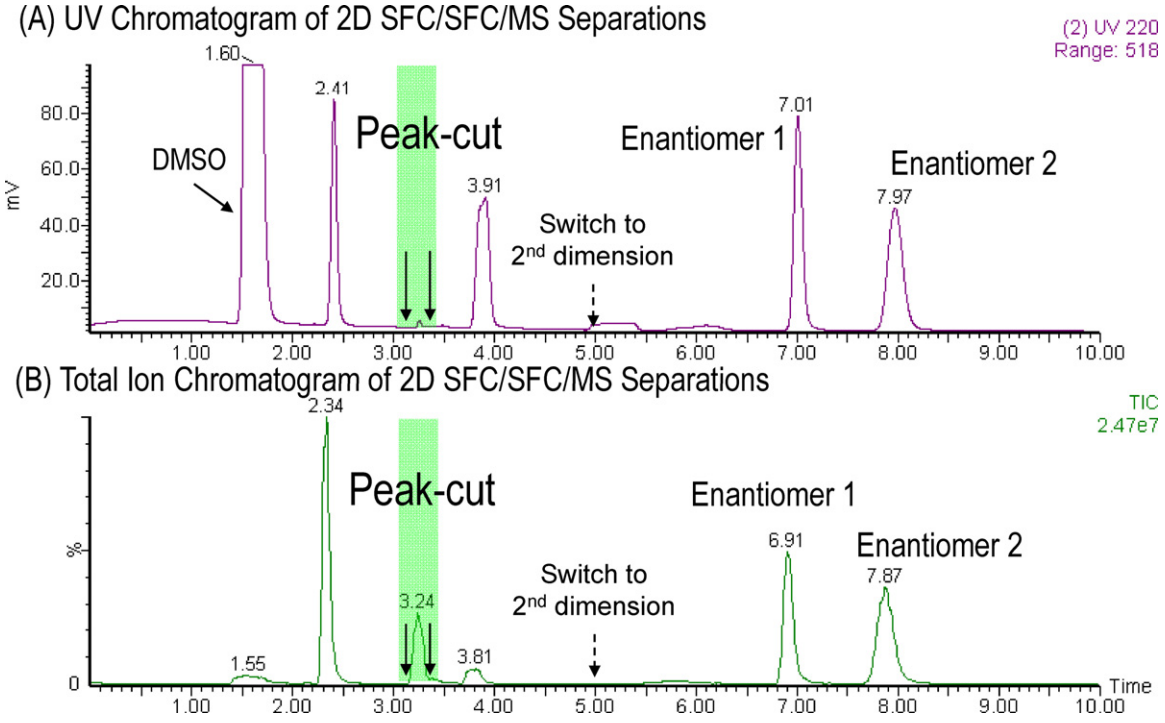

Fig. 9. The preparative two dimensional achiral–chiral SFC/SFC/MS purification of a mixture of three components (sample D, 20 mg on column), in which a targeted racemate was separated on the achiral chromatography in the first dimension followed by chiral purification and fraction collection of two enantiomers in the second dimension (see Table 1 for detailed conditions of columns, modifiers, and flow rates). (A) The UV chromatogram of achiral/chiral 2D SFC/SFC/MS showing the targeted peak being cut. (B) The TIC of preparative 2D SFC/SFC/MS with arrows to indicate the target peak, and the system switching to the second column at 5.0 min to continue the chiral chromatography from 5 to 10 min.

(warfarin) for chiral chromatography in the second dimension. The relative standard deviations of retention time over 10 replicate injections were 0.3%, 0.2%, and 0.1% for bupivacaine, warfarin, and Fmoc-Glu, respectively, in the first dimension and 0.1% and 0.2% for two warfarin enantiomers in the second dimension. The overall system recovery (including both the first and second dimensions) using a racemate standard (warfarin) was found to be 95% (average of triplicate injections).

An example of applications from the new preparative 2D SFC/SFC/MS purification is illustrated in Fig. 9. A mixture (sample D, 20 mg on column) of the targeted racemic compound with retention time at 3.24 min (Fig. 9B) and two impurities (retention times at 2.34 and 3.81 min, respectively) was separated by achiral chromatography within 5 min of the first dimension. The targeted peak was cut (arrows in Fig. 9A indicating the peak being cut) and transferred to the chiral chromatography in the second dimension, in which two enantiomers were further separated and collected.

