International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 1357-1363 www.ijprajournal.com ISSN: 2249-7781

A Review: Supercritical Fluid Chromatography - Tandem Mass Spectrometry.

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Date of Submission: 10-06-2021 Date of Acceptance: 25-06-2021

ABSTRACT:
SFC-MS tandem is a novel, fast and sensitive hyphenated technique used for the separation, determination, quantification and characterization of various chemical and natural compounds especially in the biological samples which is quite challenging due to the minute concentrations of the analytes. supercritical fluid chromatography - tandem mass spectrometry is a preferred hyphenated technique over several other analytical techniques in various fields of science. It has become an important tool in many industrial, regulatory and academic laboratories. In SFC-MS/MS super critical chromatography, a hybrid of gas chromatography and liquid chromatography that combines some of the best features of each is coupled to tandem mass spectrometry through an interface. Particularly in pharmaceutical industry SFC-MS/MS is being used for metabolite identication , chiral analysis and bioanalysis.

KEY WORDS: SFC-MS/MS, interface, bioanalysis, chiral analysis, metabolite identification.

I. INTRODUCTION:

supercritical fluid chromatography is an advanced chromatographic technique , in which mobile phase is a supercritical fluid. SFC was introduced in the early 1960s and it's usage steadily increased since the 1990s with continuous improvements in instrumentation and column technology. For certain applications it is superior to both gas chromatography and high pressure liquid chromatography. SFC is important because it permits the separation and determination of a group of compounds conveniently not handled by either GC or LC. For laboratories overwhelmed with solvent disposal and time-consuming solvent cleanup, supercritical fluid chromatography (SFC) is a beneficial alternative to purification and analytical applications. The most widely used supercritical mobile phase is carbon dioxide which makes it appealing as it fits "green chemistry" principles.SFC can also cover a broad range of analyte through the ability to"tune"the mobile phase polarity by adjusting pressure and temperature of the CO2, and the type and percentage of mobile phase modifiers provides even greater flexibility. Any MS system with atmospheric pressure ionization technique should be able to integrate with SFC. In recent times triple quadrupole(QqQ) and quadrupole -time if flight (Q-TOF) MS tandem configurations were integrated to SFC.

Principle:

Supercritical fluid chromatography:

The principle of SFC is based on adsorption and partition chromatography. SFC is a form of normal phase chromatography that uses a supercritical fluid such as carbon dioxide as the mobile phase. The principle is based on the supercritical fluid.

Supercritical fluids: A supercritical fluid is formed whenever a substance is heated above it's critical temperature. The critical temperature of a substance is the temperature above which a distinct liquid phase cannot exist, regardless of pressure. The vapor pressure of a substance at it's critical temperature is it's critical pressure. At temperature and pressures above it's critical temperature and pressure (it's critical point) , a substance is called as supercritical fluid. The supercritical fluids have densities, viscosities, and other properties that are intermediate between those of the substance in it's gaseous and liquid state.

The effluents from SFC are introduced into the MS tandem through an interface.

Tandem mass spectrometry:

Tandem mass spectrometry , sometimes called mass spectrometry- mass spectrometry (MS/MS) is a method that allows the mass spectrum of preselected and fragmented ions to be obtained. Here, an ionization source, often a soft ionization source, produces ions and some fragments. These are then the input to the first mass analyzer, which selects a particular ion called the precursor ion and send it to the interaction cell. In the interaction cell , the precursor ion can

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decompose spontaneously , react with a collision gas , or interact with an intense laser beam to produce fragments , called product ions. These ions are then mass analyzed bby the second mass analyzer and detected by the ion detector.

II. INSTRUMENTATION

1. Supercritical fluid chromatography
- ❖ Mobile phase
- ❖ Stationary phase
- ❖ Pump
- ❖ Injection system
- ❖ Column
- ❖ Oven
- ❖ Back pressure regulator (BPR)

2. Interface

3 . Tandem mass spectrometry
- ➢ Triple quadrupole:
- ❖ Ionization source
- ❖ Quadrupole mass filter (Q1)
- ❖ Quadrupole collision cell (q2)
- ❖ Quadrupole mass filter (Q3)
- ❖ Electron multiplier.
- ➢ Quadrupole - Time of flight (Q-TOF) :
- ❖ Ionization source
- ❖ Quadrupole mass filter(Q1)
- ❖ Quadrupole collision cell(q2)
- ❖ Time of flight (TOF).

