Packed-Column Supercritical Fluid Chromatography–Mass Spectrometry for Drug Discovery Applications

In the last five years, the acceptance and implementation of packed-column supercritical fluid chromatography–mass spectrometry (pSFC–MS) to drug discovery applications has gained momentum. This article describes the pros and cons of pSFC–MS and attempts to demonstrate its broad applicability to such fields as high-throughput analysis, purity assessment, structure characterization and purification. Finally, an outlook for the future of this technique is presented.

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Introduction

The analytical philosophy in drug discovery has changed drastically in recent years. Enhancing analytical capacity and efficiency is no longer the top priority as more and more effort is directed at generating high-quality data within a minimal time frame. Effectively delivering high-quality information in a high-throughput environment (1) is, therefore, of utmost importance. For more than a decade now, liquid chromatography–mass spectrometry (LC–MS) has been used predominantly in the drug discovery analytical arena for purity assessment, purification and structure analysis in a high-throughput manner. Recently, however, packed-column supercritical fluid chromatography (pSFC) has become more attractive because it offers high speed, unique selectivity and aqueous-free purification capabilities — important requirements in the drug discovery process. Consequently, pSFC is being revamped as another powerful complementary technique to LC.

In SFC, a "fluid"–either a gas or a liquid above its critical temperature and pressure–is used as mobile phase. Carbon dioxide (CO_2) is commonly applied because of its favorable critical parameters; that is, a critical temperature of 31°C and a critical pressure of 7.3 MPa. Moreover, CO_2 is cheap, non-toxic and non-flammable. The mobile phase is kept under supercritical or subcritical conditions via an electronically controlled variable pressure restrictor positioned after detection for pSFC and via a fixed restrictor positioned before a gas-phase detector for capillary column SFC (cSFC). Since its introduction in the mid-1960s, SFC has experienced several ups and downs. At present, it is widely accepted that pSFC, which uses the same injector and column configurations as LC, is more robust and thus more useful for routine operation than cSFC. In addition the hyphenation of pSFC to MS is easier than for cSFC (2).

In pSFC, the retention characteristics of the analytes are influenced by the properties of the stationary phase and the polarity/selectivity and density of the mobile phase (CO_2). The density is controlled by varying the temperature and pressure of the supercritical medium. Furthermore, by pre-column addition of polar modifiers (e.g., methanol [MeOH]), elution of very polar compounds under high densities can be achieved. pSFC instrumentation can be readily coupled to electrospray ionization (ESI)–or atmospheric pressure chemical ionization quadrupole (APCI)–MS with only a very minor modification being required: the addition of a post-column make-up fluid. There are two reasons for this modification. First, because pure CO_2 neither produces ions under any investigated conditions nor plays a direct role in ion formation as a result of the lack of CO_2-derived primary ions, it is essential to add post-column modifiers (e.g., MeOH or other solvents) into the CO_2 stream to generate MS ionization. Second, adding a make-up fluid prevents precipitation of solutes and/or peak tailing, because the fluid density and its solvating power are drastically reduced after the restriction (3).

Because of the much lower viscosity, higher diffusitivity and lower column pressure drop, pSFC can offer 5–10 times faster analysis and much higher efficiencies (3- to 10-fold) by using longer columns than LC (4). These features have made pSFC–MS a very powerful tool for high-throughput qualitative (5) and quantitative analyses (6). The unique normal phase-like separation mechanism of pSFC, which leads to a quite different solute selectivity compared with reversed-phase LC (RPLC), has long been ignored or at least received little attention. Consequently, current applications of pSFC in drug discovery still remain very limited, even in high-throughput analysis. Moreover, several interesting areas have yet to be further explored; for example, using

