Development of a Generic Gradient SFC Method for Application in Drug Discovery and Development

by Claudio Brunelli[1], Melissa Dunkle[2], Sam Morris[1] and Pat Sandra[2] -
[1]Pfizer Global R & D, Analytical R&D, Sandwich, United Kingdom
[2]Pfizer Analytical Research Centre, Ghent University, Ghent, Belgium

Key Words : Supercritical fluid chromatography, generic gradient method, validation, drug discovery, thiourea.

In recent years, packed column Supercritical Fluid Chromatography (SFC) has received renewed interest within the pharmaceutical industry. Its inherent features such as speed, efficiency and versatility make SFC the perfect complement to reversed-phase liquid chromatography (RP-LC) and hydrophilic interaction liquid chromatography (HILIC) in drug discovery and development. Moreover, the 'green' credentials of the technique are becoming more and more appreciated.

Research in SFC is principally directed towards the development of novel stationary phases and generic method development workflows. However, the pharmaceutical industry is looking at SFC with growing interest but specifically requires instrumentation that meets ever-increasing regulatory requirements. This article presents a generic gradient method applicable to the separation of analytes with a wide polarity range, the separation of complex mixtures and the trace level determination of impurities utilising the common 2-ethylpyridine and cyanopropyl SFC stationary phases. The mobile phase composition is such that several detection types including mass spectrometry (MS) may be applied.

The performance of current state-of-the-art SFC instrumentation is illustrated with a mixture composed of several compounds of pharmaceutical interest and through the determination of thiourea in a pharmaceutical intermediate at the 0.01% (w/w) level.

The current trend in pharmaceutical analysis is focused toward high throughput, high productivity or high resolution. In response to these increasingly demanding requirements, innovative technologies and improvements in instrumentation have emerged in liquid chromatography (e.g. higher pressures and elevated temperatures). Reversed-phase liquid chromatography and hydrophilic interaction liquid chromatography (HILIC) are by far the most successful LC modes presently used in drug discovery and development, for the analysis of hydrophobic (RP-LC), hydrophilic and ionisable solutes (HILIC).

In terms of throughput, productivity and resolution, supercritical fluid chromatography has much to offer but has long been considered immature or insufficiently robust for application in a routine and regulated environments. Notwithstanding this, SFC is highly appreciated for some pharmaceutical "niche" applications such as chiral analysis [1-4] and for preparative scale compound isolation [3-6]. In the former, high enantioselectivity and speed are required while in the latter, the easy removal of the volatile mobile phase allows for highly efficient recovery of the targets in terms of time and costs.

SFC appreciation, especially for applications in the pharmaceutical industry, has been reflected in the increasing number of publications and scientific works reported in recent years. The interplay of high efficiency in short analysis times, the reduced volumes of non-toxic organic solvents and the potential for unique selectivity, are highly appealing in a world were fast method development, analytical methods with high information content, cost reduction and environmental friendliness are of extreme importance.

Taylor has described the latest developments and applications of SFC in a recent publication [7]. Abbott et al. [8] reviewed the major clinical and pharmaceutical applications, which at the moment represent the leading driving forces for innovation in this field.

Despite renewed scientific enthusiasm, SFC is still failing to be accepted as a routine analytical technique. It suffers a poor reputation that stretches back more than 20 years ago (e.g. as a result of historical robustness issues). Additionally, vendor instability in the market is not helping fight a certain degree of skepticism of the technique [7].