1. Supercritical fluid chromatography:
- ❖ Mobile phase:

The most widely used mobile phase for SFC is carbon dioxide. It is an excellent solvent for various polar organic solvents. In addition , it transmits in the ultraviolet region and is odorless, non toxic, readily available, and remarkably inexpensive compared to other chromatographic solvents. It's critical temperature of 31◦c and it's critical pressure of 72.9atm permit wide selection of temperature and pressure. In some applications polar organic modifiers such as methanol are added in small concentrations to modify the selectivity factor of analytes.

- ❖ Stationary phase:

For polar solutes polar stationary phases are used. Classic polar phases include bare silica, cyano, diol and amine. In recent advancement, ethyl pyridines and a no. of other propriety phases are used. For low polarity solutes , reversed phase columns such as C18, C8, C4 and methyl stationary phases are sometimes used.

For much less polar compounds such as many natural products, including fat soluble vitamins , caretenoids and lipids C18 columns are used.

Widely used polar Stationary Phase are :
- Polysiloxanes - stable, flexible Si--O bond lead to good diffusion.
- Substituted with chemical groups for selective interaction with analyte.
- Polymethylsiloxanes - increase efficiency in separating closely related polar analytes.
- Cyanopropyl polysiloxanes - useful for compounds with –COOH

- ❖ Pump:

Flow controlling is the vital function of pumping systems. In contrast to HPLC pumping systems pressure rather than flow control is necessary and pulseless operation is more important. In general type of high pressure pump used in super critical fluid is determined by column type. For packed columns for easier blending of mobile phase or introduction of modifier fluids reciprocating pumps are generally used. For capillary super critical fluid syringe pumps are most commonly employed. Reciprocating pumps allow easier mixing of mobile phase or introduction of modifier fluids. Syringe pumps provide consistent pressure for neat mobile phase.

- ❖ Injection system:

For packed SFC a conventional HPLC system is adequate. But
for capillary column SFC pneumatically driven valves are used.

a)Loop injection: Low pressure feed pump needed to fill the loop. Mostly for preliminary tests of column performance and elution parameters

b.In-line injection: Flexibility for changing injected volume. High pressure pump required to inject feed. Injected stream dissolved in eluent flow

c.In-column injection: Permits injection of the feed solution directly onto the column. No dilutions are required.

Injectors
- ❖ Columns:

There are two types of analytical columns used in SFC,

i. Packed columns contain small deactivated particles so which the stationary phases adheres the columns are conventionally stainless steel.
ii. Capillary columns are open tubular columns of narrow internal diameter made of fused silics with the stationary phase bonded to the wall of the column.

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Packed columns are conventionally are made of stainless steel and provide more theoretical plates and handle larger volumes than open tubular ccolumns. Capillary columns are open tubular columns of narrow internal diameter made of fused silica with the stationary phase bonded to the wall of the column. Both packed columns and open tubular columns are used.Open tubular columns are preferred. Open tubular columns contain an internal coating of cross linked siloxane material as stationary phase. The thickness of coating can be 0.05-1.0µm.The length of the column can range from 10 to 20m.

- Oven

A thermostated column oven is required for precise temparature control of the mobile phase. Conventional GC or LC ovens are generally used.

- Back pressure regulator

This is a device which is used to maintain desired pressure in column by pressure adjustable diaphragm or controlled nozzle, so that, same column outlet pressure is maintained irrespective of mobile phase pump flow rate.It keeps mobile phase super critical throughout separation and often must be heated to prevent clogging . pressure restriction is placed either at the end of the column . BPR converts the eluents from supercritical fluid to a gas for the transfer.

2. Interface

Interfacing SFC with MS does not require special technical skills any more as the solutions and guidance from major instrument providers sufficiently address most situations.During hyphenation, apart from the required SFC and MS-related method parameters, the only interface-specific parameters that need to be supplied are -the flow rate and the composition of a ”make-up” solvent. Make up solvent is the liquid organic solvent(s) added to SFC mobile-phase at the column exit , to improve MS detection [1].There is also a practice of heating the interface connector employing thermal sleeve(s).Empirical guidelines are available on selecting the make-up flow rates and compositions, and also on selecting connector heater temperatures, which are helpful most of the times .Objectively, the only task of an interfacing device between chromatography and MS is to transfer the analyte molecules eluting from the column to the MS inlet, while ensuring that the separation achieved through chromatography is maintained. Such adevice can be a simple connecting tube-like in GC-MS and LC-MS. For SFC-MS, however, the situation is not so simple.SFC requires to be conducted under highly pressurised conditions and because MS is a low a pressure detector ,analytes eluting from SFC column must be depressurized before being supplied to the MS inlet.This depressurization must be done in a controlled way, otherwise it can destroy the resolution achieved by chromatography and also cause poor MS signals.This is the most complex problem related to SFC-MS interfacing and it exists because of the highly compressible nature of SFC mobile-phase.The other manifestation of the challenge of working with a compressible solvent is the requirement of employing an automated back pressure regulator(ABPR), which is a control valve employed to maintain a set pressure inside the system while allowing the system to work with any flow rates.ABPRs are employed after the column for understandable reason and may come with