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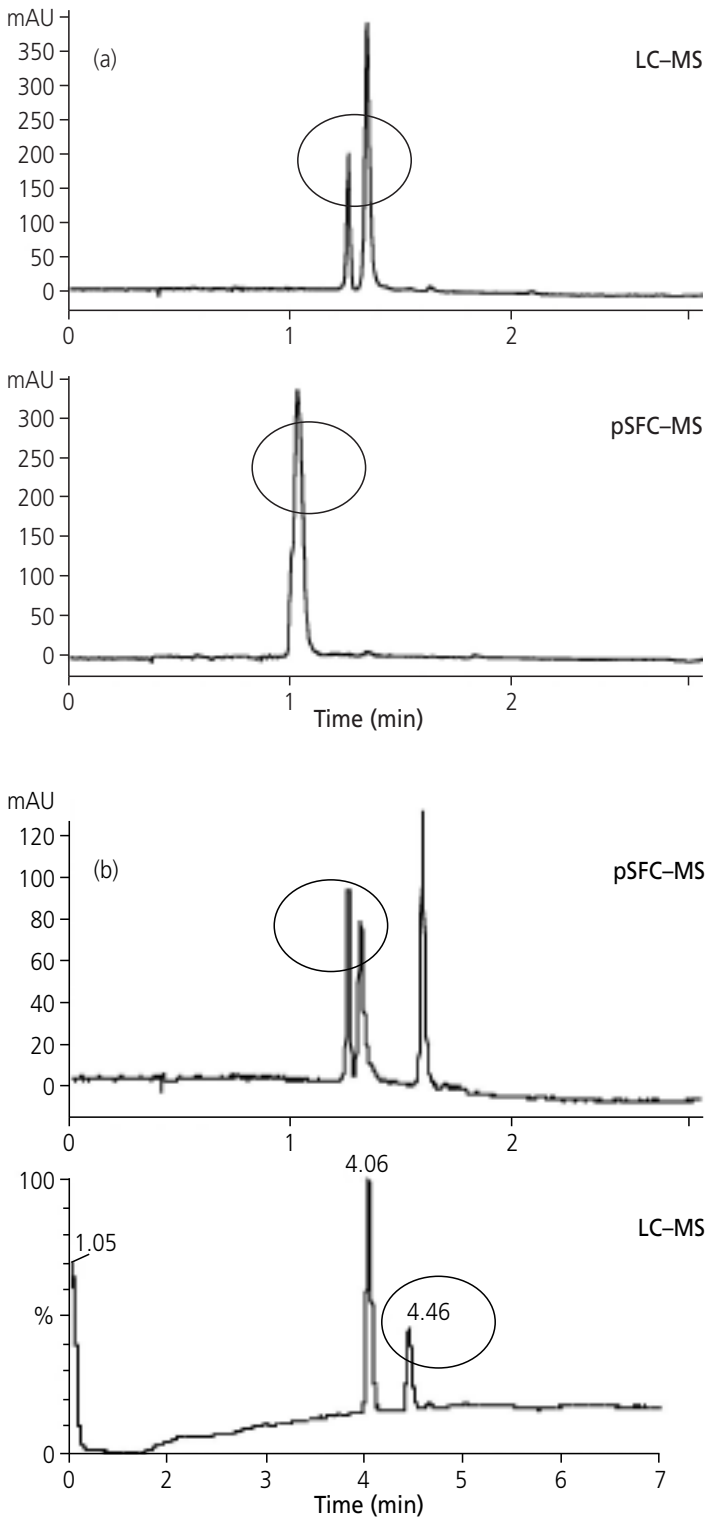

Figure 1. High-throughput analyses of samples (a) and (b) using LC–MS versus pSFC–MS. LC conditions: Phenomenex Synergi MAX-RP column, 50 × 2.1 i.d. mm, 4 µm, 0.8 mL/min flow-rate and 40°C column temperature. Mobile phase: (A): 0.1% TFA in H_2O, (B): 0.1% TFA in MeCN. Gradient: (a) (B) from 5% to 100% in 2 min, held for 0.5 min (total run time 4 min); (b) (B) from 5% to 100% in 4 min, held for 1.5 min (total run time 7 min). pSFC conditions: Princeton CN column, 150 × 4.6 mm i.d., 5 µm, 4 mL/min flow-rate, 40°C column temperature and outlet-pressure 120 bar. Mobile phase: (A): CO_2, (B): 0.2% IPA in MeOH. Gradient for (a) and (b): (B) from 5% to 45% in 1.5 min, held for 1 min (total run time 3 min).

the orthogonality of pSFC, LC and/or capillary electrophoresis (CE) for purity assessment, applying pSFC–MS–MS for structure characterization and developing new pSFC-tailored stationary phases.

The aim of this article is to illustrate the broad applicability of pSFC–MS to two key stages of drug discovery — specifically hit-to-lead and lead optimization — and to demonstrate that pSFC–MS and LC–MS are equally important in the discovery analytical arena. Apart from high-throughput analysis, some recent applications of pSFC to rapid method development, structure characterization and achiral/chiral purification also are discussed.

Experimental

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

Columns. The pSFC achiral columns were obtained from Princeton Chromatography (Cranbury, New Jersey, USA) and Thermo Electron (Bellefonte, Pennsylvania, USA) with dimensions of 150 × 4.6 mm i.d., 5 µm particles for analytical work and 150 × 20 mm i.d., 5 µm particles for preparative work unless otherwise stated. LC achiral columns were obtained from Phenomenex (Torrance, California, USA) unless otherwise stated. CE capillaries were purchased from Polymicro (Phoenix, Arizona, USA).

The chiral columns were purchased from Chiral Technologies (Exton, Pennsylvania, USA) with dimensions of 150 × 4.6 mm i.d., 5 µm for analytical purposes and 150 × 10 mm i.d. or 250 × 20 mm i.d., 10 µm for preparative purification purposes.

Analytical pSFC–MS and preparative pSFC systems. An A5000 pSFC–MS system (Berger Instruments, Newark, Delaware, USA) was used for method development and structure characterization. This system consists of a Berger SFC unit with a dual pump-control module, an FCM1200 flow-control module, a TCM2100 thermal column module and column/solvent

