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A REVIEW ON INSTRUMENTATION AND METHOD DEVELOPMENT AND VALIDATION OF LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY (LC-MS)

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ABSTRACT

Liquid Chromatography/Mass spectroscopy (LC/MS) is fast creating and it's the famous instrument of liquid chromatographers. Liquid chromatography-mass spectrum analysis (LC-MS/MS) could be a strategy that utilizes liquid normal interaction (or HPLC) with the mass spectrometry. It's a scientific science procedure that blends the actual partition abilities of liquid normal interaction (or HPLC) with the mass examination capacities of mass range investigation. (LC-MS/MS) is usually used in research centers for the subjective and estimation of medication substances, drug item and organic examples. Predominantly the LC-MS contains liquid chromatography gathering, particle age unit/ionization source, mass analyzer and mass spectrometric information securing. LC-MS is most usually utilized in biomedical sciences for pharmacokinetic investigation, hereditary examination, underlying clarification, and so forth the fundamental goal of this audit is to outline the standard, instrumentation and use of LC-MS.APCI, ESI, MALDI, Dilution techniques have been discussed.

Keywords: LC-MS, Electrospray ionization (ESI), APCI, Instrumentation, SPE

INTRODUCTION

Liquid chromatography-mass spectrometry (LC-MS) is the mix of two specific methods that permits the analytes of interest in profoundly complex

combinations to be secluded and estimated. LC separates compounds by their physio-chemical properties and MS separates compounds by mass (explicitly their mass-to-charge proportion). It is this double selectivity that makes LC-MS such an amazing insightful instrument. The mass spectrometer acts as the LC finder as well as, to some degree on a fundamental level, it gives the ability to recognize the species relating to each chromatographic peak through its one of a kind mass range. Consolidating the two partition systems of LC and MS/(MS^n) permits the investigation of intricate combinations. The subsequent selectivity permits a specific analyte or analytes to be detached from the combination and gives certainty that the right part is being estimated **[1]**.

Mass Spectrometry is likewise utilized for ID of obscure mixtures, known mixtures and to explain the design. Mass spectrometry is distant from everything else not useful for distinguishing combinations since mass spectrum mixture is really complex of covering spectra from isolated individual parts. It is hard to interface Liquid chromatography (LC) with Mass spectrometry (MS). Preparative LC-MS frameworks can be utilized for rapid mass-coordinated purification of explicit substances from such combinations that are significant in essential exploration, drug, agrochemical, food and different enterprises **[2]**.

BASIC PRINCIPLE OF LCMS

Liquid chromatography

Surface assimilation is the fundamental principle of HPLC. In HPLC, a liquid at high pressure (the mobile component) forces the sample along a column loaded with a stationary phase that is often made up of irregularly shaped or spherically formed particles chosen or modified to achieve specific separation patterns. HPLC methods have typically been separated into two distinct sub-classes that supported stationary phases as well as the required polarity of the mobile component. In procedures known as reversed part liquid natural process, octadecylsilyl (C18) and related organic-modified particles are used as stationary parts with pure or pH-adjusted water organic mixtures like water acetonitrile and water-methanol (RP-LC) **[3]**.

Flow ripping

When standard bore (4.6 mm) columns are utilised, the flow is normally separated to a magnitude ratio of -10:1. The application of various approaches in bicycles, such as MS and UV sensing area unit, is beneficial. However, if the flow is ripped toward UV, the sensitivity of spectrophotometric detectors may be reduced. At flow rates of 200 l/min or

below, the mass spectrum analysis will demonstrate improved sensitivity **[4]**.

MASS SPECTROMETRY

The analytical technique of mass spectrometry (MS) is used to determine the mass-to-charge ratio of charged particles. It's used to figure out the masses of particles, determine the elemental content of a sample or molecule, and deduce the chemical structures of molecules like peptides and other chemical compounds. MS works by ionizing chemical substances to produce charged molecules or molecule fragments, then detecting the mass-to-charge ratios of these molecules. A sample is put onto the MS equipment and vaporized in a standard MS procedure. The sample's components are ionized in one of several ways (for example, by striking them with an electron beam), resulting in the creation of charged particles (ions). In an analyzer, the ions are sorted based on their mass-to-charge ratio **[5]**.

