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Supercritical Fluid Chromatography

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Introduction

The role of supercritical fluid chromatography (SFC) for the pharmaceutical scientist is based on its enhanced performance compared with high-performance liquid chromatography (HPLC). These advantages are speed, selectivity, and efficiency. Since diffusion is faster in a fluid than a normal liquid, SFC typically is five to ten times faster than HPLC. The mobile phase is less polar than the stationary phase in most SFC analyses so that the separation mechanism is similar to normal-phase HPLC. Thus, SFC can serve as a powerful complement to the vast majority of reversed-phase HPLC analyses. In terms of efficiency, SFC does not necessarily make the column more efficient. However, the tenfold lower viscosity of the mobile phase allows columns to be joined together. As many as eleven 20 cm × 4.6 mm 5-μm packed columns have been linked to yield over 250 000 theoretical plates. Packed capillary columns have been made even longer.

In order to demonstrate the utility of SFC for pharmaceutical analysis, the range of SFC in this field needs to be understood. A brief review of mobile phases, columns, and solutes (see the SFC instrumentation section) will suggest the current limits. The modes of application are summarized, indicating current and future usage. Finally, a thorough but by no means exhaustive listing of successful solute-specific applications serve as a reference for more detailed investigation.

Range of SFC

Mobile Phases

Carbon dioxide is by far the most popular choice as the primary mobile phase component. Since most

compounds of pharmaceutical interest are more polar than carbon dioxide, a polar component will be needed in the mobile phase. Organic modifiers such as methanol, ethanol, and acetonitrile have been added to carbon dioxide to increase its elution strength. For strongly polar analytes such as amines or acids, a third component, called an additive, is needed to improve peak shape. Typical additives are primary amines for bases and trifluoroacetic acid for acids.

Solutes

SFC can separate a wide range of pharmaceutical solutes. From nonpolar fatty acid methyl esters to multifunctional acids and bases; SFC overlaps the application 'spaces' of gas chromatography (GC) and HPLC. Pharmaceuticals that are soluble only in aqueous solutions are not likely candidates for SFC. For example, purines and pyrimidines can be eluted from packed column SFC. These building blocks of DNA and RNA are polar, basic moieties but can be chromatographed, although DNA itself, has not been eluted by SFC. Similarly, amino acids can be readily analysed but proteins are not typically feasible. Recent work, however, suggests that short peptides can be eluted; further studies are in order.

Columns

The diffusivity of analytes in supercritical fluids is sufficiently high to allow both capillary and packed columns to be used. Both column types have strengths and weaknesses (refer to the instrumentation chapter). For most pharmaceutical applications, capillary columns do not offer sufficient retention time and area reproducibility to be implemented in routine use. However, some enantiomer separations are well suited to capillary SFC and warrant consideration of the technique.

Packed columns have been the predominant mode of operation for most pharmaceutical analyses. Column diameters from 50 μm up to preparative scale have been used. Particle diameters as small as 1.5 μm have found a use in packed column SFC. Since the mechanism for analyte retention is akin to normal-phase HPLC, typical stationary phases are cyanopropyl, aminopropyl, and diol-functionalized supports. Less polar columns such as octyl and octadecyl are also used. Support materials of silica, alumina, zirconia, and polymeric materials have been employed with successful results. Perhaps the most prominent application of packed column SFC is chiral separations. The majority of chiral stationary phases have been used in packed column SFC with beneficial results (refer to the chiral separations chapter).

Detectors

Numerous detectors have been modified and developed for SFC. The most prominent condensed phase detector is UV-Vis. Other condensed phase detectors are fluorescence, FTIR, radiometric, and electrochemical. Gas phase detectors such as mass spectrometry, flame ionization, and others have been interfaced to SFC.

Pharmaceutical Applications

Achiral Separations

The fundamental advantages of SFC are a normal-phase retention mechanism, rapid analysis times, tunable selectivity, and high efficiency. Since the stationary phase is more polar than the mobile phase, SFC is most similar to normal phase HPLC. Solutes are separated based on their polarity and functional groups rather than their hydrophobicity. Consequently, it is an excellent complement to reversed-phase HPLC. In addition, retention time stability is similar to reversed-phase HPLC. The effects of trace amounts of water in the mobile phase are essentially insignificant for SFC when compared with normal-phase HPLC, particularly for polar pharmaceutical compounds. For example, a mixture of steroids shows the elution profile from a silica column (**Figure 1**). The steroids elute based on the number of polar functional groups in the structure. For example, estrone (one ketone, one alcohol) elutes before estradiol (two alcohols) which elutes before estriol (three alcohols). Note the rapid speed of analysis. The column is run at 2.5 mL min^{-1}. In HPLC, the column would be run at five to ten times lower flow rate. As