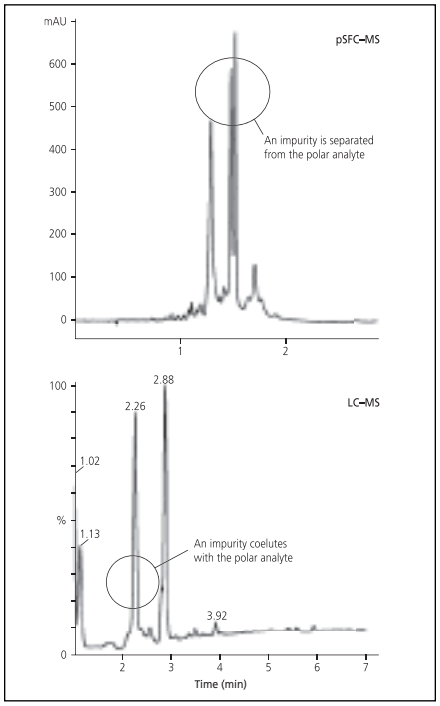

Figure 2. High-throughput analysis of a polar compound using pSFC–MS versus LC–MS. pSFC conditions: Princeton Diol column, 150 × 4.6 mm i.d., 5 µm, 4 mL/min flow-rate, 40°C column temperature and outlet-pressure 120 bar. Mobile phase: (A): CO_2, (B): 0.2% IPA in MeOH. Gradient: (B) from 10% to 40% in 1.5 min, held for 1 min (total run time 3 min). LC conditions: YMC AQ column (Milford, Massachusetts, USA), 100 × 4.6 mm i.d., 5 µm, 1 mL/min flow-rate and 25°C column temperature. Mobile phase: (A): 0.1% HCOOH in H_2O, (B): 0.1% HCOOH in MeCN. Gradient: (B) from 5% to 30% in 5 min, held for 1 min (total run time 7 min).

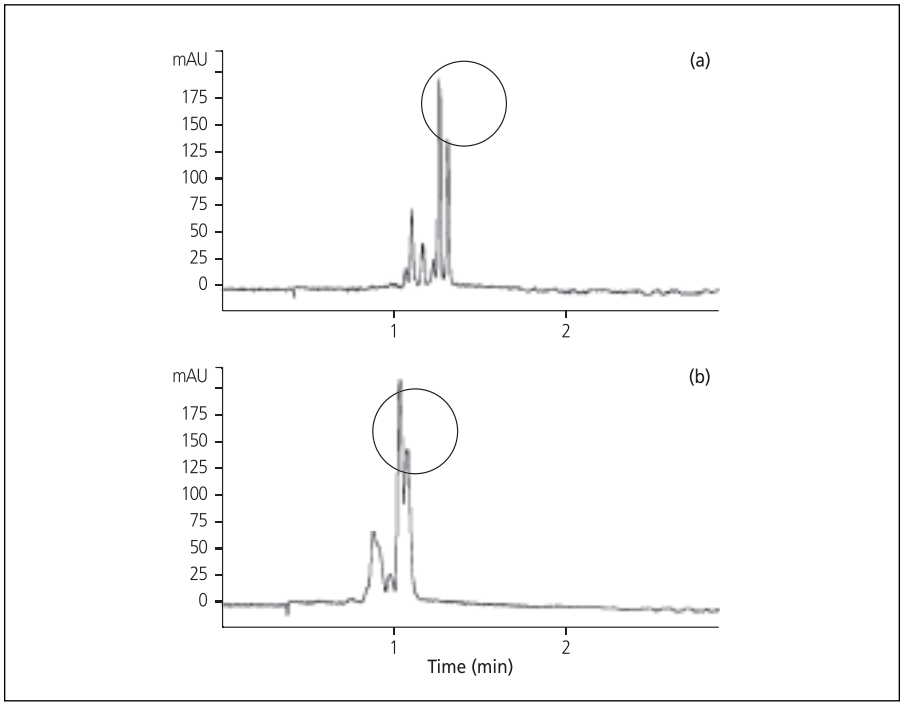

Figure 3. A comparative study of column performance for a sample made from parallel synthesis under the same conditions. pSFC condition: (a) Princeton CN column and (b) Thermo Electron Betasil CN column, 150 × 4.6 mm i.d., 5 µm, 4 mL/min flow-rate, 40°C column temperature and outlet-pressure 120 bar. Mobile phase: (A): CO_2, (B): 0.4% IPA in MeOH. Gradient for (a) and (b): (B) from 5% to 50% in 1.8 min, held for 0.5 min (total run time 3 min).

selection valves that automatically hold up to six different columns and four different modifiers. The SFC was completed with a CTC LC Mini PAL autosampler (Leap Technologies, Carrboro, North Carolina, USA), an 1100 photodiode array detector with a high-pressure flowcell (Agilent Technologies, Palo Alto, California, USA) and a ZQ™ bench-top single quadrupole mass spectrometer (Waters, Milford, Massachusetts, USA) with APCI and ESI sources. post-column make-up fluid was delivered by an 1100 HPLC quaternary pump (Agilent Technologies). Instrument control, data acquisition and analysis for both SFC and MS was performed from one software platform by integrating the SFC ProNTo™ (Berger Instruments) with MassLynx™ software (Waters).

An SF3™ analytical pSFC/LC system (Gilson, Middletown, Wisconsin, USA) was used for off-line multidimensional pSFC–LC–MS analysis. Details of the instrument configuration have been described previously (7, 8).

Two preparative SFC systems, the P7500 and P7000 (Berger Instruments), were used for large-scale and high-throughput purification, respectively. The experimental conditions are given with the figures.

Analytical LC–MS and CE–MS. A single quadrupole MSD system (Agilent Technologies) and a Deca XP iontrap MS system (Thermo Electron, San Jose, California, USA), both coupled to an 1100 LC system (Agilent Technologies), were used for the LC–MS experiments. CE–MS was conducted on an $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) coupled to a single quadrupole MSD system (Agilent Technologies) using a sheath-flow interface.