The ions are typically detected using a quantitative approach. Mass spectra are created from the ion signal. MS instruments are also made up of three modules. An ion source is a device that converts gaseous sample molecules into ions (or, in the case of electrospray ionization, move ions that exist in solution into the gas phase). A mass analyzer uses electromagnetic fields to sort ions according to their masses. A detector that calculates the abundances of each ion present by measuring the value of an indicator quantity.

Mass analyser

In LC/MS, there are a variety of mass analyzers to choose from. Single quadrupole, triple quadrupole, ion trap, time of flight (TOF), and quadrupole-time of flight are a few of them (QTOF).

Interface

For a long time, the interface between a liquid phase method that continually flows liquid and a gas phase technique that is performed in a vacuum has been tough. This changed with the introduction of electrospray ionisation. The interface is usually an electrospray ion source or a derivative such as a nanospray source, but it can also be an atmospheric pressure chemical ionisation interface. Various deposition and drying processes, such as moving belts, have also been used; nevertheless, off-line MALDI deposition remains the most frequent. A new Direct-EI LC-MS interface, which combines a micro HPLC system with a mass spectrometer coupled with electron ionisation, is still in research **[6]**.

COMBINATION OF HPLC AND MS

HPLC not only separates things, but it also gives you some insight into how a chemical is. In reality, it's difficult to tell if a peak is pure or includes only one

chemical in HPLC. When you add a Mass Spectrometry to this, you'll get the masses of all the compounds in the peak, which may be used to identify them and is also a great way to check for purity. Even a basic mass spec can be utilised as a massspecific detector for the chemical being investigated.

More advanced mass detectors, such as triple quadrupole and ion-trap devices, can be utilised to do more comprehensive structure-dependent analysis on what is eluting off the HPLC system **[4, 6]**.

INSTRUMENTATION

The Liquid Chromatography-Mass Spectrometry (LC-MS) is blend of Liquid Chromatography and Mass Spectrometry which is utilized with partition power of HPLC with detection of mass spectroscopy (MS).