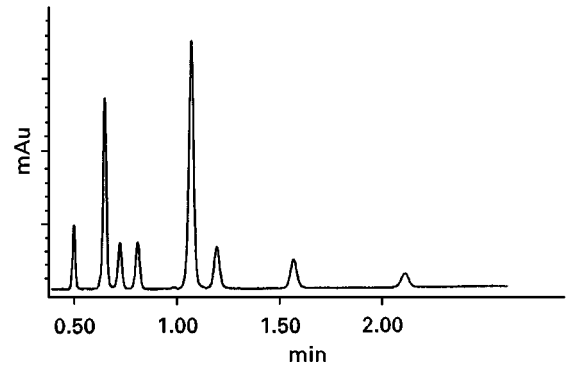

Figure 1 Steroid analysis by packed column SFC. Column 2.1 × 250 mm Lichrosphere Si-60 (5 μm particles); oven 70°C; flow rate 2.5 mL min^{-1}; outlet pressure 200 bar; modifier 20% methanol in carbon dioxide; UV detection at 210 nm. Elution order: progesterone, methyltestosterone, testosterone, estrone, estradiol, cortisone, hydrocortisone, and estriol. Courtesy Dr TA Berger, Berger Instruments.

Table 1 Achiral pharmaceutical separations

Family	*Solutes*	*Reference*
Alkaloids	Codeine, crytopine, morphine, narcotine, thebaine	*Journal of Chromatography* 437: 351
Amphetamines	Methamphetamine, amphetamine, phenethylamine, ephedrine, norephedrine	*Journal of Chromatography* 515: 385
Antibiotics	Cephalosporins	Smith RM ed. *Supercritical Fluid Chromatography*, London: Royal Society of Chemistry (1988) p 180
	Erythromycin	*Journal of Chromatography* 454: 243
	Suflonamides	*Journal of Chromatography* 363: 147
Anticancer	Taxicins	HRC 16: 666
Antidepressants	Amitriptyline, nortriptyline, protriptyline, imipramine, desipramine	*Journal of Pharmaceutical Science* (1994) 83: 281
Antipsychotics	Triflupromazine, carphenazine, methotrimeprazine, promazine, perphenazine, chloroprothixene, deserpidine, thiothixene, reserpine, acetophenazine, ethopropazine, promethazine, propriomazine, molindone	*Journal of Pharmaceutical Science* (1994) 83: 287
Barbiturates	Barbitone, butobarbitone, Amylobarbitone, pentobarbitone, talbutal, quinalbarbitone, methohexitone, phenobarbitone, heptabarbitone	*Journal of Chromatography* 481: 63
Benzodiazepines	Ketazolam, diazepam, nordazepam, cloxazolam, chlordiazepoxide, lormetazapam, estazolam, temazepam, triazolam, lorazepam, loprazolam	*Journal of Chromatography* 483: 51
Beta blockers	Betaxolol, cicloprolol, metoprolol, nadolol, pindolol, propranolol	*Journal of Chromatography* 539: 55
	Timolol, atenolol, betaxolol, pindolol, bupranolol, pronethalol, oxprenolol	*Analysis for Drugs and Metabolites*. Cambridge: Royal Society of Chemistry, (1990) p. 257
Calcium channel blockers (Dihydropyridines)	Felodipine	*Journal of Pharmacology and Biomedical Analysis* (1994) 12: 1003
Ergot alkaloids	Agroclavine, festuclavine, elymoclavine, noragroclavine, chanoclavine I, chanoclavine II, norchanoclavine II, bromocriptine mesilate, ergocryptine	*Journal of Chromatography* 363: 147
Gingkolides	Bilobalide, gingkolide A, gingkolide B, gingkolide C, gingkolide J	*Supercritical Fluid Chromatography with Packed Columns*. New York: Marcel Dekker, p. 116.
H2 receptor antagonist	Ranitidine and metabolites	*Journal of Chromatography* 683: 402
NSAIDs	Phenylbutazone and metabolite	*Journal of Pharmacology and Biomedical Analysis* (1995) 13: 59
Purines	Mercaptopurine, trimethoprim. trifluridine, zidovudine	HRC 13: 393
Sesquiterpenes	Many	*Journal of Chromatography* 779: 307
Steroids	Many	*Chromatographia* (1995) 40: 58 *Journal of Chromatography* 363: 147
Stimulants	Cocaine, amphetamine, methamphetamine, benzphetamine, phenmetrazine, phendimetrazine, methylphenidate, ephedrine, phenyephrine, hydroxyamphetamine, nylidrine, phenylpropanolamine, mephentermine, naphazoline, xylometazoline, tetrahydrozoline	*Journal of Pharmaceutical Science* (1994) 84: 489
Xanthines	Caffeine, theophylline, theobromine	*Journal of Chromatography* 363: 147