Results and Discussion

High-throughput analysis. High-throughput techniques in the hit-to-lead stage, where initial hits from a high-throughput screening campaign are assessed to provide tractable and viable leads, are required for qualitative assessment. For more than a decade, fast generic LC–MS methods using very steep gradients, high flow-rates, high temperature, short columns, small particles and acidic volatile mobile phases have been regarded as the standard approach to high-throughput analyses for purity assessment and structure confirmation. Despite its popularity and acceptable separation performance supported by sound theories (9, 10), this conventional approach often trades selectivity and resolution for high speed by leveraging the MS selectivity. As we are dealing with focused libraries that contain structurally related impurities, it is not unusual to observe co-elution of sample components.

pSFC–MS in parallel with LC–MS is proposed to provide two sets of separation profiles; that is, normal- and reversed-phase mechanisms. The orthogonal separation maximizes the selectivity of LC–MS and is extremely helpful while performing purity assessments. Neither of these two methodologies, however, is solely able to provide enough selectivity on analytes with a wide range of physicochemical properties. pSFC often is better for the

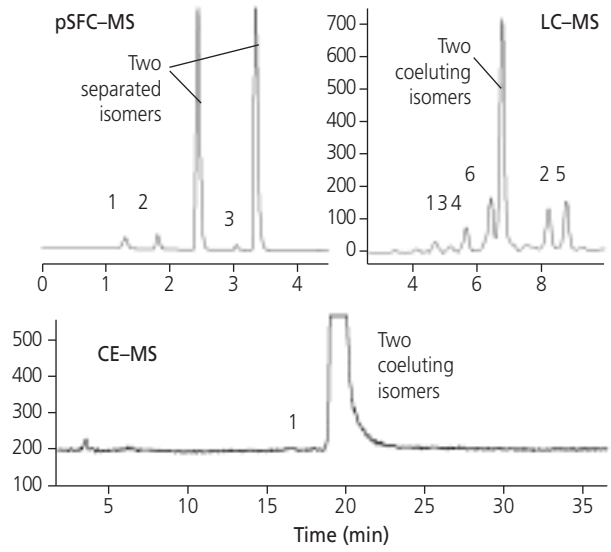

Figure 4. Separation profiles of impurities generated from LC, pSFC and CE method development platforms. LC conditions: Phenomenex LUNA C8 (2) column, 150 × 4.6 mm i.d., 5 µm, 1 mL/min flow-rate and 30˚C column temperature. Mobile phase: (A): 20 mM NH_4OAc, pH 4.5 in H_2O, (B): 5 mM NH_4OAc, pH 4.5 in MeCN. Gradient: (B) was held at 10% for 1.5 min and ramped up to 90% in 8 min, then held for 1.5 min (total run time 12 min); pSFC conditions: Princeton 2-Ethylpyridine column, 150 × 4.6 mm i.d., 5 µm, 3 mL/min flow-rate, 40˚C column temperature and back-pressure 120 bar. Mobile phase: (A): CO_2, (B): MeOH. Gradient: (B) from 5% to 45% in 2 min, held for 1.5 min (total run time 4 min). CE conditions: Capillary: 50 µm i.d. × 95 cm L (22 cm effective length). Buffer: 25 mM NH_4OAc, pH 4, 10% MeOH. Sheath liquid: 90% MeOH in water at 5 µL/min flow-rate. 5 s hydrodynamic injection, 25 kV applied at the inlet (total run time 35 min).

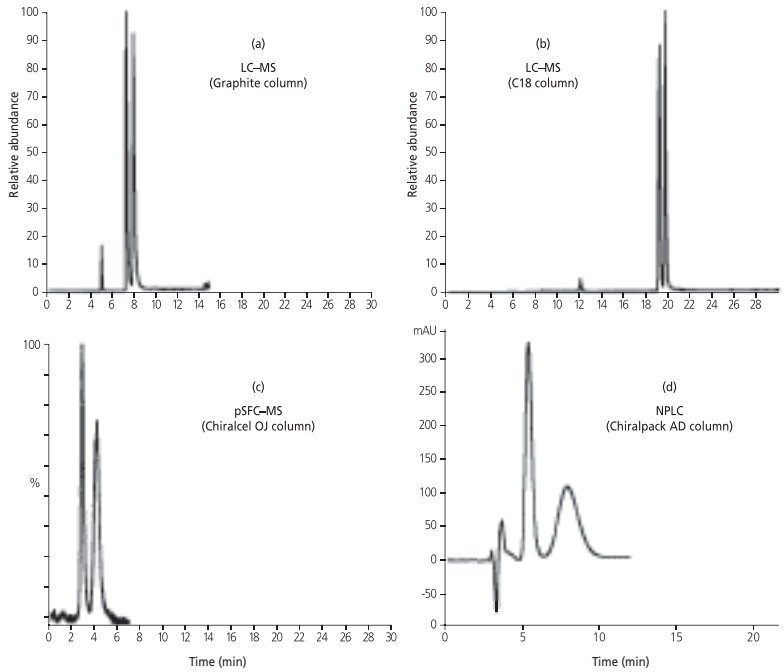

Figure 5. Diastereomer separation for the same sample using four different separation mechanisms.

separation of structurally-related compounds, isomers and enantiomers than LC; but not always. For example, Figure 1 shows the separation profiles of two samples, A and B, from parallel synthesis by LC–MS and pSFC–MS. The impurity in sample A is separated by LC but co-elutes with the parent compound in pSFC, while the opposite is observed for sample B. Figure 2 suggests that pSFC is particularly useful for the analysis of polar compounds that have less retention and/or selectivity on RPLC even with special polar-group-embedded columns. The polar impurity co-eluting in LC–MS is nicely separated from and elutes after the analyte of interest. The solute is rarely too polar to elute from the normal-phase pSFC column.