- High performance liquid chromatography
- Mass spectroscopy

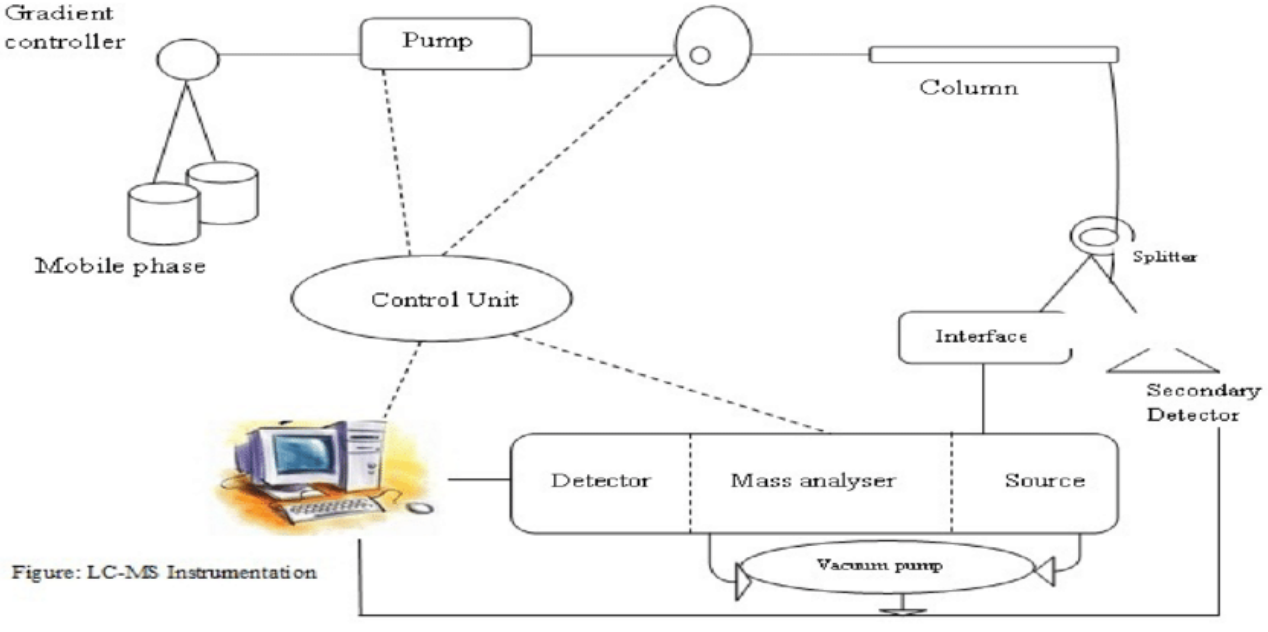


Figure: LC-MS Instrumentation

LIQUID CHROMATOGRAPHY-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Liquid chromatography uses tiny particles stuffed and working at somewhat high tension, and is alluded to as high performance liquid chromatography (HPLC); current LC-MS techniques use HPLC instrumentation, basically solely, for test. The fundamental rule in HPLC is adsorption. In HPLC, the sample is constrained by a liquid at high strain (the

mobile phase) through a section that is loaded with a stationary phase commonly made out of unpredictably or roundly molded particles picked to achieve specific kinds of separations. [7]

HPLC consists of following components

- Pump
- Sample injector
- Columns
- Detectors
- Recorder

HPLC relies upon pumps to pass a pressurized liquid and a model mix through a segment stacked with adsorbent, provoking the parcel of the example fragments. The unique fragment of the segment, the adsorbent, is consistently a granular material made of strong particles (for example silica, polymers, and so forth) 2 m to 50 m in size. The portions of the model combination are secluded from one another on account of their particular levels of association with the retentive particles. The compressed liquid is normally a mix of solvents (for example water, acetonitrile and methanol) and is known as versatile stage. Its association and temperature has a significant impact in the partition **[8]**.

Solvent Reservoir:

The substance of mobile phase are available in glass compartment. In HPLC the solvent is a combination of polar and non-polar fluid parts. Contingent upon the arrangement of test, the polar and non-polar solvents will be shifted.

Pump:

The pump attractions the mobile phase from solvent reservoir and powers it to column and afterward passes to detector. 42000 KPa is the working tension of the pump. This working tension relies upon column aspects, molecule size, and organization of mobile phase.

Sample Injector:

The injector can be a single imbuement or an automated mixture system. An injector for a HPLC structure should give implantation of the liquid specimen inside the extent of 0.1 mL to 100 mL of volume with high reproducibility and under high tension (up to 4000 psi).

Columns:

Columns are typically made of cleaned stainless steel, are 50 mm and 300 mm long and have an inward distance 2 and 5 mm. They are for the most part stacked with a stationary phase with an atom size of 3 m to 10 m. Columns with inner diameters of <2 mm are consistently implied as microbore fragments.

Detector:

The HPLC detector, situated at the finish of the column distinguishes the analytes as they elute from the chromatographic column.

Detector used in HPLC

1. UV detector
   - ✠ Single wavelength (filter)
   - ✠ Variable wavelength (monochromator)
   - ✠ Multiple wavelengths (Photodiode array detector [PDA])
2. Florescence
3. Electrochemical detector
4. Mass spectrometric **[9]**

Information Collection Devices:

Signals from the detector might be accumulated on graph recorders or electronic integrators that vacillate in diserse quality and in their capacity to process, store and reprocess chromatographic information. The PC coordinates the reaction of the indicator to each part and places it into a chromatograph.

