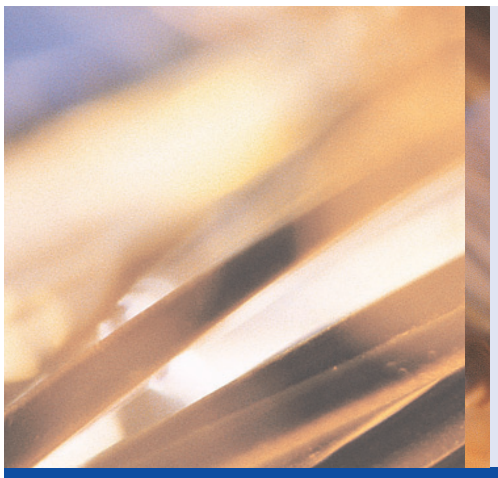

Chromatography Focus

SFC/MS/UV/ELSD: INTEGRATED HYPHENATION TECHNIQUE FOR DRUG DISCOVERY

Ziqiang Wang

Supercritical fluid chromatography (SFC) has attracted great interest as a chromatographic technique in pharmaceutical industry. Compared to high performance liquid chromatography (HPLC), SFC demonstrated higher efficiency, reduction of solvent usage and green chemistry for the safe aspect of inert carbon dioxide in the place of toxic organic solvent as in HPLC. In addition, over the past few years, analytical SFC has started to be evaluated as an orthogonal technique from HPLC for pharmaceutical analysis in both qualitative and quantitative terms.

At Pfizer Ann Arbor Discovery Laboratory we have installed the hyphenated analytical SFC system coupled with mass spectrometry (MS), ultra-violet (UV) and evaporated light scattering (ELSD). This system is used as both qualitative (screening) and quantitative analysis tool for medicinal chemistry support. The system is completely integrated, controlled by single software. The operation is straightforward and easy to use. This enables us to automate most of the routine analysis for better productivity. The systematic tests on compound characterisation and calibration by the hyphenated detection techniques (MS, UV and ELSD) from this setup are carried out, and the system performance were evaluated in both qualitative and quantitative aspects.

"OUR LABORATORY HAS BEEN ENGAGED IN THE ANALYSIS OF THOUSANDS OF CHEMICALLY DIFFERENT COMPOUNDS PRODUCED FROM MEDICINAL CHEMISTRY."

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Liquid chromatographic techniques like HPLC and LC/MS have long been the powerful tools to drug discovery for purity assessment, molecular identification, quantification as well as stability indication purposes[1-3].

The unique characteristics of LC techniques such as high efficiency, variety of stationary phases available and flexible operation modes make it capable to separate complicated mixture of acidic, basic and neutral compounds of wide range of polarities in reasonable time scale, which would be difficult to achieve by other techniques.

In particular, hyphenated LC techniques like LC/MS have become the dominant analytical technique in medicinal chemistry laboratories for its combination of selectivity from LC, high sensitivity and fast identification and characterisation capabilities from MS[4,5].

Continuous efforts in the chromatography field are being pursued in order to improve separation performance for efficiency, robustness and overall productivity enhancements. In recent years, supercritical fluid chromatography has emerged as a "new" technique for pharmaceutical analysis[6-8].

The lower viscosity of supercritical fluids, such as carbon dioxide, enables faster flow rates than HPLC. Also higher diffusivity for analytes in supercritical fluids yields greater efficiency (smaller plate heights) that gives sharper peaks or reduces column length required to resolve a sample.

This high efficiency and increase in flow rate greatly reduces chromatographic time, in others words, increasing the productivity. In addition, the additives such as trifluoroacetic acid (TFA), phosphate buffer salts and basic amines that are commonly used in HPLC to ensure reproducible performance are no longer needed in routine SFC runs.

This means the drug candidates have less exposure to stability risk from the process related to existence of these acids/bases, thus increasing the quality of the analysis. For these unique advantages SFC is amenable to improve the throughput and quality of analysis. SFC and its hyphenated techniques have therefore matured as techniques of choice for pharmaceutical analysts to complement to HPLC and even as a replacement to HPLC in some cases[9,10].

Our laboratory has been engaged in the analysis of thousands of chemically different compounds produced from medicinal chemistry. The types of analysis supported include purity assessment and scale up purifications. Since the beginning of the century SFC has been gradually introduced into the lab to complement the traditional LC techniques and has gained great success. In this article the hyphenated SFC/MS/UV/ELSD analytical instrumentation is described.