Column technology is of utmost importance and plays an indispensable role in SFC. Recently it has been addressed by several groups (11, 12) that traditional normal-phase LC (NPLC) column technology may not be the right fit for pSFC applications with respect to stationary phase chemistry, column packing techniques, etc. In other words, pSFC demands its own column series. A large set of commercial pSFC columns was screened to compare their performances (13). From this evaluation, it became clear that an in-depth fundamental study on the pSFC separation mechanism is urgently needed. An illustration of this study is shown in Figure 3. Despite good quality of both columns in normal-phase LC, the Betasil CN column (Thermo Electron) is unable to match the performance of the CN column from Princeton Chromatography in terms of column selectivity, efficiency and resolution. This difference excludes the use of the Betasil CN stationary phase for scale-up purification.

Rapid method development. After hits become leads, significant medicinal chemistry resources are used to find safe and efficacious compounds that can be selected as clinical candidates. The increasing purity requirements at this lead optimization stage necessitate detailed analysis to ensure the leads are fully characterized. It is at this stage that multidisciplinary approaches (e.g., LC–MS, pSFC–MS and CE–MS), instead of a single approach, are taken to ensure the right solution in a timely manner.

The traditional "one-at-a-time" fashion of developing methods usually starts

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with one technique (mostly LC) and one mobile-phase system with a slow gradient. However, the most effective way of developing methods, in our opinion, is to use different techniques in a parallel mode. Figure 4 presents different impurity profiles of the same sample generated from LC, pSFC and CE method screening platforms (1). None of the three techniques is able to separate all impurities. However, by combining all the information generated within the same time frame from the three different techniques, the full range of impurities in the sample is characterized. This parallel method has proven more productive than a serial one; that is, moving from one condition or technique to another.

The multidisciplinary approach also allows us to generate a set of methods using different separation conditions and mechanisms in order to meet various analytical requirements in this stage, such as in-process monitoring, degradation studies, scale-up purification and method transfer for bioassays. Figure 5 shows a typical example of developing multiple methods to separate two diastereomers of a compound for different purposes. Initially, a fast LC–MS method (6 min) was developed on a graphite column to monitor the reaction (Figure 5[a]). For stability studies, a longer LC–MS method on a C18 column was developed to separate both diastereomers and other degradants in the formulation (Figure 5[b]). To obtain each diastereomer in high quantities (10 g), a preparative scale pSFC method was developed yielding the pure compounds within a few days (Figure 5[c]); additionally, an NPLC method was generated (Figure 5[d]) allowing the transfer of this method to a contract laboratory not equipped with pSFC instrumentation.

For chiral method development, there currently are three major challenges to drug discovery:

- the number of compounds requested for chiral separation on a daily basis can be significant
- the wide variety of compounds includes starting materials, intermediates or drug substances
- the achiral impurities can be less than 10%, but sometimes merely more than 70%.

Achiral impurities can severely interfere with chiral method development.