MASS SPECTROMETRY

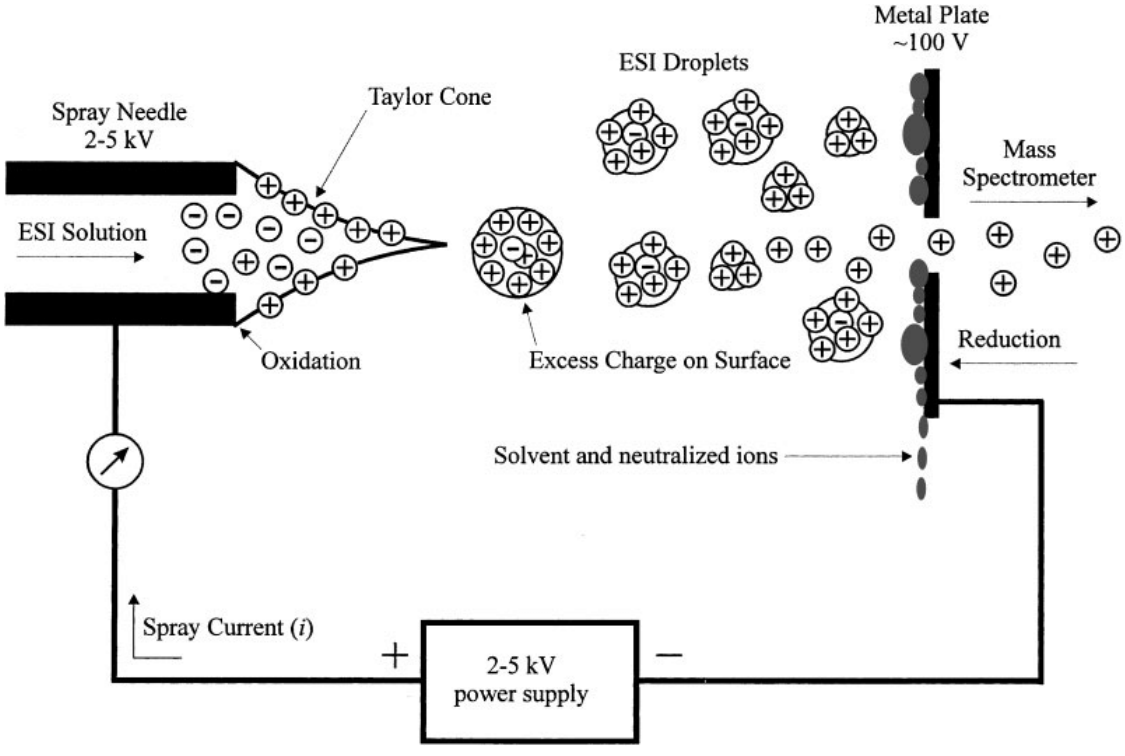
MS is an analytical technique based on the estimation of the mass to charge ratio of ionic species identified with the analyte .MS can be utilized to determine the molecular mass and basic composition of an analyte just as inside and out primary elucidation of the analyte . In LC-MS there are two key parts, ionization source and Interfaces **[10]**.

The ion source

The direct coupling of LC and MS (LC-MS) has formed into one of the most remarkable techniques for follow quantitative analysis. The main advancement was solving the issue with the incompatibility of introducing the progression of liquid mobile phase from the LC column into the vacuum required in the mass spectrometer by the utilization of atmospheric strain ionization (API) interfaces. Nowadays, electrospray ionization (ESI) and atmospheric strain chemical ionization (APCI) are the most widely recognized API techniques in routine use for quantitation of little particles by LC-MS.

Electrospray ionization (ESI)

The mechanisms involved in ion production for electrospray on the grounds that understanding how ions are created from the mobile phase into the gas-phase is invaluable in diagnosing issues, for example, loss of sensitivity and matrix impacts. There are various differing theories yet ionization happens in the liquid phase.