4. Conclusion

An analytical two-dimensional SFC/SFC/MS system was designed and implemented to streamline enantiomeric analysis and separation of complex mixtures of pharmaceutical racemate samples. The first dimension chromatography was performed on an achiral column to separate a desired racemate from the impurities and the second dimension chromatography was conducted on a chiral column to resolve a pair of enantiomers. A custom Visual Basic software program, SFC2D, interfaced directly with Waters MassLynx, AutoLynx, and Berger MassWare plug-in to control and monitor the whole process, making real-time decisions to fractionate the desired racemate peak from the first column using mass-trigger fractionation and transfer the fraction directly to the second column for chiral separation. One set of SFC pumps (one CO_2 pump and one modifier pump) was utilized to conduct 2D SFC/SFC/MS chromatography with the capability of performing gradient elution in the first achiral chromatography and isocratic elution in the second chiral chromatography to achieve the orthogonal separations for the complex sample. To support larger scale purification, a preparative 2D SFC/SFC/MS system was constructed by modifying an existing preparative LC/MS system. A custom Visual Basic software program, SFC2D Prep, was developed, and performed in a manner similar to SFC2D, which had been applied successfully to analytical 2D SFC/SFC/MS. SFC2D Prep was able to carry out multiple mass-triggered functions, both from the achiral chromatography in the first dimension as well as from the chiral chromatography in the second dimension. The applications of the 2D SFC/SFC/MS to analyses and purification of racemic pharmaceutical samples from a complex mixture demonstrated that an achiral separation (in first dimension) and a chiral separation (in second dimension) can be successfully combined into one streamlined process in both analytical and preparative scales.

Acknowledgements

The authors are grateful to Dr. Walter Keung and Mr. Mingnam Tang for providing the test compounds for the studies. The authors would also like to thank Mr. Derek B. Laskar for his technical assistance during the development of analytical 2D SFC/SFC/MS system and Mr. John Eve for his technical assistance during the development of preparative 2D SFC/SFC/MS system.

References

[1] A.C. Anderson, Chem. Biol. 10 (2003) 787.
[2] C.H. Andrade, K.F.M. Pasqualoto, E.I. Ferreira, A.J. Hopfinger, Molecules 15 (2010) 3281.
[3] P. Dugo, F. Cacciola, T. Kumm, G. Dugo, L. Mondello, J. Chromatogr. A 1184 (2008) 353.
[4] D.R. Stoll, X. Li, X. Wang, P.W. Carr, S.E.G. Porter, S.C. Rutan, J. Chromatogr. A 1168 (2007) 3.
[5] G. Guiochon, N. Marchetti, K. Mriziq, R.A. Shalliker, J. Chromatogr. A 1189 (2008) 109.
[6] C.R. Evans, J.W. Jorgenson, Anal. Bioanal. Chem. 378 (2004) 1952.
[7] R.J. Soukup, D.W. Armstrong, in: S.A. Cohen, M.R. Schure (Eds.), Multipledimensional Liquid Chromatography: Theory and Applications in Industrial Chemistry and Life Sciences, John Wiley & Sons, Inc., Hoboken, New Jersey, 2008, p. 319.
[8] R.F. Gomes, N.M. Cassiano, J. Pedrazzoli Jr., Q.B. Cass, Chirality 22 (2010) 35.
[9] H. Hasegawa, T. Fukushima, J.A. Lee, K. Tsukamoto, K. Moriya, Y. Ono, K. Imai, Anal. Bioanal. Chem. 377 (2003) 886.
[10] Q.B. Cass, A.L.G. Degani, N.M. Cassiano, J. Pedrazolli Jr., J. Chromatogr. B 766 (2001) 153.
[11] Q.B. Cass, V.V. Lima, R.V. Oliveira, N.M. Cassiano, A.L.G. Degani, J. Pedrazzoli Jr., J. Chromatogr. B 798 (2003) 275.
[12] A. Medvedovici, F. Albu, C. Georgita, D.I. Sora, T. Galaon, S. Udrescu, V. David, J. Chromatogr. B 850 (2007) 327.
[13] B. Kammerer, R. Kahlich, M. Ufer, S. Laufer, C.H. Gleiter, Anal. Biochem. 339 (2005) 297.
[14] H. Tian, J. Xu, Y. Xu, Y. Guan, J. Chromatogr. A 1137 (2006) 42.
[15] I.L. Davies, B. Xu, K.E. Markides, K.D. Bartle, M.L. Lee, J. Microcolumn Sep. 1 (1989) 71.
[16] K.M. Payne, I.L. Davies, K.D. Bartle, K.E. Markides, M.L. Lee, J. Chromatogr. A 477 (1989) 161.
[17] Z. Juvancz, K.M. Payne, K.E. Markides, M.L. Lee, Anal. Chem. 62 (1990) 1384.
[18] Y. Hirata, T. Hashiguchi, E. Kawata, J. Sep. Sci. 26 (2003) 531.
[19] P. Sandra, A. Medvedovici, F. David, LCGC Eur. (2003) 2.
[20] K.W. Phinney, L.C. Sander, S.A. Wise, Anal. Chem. 70 (1998) 2331.
[21] A.J. Alexander, A. Staab, Anal. Chem. 78 (2006) 3835.
[22] C.J. Welch, M. Biba, J.R. Gouker, G. Kath, P. Augustine, P. Hosek, Chirality 19 (2007) 184.
[23] L. Zeng, L. Burton, K. Yung, B. Shushan, D.B. Kassel, J. Chromatogr. A 794 (1998) 3.
[24] L. Zeng, D.B. Kassel, Anal. Chem. 70 (1998) 4380.
[25] D.B. Kassel, Chem. Rev. 101 (2001) 255.
[26] L. Zeng, R. Xu, D.B. Laskar, D.B. Kassel, J. Chromatogr. A 1169 (2007) 193.
[27] D.B. Laskar, L. Zeng, R. Xu, D.B. Kassel, Chirality 20 (2008) 885.