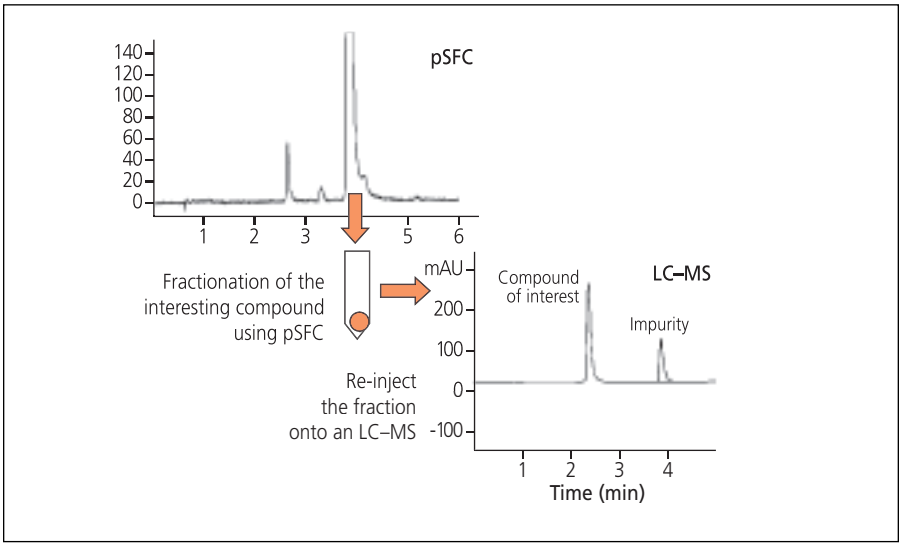

Figure 6. An example of performing a two-dimensional (off-line) pSFC–LC–MS for purity assessment and structure analysis. pSFC conditions: Thermo Electron Betasil CN column, 150 × 10 mm i.d., 5 µm, 5 mL/min flow-rate, 40°C column temperature and back-pressure 120 bar. Mobile phase: (A): CO_2, (B): 0.2% DEA in MeOH. Gradient: (B) from 10% to 40% in 3 min, held for 2 min (total run time 6 min). LC conditions: (A) Thermo Electron Hypercarb column, 100 × 4.6 mm i.d., 5 µm, 1 mL/min flow-rate and 25°C column temperature. Mobile phase: (A): 0.1% HCOOH in H_2O, (B): 0.1% HCOOH in MeCN. Gradient: (B) from 10% to 50% in 5 min, held for 0.5 min (total run time 6 min).

pSFC currently is considered the primary choice for chiral separation because, from an industry perspective, pSFC has proven to be fast, cost-effective and environmentally friendly with its reduced solvent consumption. A chiral screening strategy (14) based on pSFC–MS was developed to screen on four chiral stationary phases under a total of 32 conditions. Sample-pooling was used to substantially increase the throughput. By using the extracted ion chromatogram (EIC) function of the MS it is possible to distinguish the enantiomers without fear of interferences from achiral impurities, with the exception of structural isomers. The technical details have been described elsewhere (15).

Purity assessment and structure characterization. For most purity assessments, the general requirement is to prove that no co-eluting peaks are hiding under the compound of interest; less attention is paid to compounds of no interest as long as they are separated from the major compound of interest. Multidimensional separations operated in either on-line (column-switching) or off-line (fraction collection) mode are able to provide the optimal efficiency and selectivity for separations of the component of interest while simultaneously minimizing the analysis time by decreasing the time spent on separating components of no interest (1). Krstulovic et al., (16) and Venkatramani et al., (17) recently proposed several on-line LC–LC column-switching designs for separating co-eluting impurities. Sandra et al., (8) introduced an on-line and off-line SFC–SFC system for both analytical and semi-preparative purposes. The approach in the author's laboratory is partly based on Sandra's design aiming to set up a simple off-line pSFC fractionation and then re-injecting the fraction onto either a second SFC–MS or LC–MS. Figure 6 presents an example in which some minor impurities (<2% in total) are separated from the compound of interest by pSFC in the first dimension, followed by re-injection of the major peak fraction onto a second dimension LC–MS. The final result shows a clear separation of a significant amount of impurity (30%) that was co-eluting with the major peak in SFC. As fractionation by pSFC results in a much smaller fraction size than LC because of the use of CO_2 and methanol, sample preconcentration on the secondary column becomes much more effective.

pSFC–MS also has shown great potential in structure characterization for metabolites; however, only a few reports were published in the last decade (18). The advantages of using pSFC–MS for metabolite characterization (instead of LC–MS)

are its unique selectivity for structurally-related metabolites or solutes and its normal-phase mechanism to retain polar metabolites. In addition, we experience less signal interference from biological fluids on pSFC than on LC because polar protein-type components can easily be trapped on a pSFC guard column with a normal-phase packing. As illustration, Figure 7 shows the separation of three isomers. Based on their MS fragmentation pattern, their structures were characterized as hydroxylated forms on three different rings.

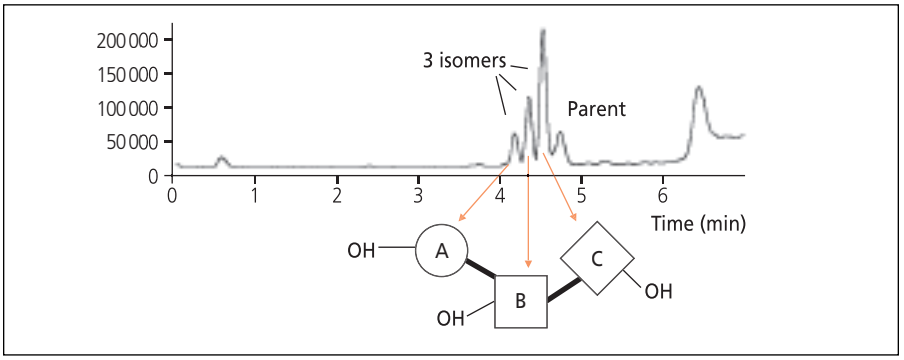

Figure 7. Structure characterization of isomers by pSFC–MS. pSFC–MS conditions: Princeton 2-Ethylpyridine column, 150 × 4.6 mm i.d., 5 µm, 3 mL/min flow-rate, 40°C column temperature and back-pressure 120 bar. Mobile phase: (A): CO_2, (B): MeOH. Gradient: (B) from 5% to 45% in 4 min, held for 1.5 min (total run time 6 min). ESI-MS scan range: 200–500 Da, positive mode. Make-up fluid: 0.05% HCOOH in MeOH at 0.1 mL/min flow-rate.