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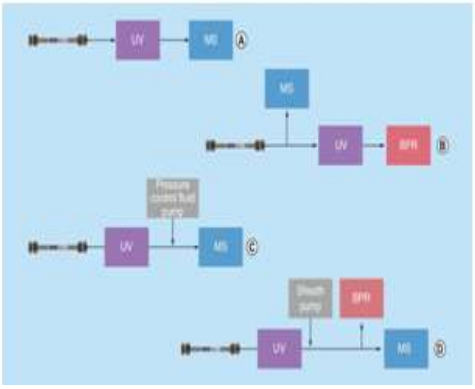
voluminous designs that can add dispersion to analyte bands.To address this issue sometimes a part of the mobile-phase is diverted to the MS by splitting the mainstream. Most challenges related to SFC-MS interface design can be categorized under (a)how to design a pressurized system where the pressure controlling device is not contributing significantly to system dispersion, and
(b) how to manage solvent decompression properly so that chromatographic fidelity is not compromised.Solutions adopted to address SFC.
SFC-MS coupling strategies:
One of the main issues that influenced strategies to fluidically couple SFC with MSbwas of maintaining a set pressure inside SFC system while not adding a significant volume by placing a bulky pressure-controlling device before MS. The design approaches taken to accomplish this task can be divided in two broad categories :
(a)full-flow introduction, and
(b)splits flow introduction



Schematic representations of the four most common supercritical fluid chromatography-MS interfaces. (A) Direct coupling interface, (B) pre-UV and BPR splitter without sheath pump interface, (C) pressure control fluid interface and (D) pre-BPR splitter with sheath pump interface. BPR: Backpressure regulator.
Full flow introduction:

Directing the full flow from SFC to MS was the earliest design adaptation for SFC-MS interfacing, reported by Randall and Wahrhaftig[1].Referring the design as dense gas chromatography /mass- spectrometer interface, the authors expanded the dense gas in a nozzle skimmer-collimator system.The advantage of full-flow is the ability to introduce all or most of the analyte molecules for detection-potentially increasing sensitivity for mass-flow sensitive ionization methods e.g.APCI (atmosphericpressurechemicalionization). The challenge, however, is to design an interface which is low volume,robust and does not put any constraint on SFC method design.Based on the reported design alternatives,full-flow is achieved through (1)employing a capillary restrictor,
(2)employing a liquid pump to control SFC system pressure ,(3)employing low volume ABPR

Split flow introduction:

In split-flow configuration a minor portion of the effluent from SFC column is diverted to MS inlet, where as the major portion is passed through the a ABPR. The main advantage is lesser constraint on the ABPR design.The main disadvantage ,however, is that only a minor fraction of the column output is directed to MS. Flow-spliting configurations may affect MS response.E.g.for mass-flow sensitive ionization methods, like APCI, flows split leads to lower response compared to the possibility of full flow to MS. For concentration dependent techniques e.g.ESI, however, loss in mass-flow is not a concern.Another issue that can lead to problems with quantitation is that the split ratio directed towards MS varies with mobile phase pressure or with any other changes that can vary the connector resistance vis-a-vis the main line resistance.

MS split before UV: This is the configuration that is most often found with instrument manufacturers/ vendors. This configuration splits the flow after the column, such that most of the column flow goes to the UV detector and only a portion goes to the MS (similar to LC-MS) setups. The BPR is placed in-line after the UV detector. This approach is easily controlled by the instrument software and generally maintains good chromatographic fidelity.There are however, a few disadvantages, one being only a portion of the effluent is diverted to the source, which can affect sensitivity when using mass dependent ionization sources.
MS - split after UV with make up pump: In this configuration, the column outlet isvfirst taken through a UV detector to a mixer.Flow of liquid organic make-up solvent(s), is added to the mobile phase through this mixer.The flow from the mixer is then taken to a splitter where a minor fraction of the flow is led to the MS and the major fraction to the ABPR.
3. Tandem mass sspectrometry