This instrumentation is designed to support achiral method development for medicinal chemistry and for final purity assessment and molecular characterisation in support of pharmaceutical compound registration.

The system design and optimisation is discussed; characteristics of the SFC/MS capability will be demonstrated; evaluation of the quantification performance is carried out with commercial standard compounds, the result of these tests will be discussed. Finally, future development plan of the technique will be outlined.

EXPERIMENTAL

Materials

Chemicals: Methanol, trans stilbene oxide (TSO, MW197), ketoprofen (MW254), amcinonide (MW503) and, Hydroxyethyl theophylline (HETP, MW224.2) are purchased from Sigma (St. Louis, MO, USA), carbon dioxide gas is supplied by Linde Gas (Maumee, OH USA)

Columns: Kromasil silica column is from Eka Chemical (Dobbs Ferry, NY, USA). Diol, cyano, and pyridine are all from Zymor Chromatography (NJ, USA). All columns are of 4.6mm x 250mm, 5µm, 100A.

Instrumentation

Thar's SFC-MS –ELSD-VWD system with MassLynx control software

SFC pumping: The system consists of Thar's Automated Method Development System (AMDS) as the chromatographic inlet that includes SFC/modifier pump module, an autosampler unit and a column oven and 6-column selector unit. The pressure was held at 100 bar and 40°C with a flow rate of 4 mL/min. A general gradient program is applied as follows: it holds 5 % modifier for 1 min, then increase to 40% over 5 min., hold for 1 min., decrease back to 5 % in 1 min. and hold for 1 min. The total run time is 9 min.

MS: The Waters' ZQ quadruple mass spectrometer, capable to run in both ESI and APCI mode.

UV: The Gilson VWD was monitoring 254nm at a sensitivity of 0.01.

ELSD: A SEDEX 55 ELSD is used in the setup. N_2 gas flow for nebulization and evaporation is set at 2.0 bar and Evaporator temperature programmed to 70 °C.

Both UV and ELSD signal are recorded via analog channels, that is, UV at Channel 1 (An1) and ELSD at Channel 2 (An 2)

Experiment

Standard Calibration Test: Standard solutions of TSO, Ketoprofen, Amcinonide and HETP were prepared by weighing 20mg (TSO is 100mg) of compound separately into vials with 4 ml Methanol, this is stock solutions of 5mg/ml concentration that will be used for all subsequent dilutions of different concentrations. The calibration mixtures of 1 mg/ml, 0. 5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml concentrations were then prepared by pipetting each of the four standard stock solutions into vials according to dilution ratio, and then add calculated volume of methanol to desired concentrations. That is, at first, a 5:1 dilution is made to make 1 mg/ml; then the dilution of 2:1 for all subsequent concentrations from the solutions prepared at previous step.

All vials are injected in triplicates, as well as blank methanol.

Mass Spec. Operation conditions: APCI mode: Corona: 10uA, Cone: 30 V, Extractor: 2 V, Source Temp: 150°C, desolvation temp: 600°C, Desolvation Gas: 500 L/hr, RF Lens: 0.2 V,

ESI mode: capillary: 3.4 KV, cone: 50V, Extractor: 5 V, RF lens: 0.1V, source temp. 90°C, desolvation temp. 120°C. Desolvation Gas: 270L/hr.

All data shown in this article are obtained from injections onto the Kromasil silica column.

RESULTS AND DISCUSSIONS

1. SFC Plumbing Considerations

In hyphenated chromatographic instrumentation the effluents from the column always pass through the UV detector first if present, since it is the most commonly used one in any such setup, then splitting to other detectors for further measurement. This arrangement works well with HPLC instruments since the mobile phase is homogeneous all along the flow path, the splitting ratio is in a relatively small range (1:1 to 3:1), the signal readout from all detectors therefore have compatible sensitivities. This same plumbing arrangement may introduce issues with SFC instrumentation. While the mobile phase, supercritical carbon dioxide with liquid modifier, is in homogeneous state in the UV detector flow cell with pressure regulator at its back, it is no longer in the same kind of state when it is splitting to other detectors than when it hits the atmospheric pressure like in MS and ELSD. This brings up two issues that need to be addressed.