cited previously, columns can be linked to increase efficiency or tune selectivity. In the latter case, columns of different stationary phase can be joined to change selectivity. An example of this appears in the chiral section.

Table 1 contains a summary of achiral pharmaceutical separations. Where practical, the actual solutes chromatographed are listed. The majority of these separations have been done on packed columns. SFC easily analyses basic compounds such as phenothiazine antipsychotics, tricyclic antidepressants, and stimulants. Nevertheless analysis of acids is also possible. Berger has demonstrated the elution of benzene derivatives with up to six carboxylic acid functional

groups. A ternary mobile phase of carbon dioxide, methanol, and trifluoroacetic acid was required to elute these strong acids.

An extremely valuable application of SFC is as a compound purity measurement tool. Gyllenhal *et al.* demonstrate the utility of SFC on four separate pharmaceuticals. All four examples indicate that SFC either equalled or improved the existing HPLC methods. For instance, packed column SFC was able to separate two impurities from metoprolol that HPLC was unable to achieve. Once again, the complementary nature of a reproducible, normal phase-like separation enhances the more traditional reverse phase HPLC results.

Chiral separations

Perhaps the most important role SFC currently plays in pharmaceutical analysis is as a chiral resolution technique (refer to Chiral Separations/Supercritical Fluid Chromatography). In this arena, the normal-phase retention mechanism is a tremendous asset. Since most chiral separations rely on polar solute–stationary phase interactions, a normal-phase environment is much more conducive to chiral recognition. Normal-phase HPLC chiral separations typically can be replaced by packed column SFC and enjoy the benefits of faster analysis times, lower solvent consumption, and more reproducible retention times. SFC also offers greater efficiency by allowing columns to be coupled.

Similar and dissimilar stationary phases can be coupled together to permit selectivity tuning. **Figure 2** shows an achiral/chiral column pair used to separate ibuprofen enantiomers in a urine matrix. The sample is initially injected onto the cyano column. The large peak at approximately 1 min is ibuprofen. When this peak has passed into the switching valve, the valve is turned and the loop contents are injected into the chiral column. The advantage of this approach is that on the achiral column, both enantiomers will coelute. With the valve arrangement used, the more polar urine components are never introduced into the chiral column. The peaks at approximately 7 min are the ibuprofen enantiomers. Note that ibuprofen is sold as the racemate of the enantiomers. The larger second peak is the S form of ibuprofen, while the barely visible first peak is the R form. In the body, the R form is inverted to the S form. Sandra et al. investigated a 'universal' chiral column by coupling three different chiral columns in series. In 90% of the successful separations on a single phase, the column triad was able to perform as well.

Preparative Separations

A natural extension of SFC is as a preparative separation technique. The column capacity is a function of the packing material and is essentially equivalent to liquid chromatographic systems. Consequently, scale-up procedures are similar. A major advantage for SFC is the solvent. Upon expansion, the mobile phase becomes gaseous or gaseous with a small amount of liquid phase, simplifying the recovery of analytes. The bulk of the mobile phase is nontoxic