A drawback of this approach is the relatively lower MS sensitivity of pSFC compared with RPLC–MS. There may be two reasons for this. First, the ionization efficiency of a compound in the pSFC eluent (especially in the presence of basic additives such as DEA) may not be as high as expected in spite of its high volatility and seemingly efficient nebulization. Second, to use ESI–MS, a make-up fluid must be added to aid ion formation, diluting the analytes. APCI may offer a better alternative than ESI. Nonetheless, pSFC–MS–MS has been successfully used for bioanalytical quantification, achieving LC-comparable sensitivities (ppt level) (19). The ionization mechanism in the pSFC eluent so far still remains unclear, substantially affecting the application of pSFC–MS to drug metabolism and pharmacokinetic (DMPK) studies. For chiral separations in biological matrices, pSFC–MS remains superior to LC–MS (particularly NPLC–MS). As illustrated in Figure 8, pSFC–MS is more efficient and sensitive than NPLC–MS to quantitatively monitor the compound chiral inversion rate in biological fluids (Figure 8[c]).

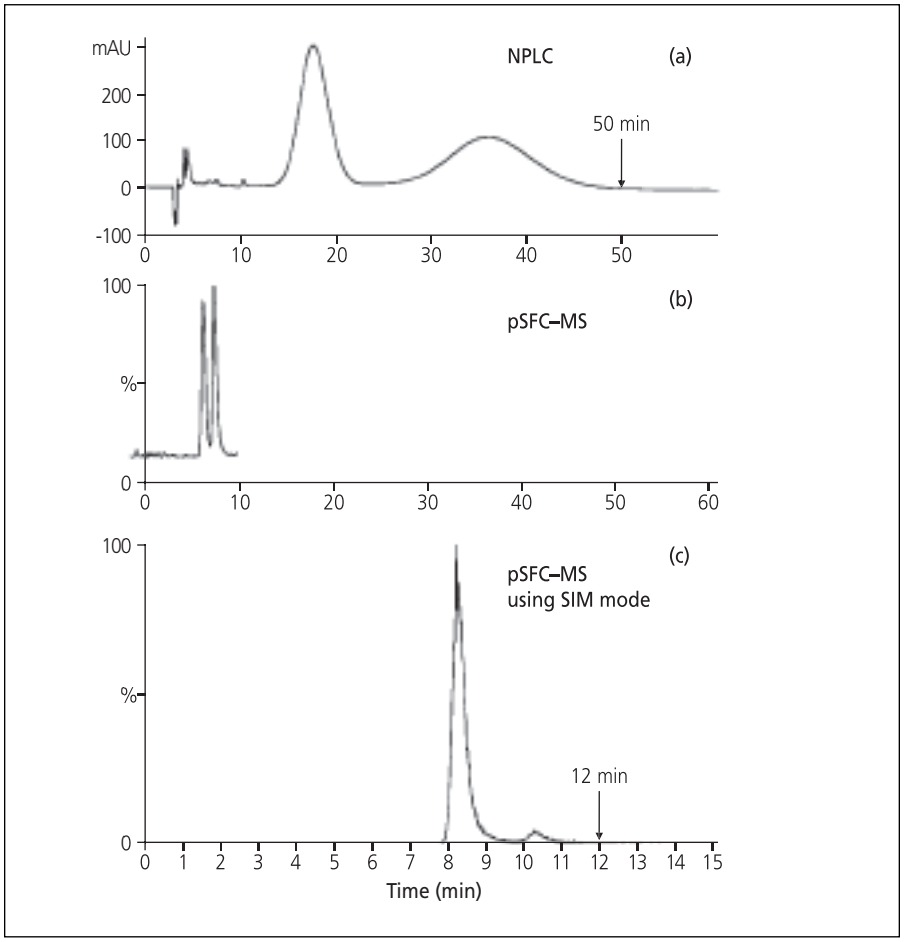

Figure 8. Chiral separation of a drug compound by (a) NPLC, (b) pSFC–MS and (c) pSFC–MS in biological fluids using single ion monitoring (SIM). NPLC conditions: Chiralpak AD column, 250 × 4.6 mm i.d., 10 µm, 0.8 mL/min flow-rate and 25 °C column temperature. Mobile phase: Hexane:Isopropanol:DEA (90:10:0.2); pSFC conditions: Chiralpak AD column, 250 × 4.6 mm i.d., 10 µm, 3.2 mL/min flow-rate, 40 °C column temperature and back-pressure 120 bar. Mobile phase: CO_2:MeOH (0.2% IPA) (92:8)

Purification. One of the most difficult steps in drug discovery is the purification process. The successful introduction of automated preparative LC–MS (prepLC–MS)-based high-throughput purification (HTP) platforms in the late 1990s (20, 21) have enabled purification to be achieved quickly and simply. The biggest bottleneck remains in the post-purification process, which involves multiple steps, (e.g., transferring fractions, solvent evaporation, purity assessment and weighing) to achieve the final products. This is a very time-consuming and labor-intensive process, especially for solvent evaporation in LC. Preparative SFC (prepSFC) has started to make a big impact in this process because it only generates volatile non-aqueous fractions with very small volumes.