- Triple quadrupole:

A triple quadrupole mass spectrometer (TQMS), is a tandem mass spectrometer consisting of two quadrupole mass analyzers in series, with a (non-mass-resolving) radio frequency (RF)–only

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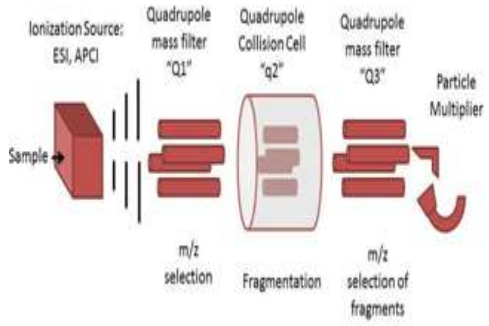
quadrupole between them to act as a cell for collision-induced dissociation. In this the sample is introduced into a soft ionization techniques such as atmospheric pressure photo ionization (APPI) ,atmospheric pressure chemical ionization(APCI) , electrosoray ionization which take place under atmospheric pressure. The ions are then accelerated into quadrupole 1(Q) , which is an ordinary quadrupole mass filter. The selected fast moving ions pass into quadrupole 2 (q) , which is a collision chamber where dissociation of the ions selected by quadrupole 1 occurs. This quadrupole is operated in a radio frequency only mode in which no DC voltage is applied across the rods. This mode basically traps the precursor and product ions in a relatively high concentration of collision gas so that collisionally activated dissociation (CAD) can occur. Quadrupole 3 (Q) then allows mass analysis of the product ion formed in the collision cell. The ions are then detected by electron multiplier detector.



Schematic diagram triple quadrupole

➢ Quadrupole - Time of flight (Q-TOF)

Q-TOF-MS is an analytical technique that advantageously combines the benefits of two different mass analysers. Utilising the high compound fragmentation efficiency of quadrupole technology in combination with the rapid analysis speed and high mass resolution capability of titime-of-flight.Q-TOQ-TOF-MS is an analytical technique that advantageously combines the benefits of two different mass analysers. Utilising the high compound fragmentation efficiency of quadrupole technology in combination with the rapid analysis speed and high mass resolution capability of titime-of-flight.Q-TOF-MS instrumentation closely resembles that of a triple-quadrupole mass spectrometer, though the third quadrupole has been replaced by a time-of-flight tube. The first quadrupole (Q1) is capable of operating as a mass filter for the selection of specific ions based on their mass-to-charge ratio (m/z), or in radio frequency (RF) only mode where all ions are transmitted through the quadrupole. The second quadrupole (Q2) acts as a collision cell where ions are bombarded by neutral gas molecules such as nitrogen or argon, resulting in fragmentation of the ions by a process known as collision induced dissociation (CID). The Q2 can also act in RF-only mode without subsequent fragmentation of ions. After leaving the quadrupole, ions are reaccelerated into the ion modulator region of the time-of-flight analyser where they are pulsed by an electric field and accelerated orthogonally to their original direction. All ions having acquired the same kinetic energy now enter the flight tube which is a field free drift region where mass separation occurs. Ions exhibiting a lightermass will have a shorter time of flight, whereas heavier ions will take longer to traverse the flight path towards the detector. Modern time-of-flight analysers also utilise a reflectron device which serves to correct for kinetic energy dispersion and spatial spread of ions that exhibit the same m/z, but have varying velocities. This reflectron correction allows ions of the same m/z to arrive at the detector at the same time. The reflectron device also increases the flight path length which improves mass resolution. Since Q-TOF-MS utilises quadrupole technology in conjunction with a time-of-flight analyser, two distinct scan types can be used for data acquisition. The first mode known as single MS mode uses the first and second quadrupole in RF-only mode to provide an accurate mass scan of the unfragmented precursor ion. The Q1 may also be used to select a specific mass or range of masses for transmission to the TOF analyser. The second mode (MS/MS) can utilise the Q1 in RF-only or mass filter mode to transmit ions into the collision cell (Q2) where CID occurs. The subsequent product ions and any unfragmented precursor ions are then transmitted to the TOF analyser where accurate mass measurement occurs. Alternating between these modes allows the Q-TOF-MS to simultaneously collect both precursor and product ion information. Detection of ions is achieved by a detector system known as a time to-digital converter which converts the flight time of the ion into a mass signal.