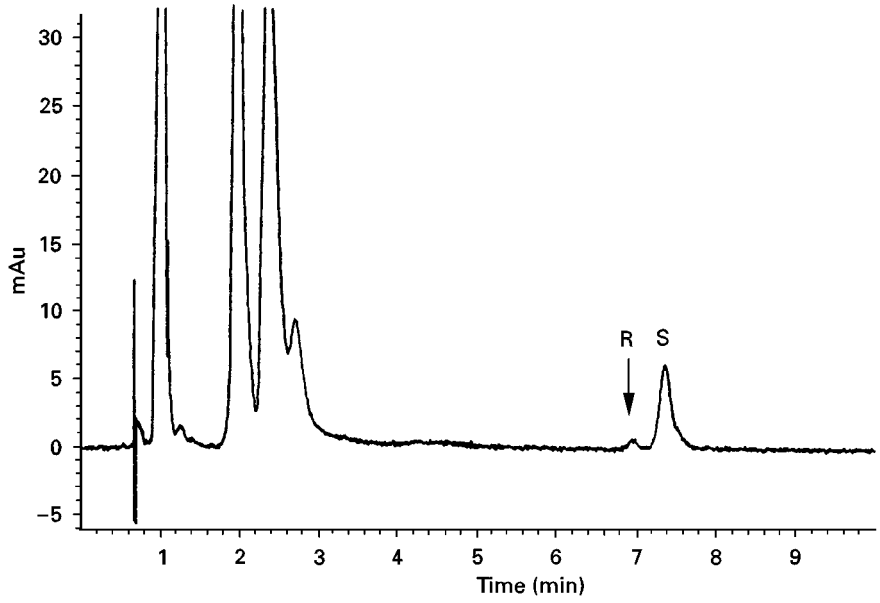

Figure 2 Chiral separation of ibuprofen in a biological matrix. Columns 4 × 125 mm Lichrosphere CN (5 μm particles) and 4.6 × 250 mm Chiralpak AD (10 μm particles); oven 35°C, flow rate 2 mL min^{-1}; outlet pressure 150 bar; modifier 5% methanol in carbon dioxide, UV detection at 210 nm; injection volume 5 μL. Courtesy Dr TA Berger, Berger Instruments.

(carbon dioxide) and, at large enough scale, can be recycled.

The differences between using normal liquids and supercritical fluids for preparative separations present advantages and disadvantages in both cases. For isocratic mobile phases, HPLC is probably easier in terms of mobile phase handling (e.g. no back-pressure regulator required). Mobile phase recycling is straightforward in HPLC since the column effluent is merely redirected to the pump inlet reservoir. SFC requires some repressurization apparatus at the outlet of the back-pressure regulator in order to recycle the mobile phase. However, SFC can use pressure as a separation variable whereas HPLC cannot. Although pressure programming in SFC is not as powerful as composition programming, it still affords the user an additional degree of flexibility. Recovery to initial chromatographic conditions from a pressure programme in SFC is very rapid. For composition programming or in HPLC terms, 'gradient programming', HPLC is more difficult to use. The time for column recovery is substantially longer in HPLC than in SFC, particularly for normal-phase separations. In addition, mobile phase recycling is complicated in gradient HPLC. The mobile phase depressurization actually benefits SFC in this scenario. By depressurizing the mobile phase, the concentration of modifier can be reduced to some low, reproducible value. This fluid can then be repressurized and fed to the pumping system with the modifier added to restore the desired initial composition. Column re-equilibration is very fast in SFC, thus minimizing recovery time.

Jusforgues *et al*. have described three applications of preparative SFC which demonstrate some of the merits of this approach. The excellent retention time and area reproducibility possible with SFC is demonstrated with an insecticide. For the discrete peaks, the retention time reproducibility ranges from 0.17% to 0.19% relative standard deviation. In terms of peak area, the range is 1.17–1.54% relative standard deviation. An additional example given by these authors shows the separation of enantiomers by preparative SFC. The separation cycle takes less than 5 min and the yield of $> 99\%$ purity compounds is 29 g day^{-1}.

Laboratory-scale preparative separations, typically in the milligram range, are attractive in conjunction with combinatorial chemistry. The aforementioned rapid analysis time, simple mobile phase elimination, and loading capacity make SFC an attractive option for small-scale purification.

Rapid Screening

The extensive growth of combinatorial chemistry has brought about significant changes in pharmaceutical analysis. Synthetic production is now discussed in terms of thousands of compounds per year. With this explosion of organic syntheses, the companion techniques must also develop to handle the load. Parallel synthesis must be met with rapid, parallel analysis to maintain the pace. SFC is well suited for interfacing with this demanding need.

The current workhorse for combinatorial analysis is still reversed-phase HPLC. As discussed previously, packed column SFC is both complementary to reversed-phase HPLC and potentially an order of magnitude faster. Not only is this speed apparent in the analysis times but more importantly, in the total cycle time on the system. **Figure 3** illustrates the impressive speed of gradient elution by packedcolumn SFC. The