PrepSFC has been used to purify fatty acid esters, synthesis intermediates, steroids, fullerenes and, most importantly, chiral compounds (22, 23). Most systems have been designed to use pure CO_2 as the mobile phase and are equipped with

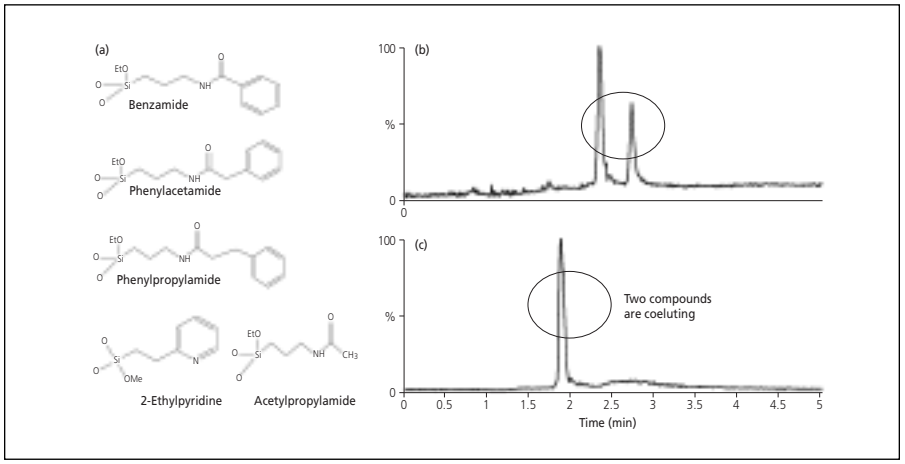

Figure 9. (a) Some new pSFC stationary phases under investigation, (b) an example of using 2-Ethylpyridine vs. (c) Diol column for basic compounds. pSFC conditions: (B) Princeton 2-Ethylpyridine column, 150 × 4.6 mm i.d., 5 µm. Mobile phase: (A): CO_2, (B): MeOH, (C) Princeton Diol column, 150 × 4.6 mm i.d., 5 µm. Mobile phase: (A): CO_2, (B): 0.2% IPA in MeOH. Both were set at 3 mL/min flow-rate, 40°C column temperature and back-pressure 120 bar. Gradient for (b) and (c): (B) from 5% to 45% in 2 min, held for 2 min (total run time 5 min).

a cyclone separator to collect a single or a few components from an unchanging feedstock. There are numerous instances of tens of milligrams–scale pSFC using CO_2 with a polar modifier (e.g., MeOH) for complex mixtures and enantiomeric purification (8); however, from the beginning they all suffered from a loss of 30–50% of analytes, as aerosols, during fraction collection. These losses occur because of the difficulty in separating and trapping quantitatively the sample from the eluent. During rapid depressurization at the system outlet, the sample is micronized and tends to be carried away with the large volume of the high-speed gas phase (22). Recently, Berger et al., (24) and Perrut et al., (22, 23) came up with new hardware designs to effectively trap the samples on-line for semi- and large-scale purification (achieving >90% recovery). Through these developments, prepSFC became useful for medicinal chemistry compound purifications.

At present, two critical issues must be resolved before prepSFC can surpass prepLC for HTP. The first issue is how to integrate the prepSFC technology into the existing prepLC-based HTP platform, as current prepSFC purification is still a UV-triggered system compared with the well-established MS-triggered prepLC–MS system. Although breakthroughs recently have been reported on the development of a parallel MS-triggered prepSFC–MS purification system that aims to maximize the purification throughput (25, 26), commercialization and hardware/software reliability still have a long way to go. The second issue is how to introduce a new generation of pSFC column stationary phases that are able to achieve unique selectivity, good peak symmetry and, most significantly, non-additive purification (avoid using any basic or acidic additives such as DEA and TFA). With this remarkable feature of non-additive purification, collected fractions only stay in pure methanol before drying down. This offers a huge advantage over prepLC, which commonly uses problematic TFA or other additives. This new feature will also allow the wide acceptance of coupling pSFC to quantitative chemiluminescence nitrogen detection (CLND), which is incompatible with nitrogen-containing solvents. Fortunately, major progress has been made on new pSFC column stationary phases offering all those desired features (10). Figure 9a presents some novel stationary phases among which the ethylpyridine-type has proven capable of replacing traditional CN and diol phases for HTP (27, 28). No tailing and even better selectivity compared with traditional LC phases have been experienced [Figure 9(b)] with only methanol as the modifier. More theoretical studies presently are being conducted to provide an in-depth understanding of solute–stationary phase interaction under pSFC conditions.

Future Outlook

pSFC-related technologies have been implemented extensively in almost every stage of the drug discovery analytical arena. However, to make it as successful as LC, collaborative efforts from instrument vendors, academia and end-users must be made to redesign instrument hardware, conduct more theoretical research and generate unique applications.

For decades, pSFC instrument design has been based on LC designs with some specific modifications; that is, pSFC was treated as a "by-product" of LC. Consequently, the more applications that are identified, the more instrument limitations are recognized by the practitioner. This has seriously hampered the technique's further development in a sustainable manner. pSFC must have its own hardware framework with respect to a new integrated design of the pumping system, nozzle, separator and even detectors, into which the robustness and reliability of the technique must be built. In addition, the academic world must become re-involved to conduct theoretical studies on several important issues of pSFC; for example, column stationary phases and formats, MS ionization mechanism in the SFC eluent, ultra-fast pSFC separation using monolithic columns and the feasibility of pSFC–NMR (nuclear magnetic resonance). Nevertheless, there is no doubt that it is just a matter of time before pSFC becomes the first choice for purification, chiral separation and high-throughput bioanalysis in the future of analytical drug discovery.

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