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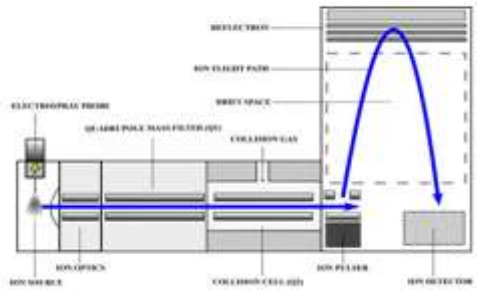

Schematic diagram of quadrupole -time of flights

III. APPLICATIONS:

- supercritical fluid chromatographytandem mass spectrometry (SFCMS/MS) method was developed and validated for simultaneous measurement of five (PGD2, PGE2, PGF2α, 6KetoPGF1α and LTB4) AAmetabolites in biological samples[2]
- Agarwal et al. had developed a validated method for simultaneous estimation of metformin and gliclazide in human plasma supercritical fl uid chromatography followed by tandem UPC2TM BEH, 2-EP column (100 × 3 mm, 1.7 µm) at a flow rate of 1.0 mL/min and multi-reaction monitoring (MRM) was performed for determination of the analytes and internal standard (IS) in polarity switching mode [6].
- preclinical pharmacokinetic evaluation of new formulation of a bifendate solid dispersion was conducted using SFC-MS/MS. Plasma samples were subjected to liquid–liquid extraction with ethyl acetate. Separation of bifendate and diazepam (internal standard, IS) was performed on an HSS C18 SB column (3 × 100 mm, 1.8 µm) with a mobile phase consisting of CO2 (≥99.99%) – methanol (95:5, v/v) at a flow rate of 2 mL/min and the compensation solvent was methanol with 2% formic acid at a flow rate of 0.2 mL/min. A tandem triple quadrupole mass spectrometer was operated in multiple reaction monitoring (MRM) mode with an electrospray ionization (ESI) source.[7].
- SFC-MS/MS has been extensively applied lipidomics[8,9].
- SFC-MS/MS method was developed for the determination of azacitidine in rat plasma. Azacitidine was completely separated from the endogenous compounds on an ACQUITY UPLC™ BEH C18 column using isocratic elution with CO2/methanol as the mobile phase. The single-run analysis time was as short as 3.5 min. The sample preparation for protein removal was accomplished using a simple methanol precipitation method [10]
- SFC-MS/MS was used to quantitate vitamin D and it's main metabolites in breast milk. A small volume of sample (1 mL) was subjected to ethanolic protein precipitation and liquid-liquid extraction. Final extracts were derivatized with 4-phenyl-1,2,4-triazoline-3,5-dione and vitamin D derivatives analyzed by supercritical fluid chromatography hyphenated to tandem mass spectrometry with atmospheric pressure chemical ionization [11]
- A SFC-MS/MS method was developed and validated for identification and simultaneous estimation of isoniazid and pyrazinamide in fix dosage combination. The separation of INH and PYZ was achieved in less than 5 min on a C18 reverse-phase fused-core column (Inertsil ODS-5 µm C18, 150 mm × 4.6 mm) using supercritical carbon dioxide (SC-CO2) as mobile phase at a flow rate of 2 ml/min and modifier as dichloromethane:methanol:formic acid (50:50:0.1 v/v/v) at a flow rate of 0.3 ml/min.[12]
- A simple, fast and efficient environmentally friendly supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS) method was developed and validated for simultaneous measurement of five (PGD2, PGE2, PGF2α, 7 6KetoPGF1α and LTB4) AA-metabolites in biological samples.
- supercritical fluid chromatography-tandem mass spectrometry (SFC-MS/MS) was developed and validated for the determination of oxcarbazepine (OXC) and its chiral metabolite licarbazine (Lic) in beagle dog plasma using carbamazepine as internal standard. Chiral analysis in a run time of only 3 min was performed on an ACQUITY UPC2 ™ Trefoil™ CEL2 column (3.0 × 150 mm, 2.5 µm) at 50 °C by isocratic elution with a mobile phase of supercritical carbon dioxide (purity ≥ 99.99%) and methanol (60:40, v/v) at a flow rate of 2.3 mL/min[13].

IV. ACKNOWLEDGEMENT:

I want to acknowledge our beloved principal Prof. M. Sumakanth RBVRR women's college of pharmacy for giving me the opportunity for writing this review paper.

International Journal of Pharmaceutical Research and Applications
Volume 6, Issue 3 May - June 2021, pp: 1357-1363 www.ijprajournal.com ISSN: 2249-7781

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