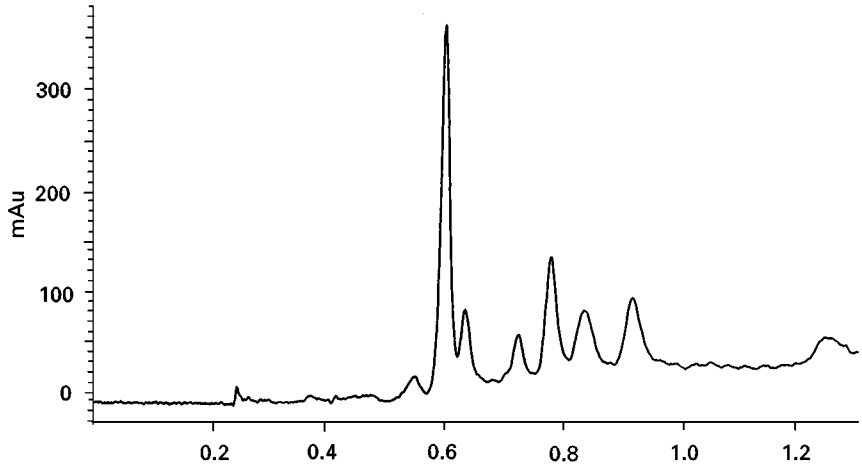

Figure 3 High-speed packed column SFC for combinatorial screening. Column 4 × 125 mm Lichrosphere CN (5 μm particles); oven 35°C; column flow 4 mL min^{-1}; outlet pressure 120 bar; 15–65% modifier (methanol + 0.4% isopropylamine) programmed at 45% min^{-1} in carbon dioxide, UV detection at 230 nm; 10 μL injection from a 96-well plate. Courtesy Dr TA Berger, Berger Instruments.

column is a 125 mm × 4 mm × 5 μm Lichrosphere cyano (these are typical column dimensions for a standard HPLC separation). The sample is drawn directly from a titre plate and injected. The modifier is programmed from 15% to 65% at 45% min^{-1}. The analysis takes 1.2 min to complete and an additional 0.68 min to re-equilibrate. Consequently, the system is able to run over 760 samples per day. On a similar column by reversed-phase HPLC, the system is capable of two to three runs per hour or at best, 72 samples per day.

Note that the chromatogram shows several peaks in the sample. Since the goal of this synthesis was a single component, there are some obvious complications. With the column used, the system has sufficient peak capacity to separate the seven components identified. For a similar speed of analysis by HPLC, the column would have to be substantially shorter or have much smaller particles. In either case, the peak capacity would not be the same at the SFC separation. As a result, the HPLC system may be unable to resolve all of the peaks. The speed and efficiency of SFC is better suited to these high volume analyses than HPLC.

Summary

The rapid analysis speed, complementary selectivity, and achievable high efficiency of SFC make it a valuable technique for pharmaceutical analysis. Because of its normal-phase-like retention mechanism, SFC affords users of reversed-phase HPLC an orthogonal separation system. In addition, chiral separations are clearly a strong application for SFC. The renewed interest in SFC for laboratory-scale purifications and combinatorial screening has breathed new life into this technique.

A valid point raised by Wilson *et al.* is that the current literature still demonstrates the potential of SFC rather than fully developed methods. This situation has begun to change. Although the literature may not reflect it yet, more and more pharmaceutical workers are using SFC as a routine technique.

Further Reading

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Thin-Layer (Planar) Chromatography

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In 1938, Izmailov and Schraiber used aluminium-coated plates to separate coloured plant extracts. Further development of thin-layer chromatography (TLC) as a semiquantitative analytical technique and a tool for identity testing took place in the mid-1950s, especially by Stahl. Soon after this pioneering work, TLC found its way into pharmaceutical analysis.

Surveys of the analytical literature from the late 1980s to the mid-1990s show that approximately 25–30% of all articles published in the field of TLC described pharmaceutical applications. These figures may give some idea about the status of TLC in pharmaceutical analyses.

The rapid development of liquid chromatography (LC, especially high performance liquid chromatography (HPLC)) in the mid-1970s has made LC the predominant analytical method in pharmaceutical analysis. Nevertheless, even today TLC must be considered the most widely used pharmacopoeial chromatographic technique.

TLC in the Pharmacopoeias

An early example of pharmacopoeial use of TLC (**Table 1**) was for impurity testing of corticosteroids in the *British Pharmacopoeia* (BP) addendum 1966 and as a general method featured in the BP of 1968. A typical specification for an active pharmaceutical ingredient until then would require verification of identity and then rely on a usually nonspecific assay supplemented by traditional limit tests, e.g. for heavy metals and chloride. For most organic substances a reasonably sharp melting point was a crude but generally accepted measure of purity. The introduction of TLC to the BP 1968 had great expectations of this technique, as it refers to 'greatly increased