Although some issues still have to be addressed to make SFC a technique with the

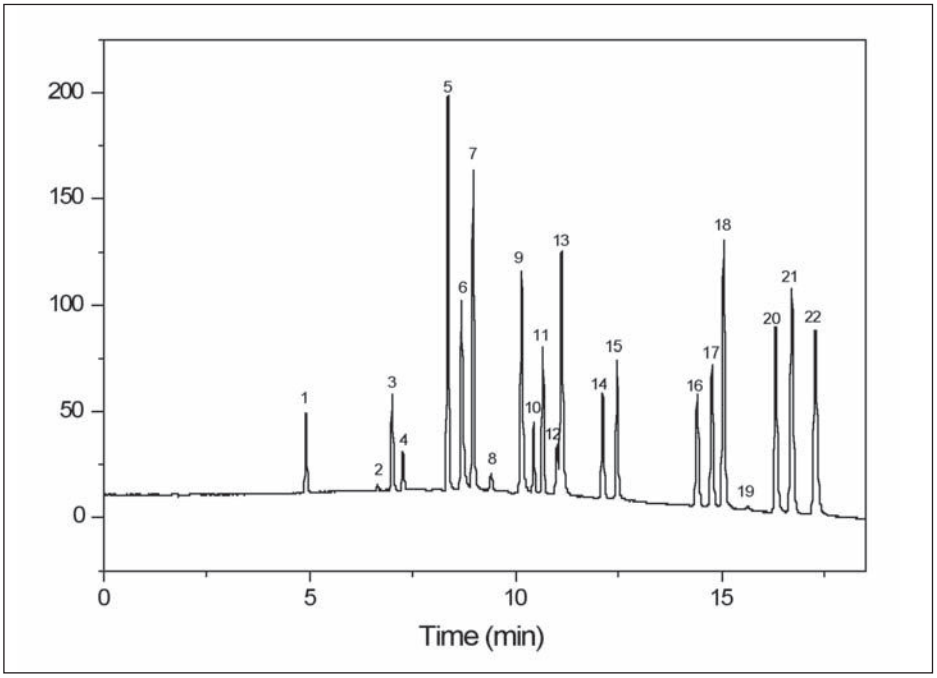

Figure 1. SFC chromatogram of a 22 component mixture using the generic gradient conditions. For compound numbering see Experimental section.

same maturity as LC, more and more systems are being delivered with detailed user requirement documentation (IQ/OQ/PV). This is an essential prerequisite for operation in a regulated environment. There have been attempts to list performance standards for SFC [9]. More recently Dejaegher et al. [10] described different approaches to robustness testing of an SFC method but due to the rapid evolution of the other separation techniques i.e. in gas and liquid chromatography, these parameters and acceptance criteria require review.

Only a few examples of validated SFC methods can be found in the literature and operating conditions are very simple (i.e. isocratic methods with high organic modifier content). Coe et al. [11] described a validated SFC-MS bioanalytical method for the determination of warfarin in human plasma. Mukherjee [12] recently described the validation studies of an enantioselective method with direct injection of an aqueous formulation.

In drug discovery, isocratic conditions are rarely applicable for the separation of analytes with a wide polarity range or for complex mixtures. As a result gradient conditions are applied which in turn puts much higher demands on the acceptance criteria of SFC.

This study aimed to develop a generic gradient method and validate it for a pharmaceutically relevant application - the determination of thiourea in a pharmaceutical intermediate at low level (0.01% w/w). In this work, the applied method validation acceptance criteria were consistent with those used for Active Pharmaceutical Ingredient (API) late stage development and regulatory submission. This suggests that with some further research and instrumental developments SFC can potentially reach the same maturity as GC and LC.

Experimental

Materials

A 22-component test mixture containing acidic, basic and neutral pharmaceutical compounds was used for developing the generic gradient SFC conditions. The following compounds were purchased from Sigma-Aldrich (Munich, Germany): acidic solutes ibuprofen (2), fenoprofen (8) and flurbiprofen (9); basic solutes caffeine (1), theophylline (3), theobromine (4), thymine (5), adenine (6), uracil (7), cytosine (12) and hypoxanthine (13); neutral solutes cortisone (10), prednisone (11) hydrocortisone (14), prednisolone (15), sulfamerazine (16), sulfamethoxazole (17), sulfadimenthoxine (18), estriol (19), sulfaguanidine (20), sulfaquinoxaline (21), sulfamethizole (22). The number in parentheses after the compound name was used for peak labeling in Figure 1. Solutions were prepared in methanol and injected quantities were ca. 100 ng.

For the trace analysis work, thiourea was purchased from Sigma-Aldrich while the pharmaceutical intermediate (PI-X) was a Pfizer proprietary compound obtained from Pfizer Global R&D, Sandwich, UK. Solutions were prepared in methanol.