First, this kind of the splitting is considered as a "leak" from the pressurised flow path that will cause back pressure holding problematic or even impossible, therefore the splitting ratio has to be controlled to be larger that only a small fraction (5:1 or even higher) flow will be allowed to direct to other detectors. Second, this smaller than usual effluent brings sensitivity issues with other detectors that may not be as sensitive as UV detectors such as the ELSD, simply because not enough numbers of analyte molecules are available in the flow. With some distinct detector design from specific vendors, this very small stream of molecules may even not be able to reach the detector, or cause other kinds of problems as we found out.

So to address these issues, the plumbing has to be adjusted so that the splitting ratio needs to be kept low enough to ensure the detector sensitivity while still holding the back pressure for the system. This can be done by testing different dimensions of capillaries as the splitter. In our setup it is found that the system is able to split to about 4:1 to ELSD with 75 µm capillary while still holding pressure with 0.010" (~250 µm) i.d. SS tubing used in the main stream. This implies that although it is critical to have certain dimension (id and length) requirements for pressure holding concern as a vendor claimed[13], it is not necessary to see it as the only workable solution; the dimensions of the splitter are in fact quite adjustable and users should play around with them to get the optimum sensitivity desired while still holding the back pressure for whole system.

The ELSD splitting is also arranged right after the column and upstream to the UV. This is to avoid further extra column dilution and decrease signal delay by the slower flow to ELSD. In experiments this arrangement performs quite well to the satisfactory, as can be seen in posted chromatograms in this article.

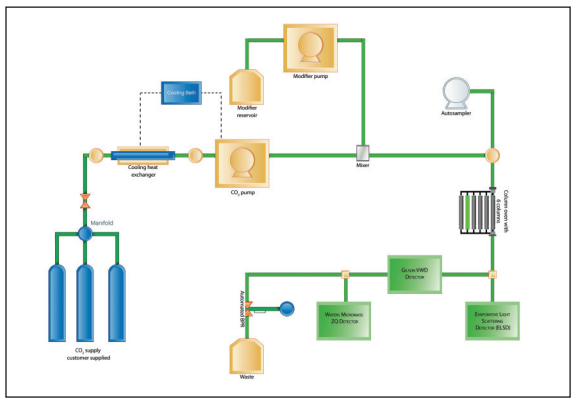

Figure 1. Schematic diagram of the instrumentation setup.

2. MS Detector And Software Control

Waters ZQ mass detector is successfully integrated in the system. The operation is totally controlled from MassLynx (version 4.0). The SFC gradient and flow control is achieved through the "Inlet Method" tab in MassLynx; it can have 6 different co-solvents and 6 column selections so it is capable of performing highly automated screening. The MS configuration is done the same way as it would in LC/MS. The analysis setup is straight forward for any experienced user who has used ZQ before with LC.

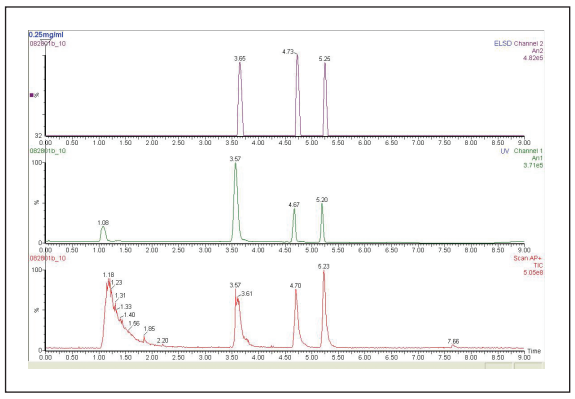

Figure 2. SFC/MS/UV/ELSD Chromatogram of Standard Mixtures;

The splitting related sensitivity issue discussed above are also seen from the MS detector. While in LC/MS most people found that the electrospray (ESI) mode performs better on the ZQ for small molecules, it is found that on SFC setup there is not enough signal from ESI mode,. The flow is only fractional compared to the one in LC, and most of it is CO_2 that cannot help ionization. In reality it is found APCI mode shows much better overall sensitivity (data not shown) than ESI mode; thus as the result APCI mode is chosen as the default MS ionisation mode for all of our routine screenings.