The mobile phase CO_2 (grade 4.5) purchased from Air Products (Crewe, UK). Methanol (MeOH) HPLC grade used as modifier and ammonium acetate (AmAc) 99.00% used additive were purchased from Fluka (Steenheim, Germany).

Instrumentation

Generic method development

A Berger SFC Minigram system, equipped with UV DAD detector at 254 nm was utilized for the development of the generic conditions on a 2-ethylpyridine column (Berger Instruments, Inc., Mettler-Toledo Co Newark, DE). The 2-ethylpyridine column used for this generic method was selected based on previous screening work and statistical analysis of the data (data not published). System operation was controlled with SFC-ProNTo software (Berger Instrument) and data were analyzed using ChemStation software (Agilent Technologies, Brussels, Belgium). The injection volume was 5 µL. The column was 25 cm x 4.6 mm i.d.. and packed with 3.0 µm particle size 2-ethylpyridine silica.

Trace analysis and validation

Thiourea analyses were performed using a Jasco SFC-2000 Series (Jasco Corporation, Tokyo, Japan), equipped with UV detection at 254 nm and a 5 µL injection loop. The system was controlled by EZChrom (1 Version 3.2.1). The column was 25 cm x 4.6 mm i.d. packed with 5.0 µm particle size cyanopropyl silica, L10 according to USP nomenclature, (Princeton Chromatography, Cranbury, NJ, USA).

Method conditions

On both systems the following generic conditions were applied: mobile phase CO2 with organic modifier MeOH containing 20 mM AmAc. The modifier was programmed from 5%, hold 1min, to 40% at 2%/min. The pressure was 150 bar, the temperature: 40°C and the flow rate 2.0 mL/min.

Results and discussion

At present, complete SFC instruments are available from Thar SFC, a Waters company, (Pittsburgh, PA, USA) and Jasco Corporation (Tokyo, Japan). Modular SFC pumps including back pressure regulators and mixing chambers as accessories that can be installed on existing LC instruments have recently been introduced by SandraSelerity Technologies (Salt Lake City, UT, USA) and Aurora SFC Systems (Sunnyvale CA, USA).

We are currently evaluating these systems with respect to the performance standards required for implementation in regulated environments such as pharmaceutical laboratories.

In the first instance a 'generic' gradient method approach was developed for separation of a standard mixture comprising acidic, basic and neutral solutes. 'Generic

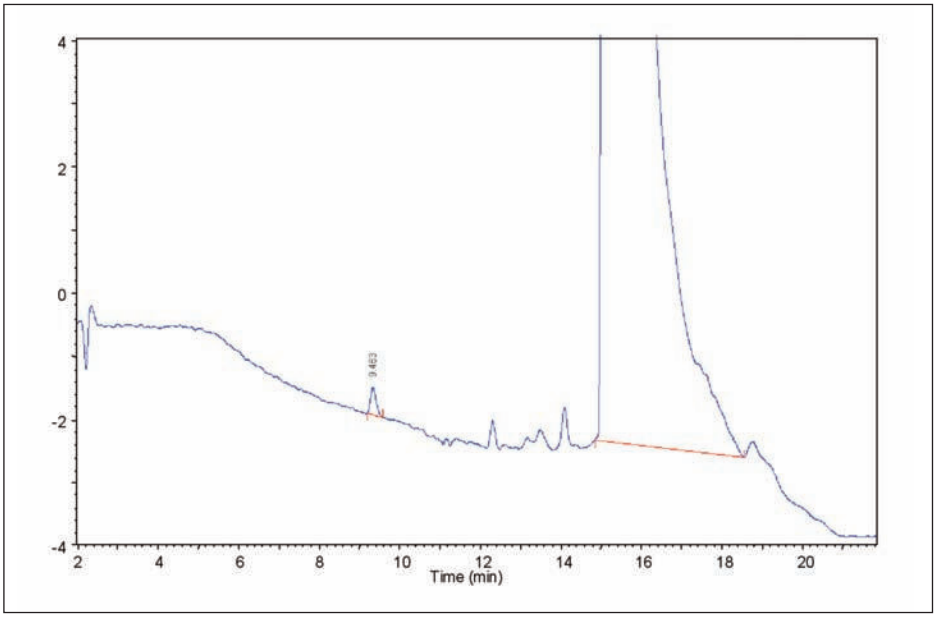

Figure 2. SFC chromatogram for the determination of thiourea in PI-X at LOQ level (0.01% = 1.5 µg/mL)

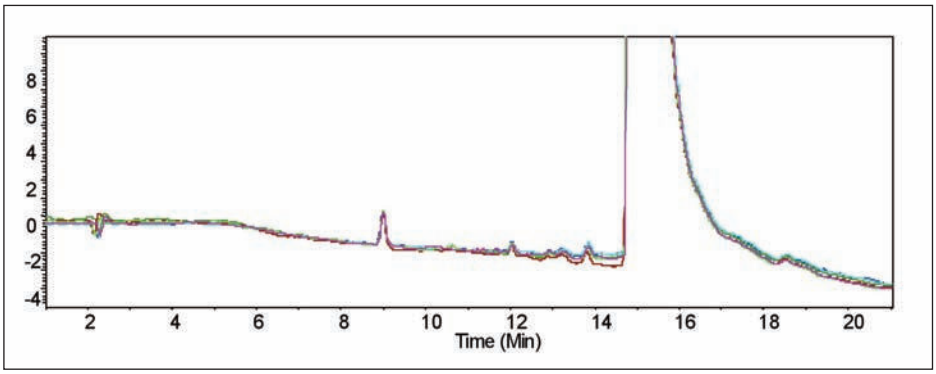

Figure 3. Overlay of six repeated injections of thiourea at specification level of 7.5 µg/mL (0.05% w/w) in the presence of PI-X.

Approach' in this context means a set of default operating conditions (i.e. pressure, temperature and flow rate) applied with a gradient program for organic modifier content, which is expected to provide adequate performance for large proportion of analytical problems. Methanol is used as organic modifier in combination with ammonium acetate as generic additive. We recently described that ammonium salts can replace the basic and acidic additives commonly used in SFC (i.e.: diethylamine, isopropylamine, formic acid and TFA) [13]. Another advantage of ammonium acetate as an additive is that this is the preferred volatile salt for SFC-MS. Furthermore the generic conditions can be fine-tuned for the separation of critical pairs by mixing methanol with acetonitrile in appropriate ratios [14]. Figure 1 shows the separation of the 22 component mixture. All solutes elute with good peak shape in less than 20 minutes.

From a chromatographic point of view, this is a remarkable separation. It illustrates that there should be a place for SFC in pharmaceutical analysis if traditional HPLC validation acceptance criteria can be met. To test this hypothesis, SFC was subsequently applied to the trace-level analysis of thiourea in an API using the generic conditions.

Thiourea is classified as a category 3 carcinogen [15] and therefore a method is required to determine thiourea at appropriate levels. Different LC modes were investigated but for the given application, significant limitations were observed. In RP-LC, thiourea was not retained while HILIC provided sufficient retention for thiourea but insufficient resolution between process related impurities and PI-X. Poor peak symmetry was observed when normal phase LC (NPLC) was applied.

With the SFC generic approach, the analysis of thiourea and PI-X was successful in terms of analyte resolution and peak symmetry. Gradient programs in SFC have traditionally been discouraged because the dynamic mixing of supercritical CO_2 with an organic modifier often lead to baseline instability and irreproducibility of the mixing process. As

mentioned previously, for this reason, SFC methods currently adopted in a QA/QC environment or reported to regulatory agencies are typically isocratic adopting high concentrations of organic modifiers.

The performance of the generic method was therefore submitted to the standard validation exercise for established analytical tools such as LC or GC. Only those aspects of validation which are specific to instrumentation performance and not to analysts (such as intermediate precision) are discussed.

Initial experiments were carried out on the 2-ethylpyridine column but some API related impurities could not be resolved. Subsequently, a cyanopropyl silica column was selected from screening of alternative stationary phase with the same generic conditions. The cyanopropyl column also gave slightly improved peak shape for PI-X over the 2-ethylpiridine column. A mixture containing PI-X and thiourea each at 0.1 mg/mL in MeOH was analysed using the generic conditions. Thiourea and PI-X were baseline resolved from other process related impurities using these conditions.