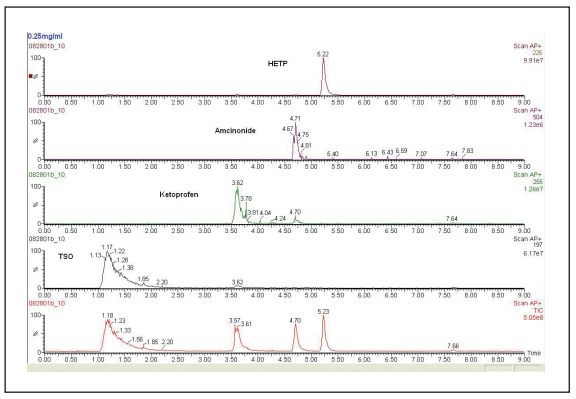

Figure 3. EIC Chromatogram of Standard Mixtures

3. Analytical Screening Procedure

A 4-column screening procedure including silica, diol, pyridine and cyano phases is adapted for all routine analysis. 5-minutes gradient with modifier from 5-40% is used with total run time of 9 minutes, including column equilibration step, and is identical to our general procedures previously reported[11,12].

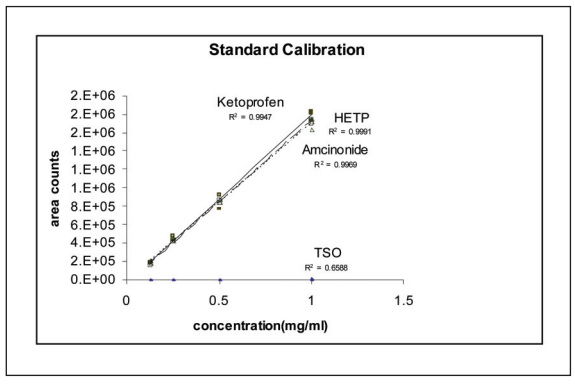

Figure 4. Linear regression of standard calibrations.

4. Standard Calibration By ELSD

The data points range from 0.0675mg/ml, 0.125mg/ml, 0.25 mg/ml, 0.5mg/ml to 1mg/ml concentration levels for each compound in the mixture (TSO concentration is five time higher than other three compounds in the same vial, as described above). The injection volume is 5 µL. All injections are in triplicates as well as for blank (methanol) injections. The area count of each peak from the ELSD trace (channel An 2) is integrated by MassLynx with its default ApexTrack Peak Integration utility and plotted against the correspondent concentrations, no further data manipulation is performed.

It can been seen that except for TSO which has very poor response from ELSD, all other three compounds show good linear coefficients (>0.99) within tested concentration range; the calibration lines are very close to each other, this means that the signal response from ELSD is dependent only on concentration and not on the nature of the compounds, which confirms the "universal" feature that is desired for quantification purpose. It is interesting to note that unlike in HPLC, where people reported significant response factor change with mobile phase gradient compositions, this makes quantification process more complicated and needs more customised software development[14]. In SFC this variance in response factor looks much less serious and can be illustrated by the closeness of linear regression lines in *Figure 4*. The peaks come out at different gradient levels, yet the response factor in terms of area count is very similar to each other, given the sample concentrations and injection volume are identical. It is believed that the explanation comes from the efficiency of the evaporation process from ELSD.

In HPLC, it is usually harder to evaporate aqueous mobile phase out than the volatile organic counterpart in ELSD, so the efficiency is lower at the early stage of a gradient programming, where the water content is high, this results in small signal response factor at the time; if the same peak elutes at higher organic content such as the later stage of the gradient, there is less water to evaporate so the efficiency gets higher, consequently the signal is much higher, although the injection is identical. In SFC, there is no water content in the gradient; instead, CO_2 evaporates itself immediately after passing through the splitting point into the ELSD, the small fraction of the organic modifier poses much less stress on evaporation and so the efficiency difference from gradient program is much less, the response factors are more uniform, though there maybe still a little variance from specific brand design. This makes quantification process less complicated and more accurate results can be expected. We therefore believe this improved linear response feature is one of the advantages that SFC is superior over HPLC in terms of universal quantification capability.

5. Future Development

SFC has demonstrated great potential in pharmaceutical analysis; its high efficiency can be utilised into more fields such as impurity profiling in pharmacokinetics and quality control, drug stability and safety studies. It is also a promising tool to get it directly into the hands of medicinal chemists by putting it in the format of open access mode. The ease of use, high efficiency and high degree of automation will be the keys to make it feasible and successful.

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