A limit of quantitation (LOQ, S/N ≥ 10) of 1.5 µg/mL was estimated from the S/N ratio obtained from the 5 µl injection of a 10 µg/mL thiourea solution. This was experimentally verified by injecting a 1.5 µg/mL thiourea solution, which corresponds to 0.01% (w/w) of nominal loading of 15 mg/mL of PI-X. Figure 2 shows the chromatogram of 15 mg/mL of PI-X spiked with thiourea at 1.5 µg/mL

The system suitability and precision based on six injections of a 7.5 µg/mL thiourea (0.05% w/w) solution showed an RSD of 3.13% for peak area and of 0.09% for retention time. Figure 3 shows the overlay of the six replicate injections at the specification level (0.05%) and illustrates excellent chromatographic reproducibility.

The linearity of the thiourea response was evaluated with a 5 point calibration curve in the range 1.0 to 15.0 µg/mL. This range covers a LOQ up to twice the specification limit. A linear response was observed over this interval with a correlation coefficient r = 0.999 and a statistically insignificant intercept.

The accuracy and repeatability were verified by six replicate injections of thiourea solutions at 3 concentration levels (1.5, 7.5 and 15 µg/mL) in the presence of PI-X at nominal concentration (i.e. 15 mg/mL). The data are summarized in Table 1. Mean recovery and RSD values demonstrate acceptable accuracy and repeatability across the range investigated.

Level (g/mL)	Mean % Recovery	% RSD
1.5 (0.01%)	109.6	11.7
7.5 (0.05%)	97.9	5.8
15.0 (0.1%)	94.0	3.2

Table 1. Accuracy and repeatability data

Conclusions

The generic gradient SFC method discussed in this work is applicable to the separation of a large number of pharmaceutical compounds. Informed selection of the appropriate stationary phase to which the generic gradient is applied is an essential starting point for method development.

The study also demonstrated that, for the selected application, namely the determination of thiourea in an API intermediate, late stage development and regulatory submission performance criteria were met.

Acknowledgements

The authors wish to acknowledge Jasco corporation (Tokyo, Japan) and Mettler Toledo (SFC product line now owned by Thar SFC, a Waters Company) for providing the instrumentation and technical assistance for this work. The authors also wish to thank Lucie Alcaraz (Université d'Orleans, Orleans, France) and Agnes Ouattara (École Supérieure de Chimie Organique et Minérale, Paris, France) for their contribution.

References

[1] D. Mangelings, Y. Vander Heyden, J. Sep. Sci. 31, (2008), 1252 – 1273.
[2] G. Felix, A. Berthod, P. Piras, C. Roussel, Separation and Purification Reviews, 37 (3), (2008), 229-301.
[3] DR.Wu, L. Leith, Am. Pharm. Rev. 10, (2007), 84–87.
[4] L. Taylor, J. of Supercritical Fluids 47 (2009) 566–573.
[5] R.T. McClain, A. Dudkina, J. Barrow, G. Hartman, C. J. Welch, J. Liquid Chrom. & Rel. Techn, 32(4), (2009), 483-499.
[6] L. Miller, M. Potter, J. Chromatogr. B, 875(1) (2008), 230-236.
[7] L. Taylor, Anal. Chem., 80 (12), (2008), 4285-4294.
[8] E. Abbott, T.D. Veenstra, H.J. Issaq, J. Sep. Sci. 31, (2008), 1223 – 1230.
[9] K. Anton, C. Siffrin, Analusis, 27 (8), (1999), 691-701.
[10] B. Dejaegher, Y. Vander Heyden, J. of Chromatogr A, 1158 (2007) 138–157.
[11] R.A. Coe, J.O. Rathe, J.W. Lee, J. Pharm. Biomed. Anal. 42 (2006) 573.
[12] P.S. Mukherjee J.Pharm. & Biomed. Anal., 43, (2007) 464–470.
[13] M. Dunkle, C. Brunelli, W. Farrell, P. Sandra, submitted for publication to J. Sep. Sci.
[14] C. Brunelli, Y. Zhao, M. Hanna-Brown, P. Sandra, J. Sep. Sci., 31, (2008), 1299-1306.
[15] International Agency for Research on Cancer (IARC), 79, (2001), 703.