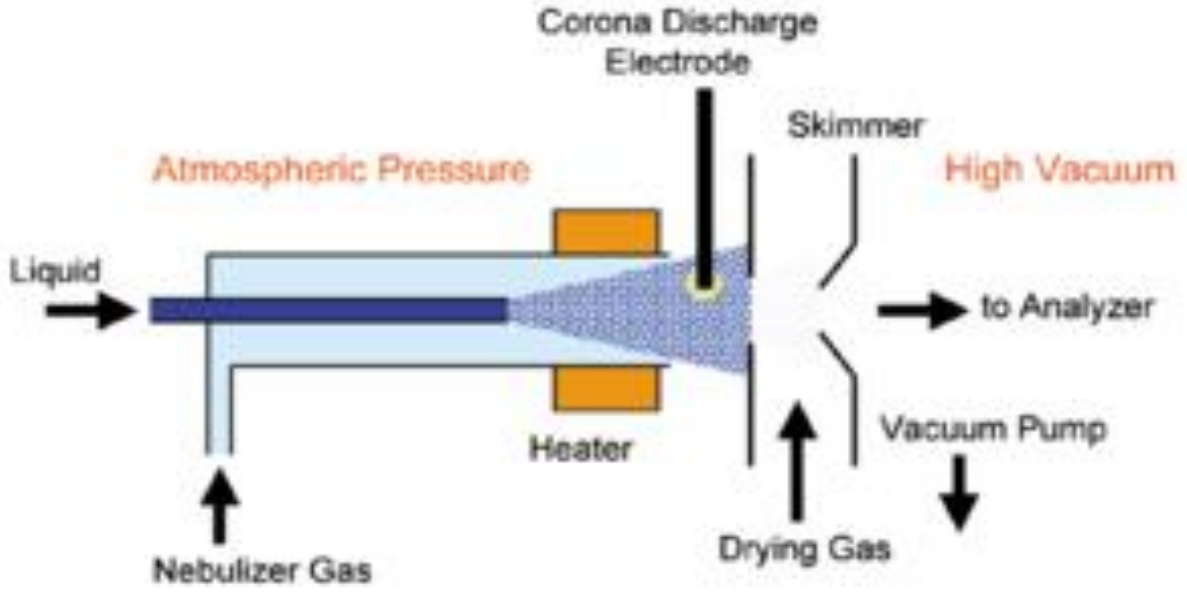


Electrospray is created by applying a solid electric field to a liquid passing through a capillary tube with a frail motion. This produces charged huge droplets which is then exposed to solvent evaporation. Increase in charge density resulting from solvent evaporation makes coulombic repulsion conquer the liquid surface tension, resulting in arrival of ions from droplets. This is the principle of ion formation using this technique **[11]**.

Due to the increase in charge density caused by solvent evaporation, coulombic repulsion overcomes the liquid surface tension, allowing ions to escape from droplets. This is the ion generation concept in this approach. The detection sensitivity of this approach is restricted to 10-8 l, and it requires a big volume to maximise detection sensitivity. Non-volatile molecules and liquids can be ionised using an ESI high mass sample, although this source of ionisation has limited sensitivity, low fragmentation, and is unstable.

Atmospheric tension chemical ionization (APCI):

Principle of this technique involves nebulization of the mobile phase with nitrogen gas and vaporization by heating it to high temperature. The resulting fume is then exposed to a corona discharge electrode to make ions. APCI is most regularly utilized ionization source utilized in LC-MS. APCI are utilized for analysis of pharmaceutical, environmental, toxicological, clinical and chemical industrial and laboratory samples **[12]**.



MATRIX-ASSISTED LASER DESORPTION/IONIZATION

MALDI stands for matrix-assisted laser desorption/ionization and it's a technique for ionising big and/or labile molecules including peptides, proteins, polymers, dendrimers, and fullerenes. This method entails embedding analytes in a matrix that absorbs energy at the laser's wavelength. To create analyte ions, nitrogen ultraviolet (UV) lasers (337 nm) are delivered over the matrix in vacuum. The process for ionisation is unknown, however three alternative hypotheses have been proposed to explain matrix-sample material desorption from the crystal surface.

Preparation of matrix/sample perpetration for MALDI mass spectra:

Dried-droplet, thin-layer, and sandwich methods are often used to create MALDI mass spectra samples. The matrix-to-sample ratio in the dried-droplet approach is around 5000:1, and an aliquot (0.5–2.0 L) of this mixture is then applied to the sample target and left to dry. The sample will be put to the matrix and absorbed by the matrix in the thin layer technique. The sensitivity, resolving power, and mass accuracy of this approach are all excellent. Nitrocellulose (NC) is utilised as a matrix in the thin layer process. In the sandwich technique, thin layer matrix crystals are created, then droplets of aqouse TFA (solvent/ trifluroacetic acid), sample, and matrix are added.

The mass spectrometer analyzer

The analyzer is part of the mass spectrometer that takes ionized molecules

and isolates them dependent on charge to mass ratios and yields them to the detector where they are distinguished and later changed over to a digital yield **[12]**.

MASS SPECTROMETER TYPES [1]

- Single quadrupole
- Triple quadrupole
- Ion trap (low resolution)
- Ion trap (high resolution)
- Time of flight (TOF)
- qTOF

Quadrupole Mass analyzer:

When ions are moved down the middle axis of four parallel equidistant poles, electric fields are used to separate them according to their mass to charge ratio. The stability of their trajectories under the fluctuating electric fields supplied to the rods is used to separate ions in a quadrupole. Two pairs of metallic rods make create a quadrupole. An electrical connection is made between each opposing rod pair, and a Radio Frequency (RF) voltage is provided between one pair of rods and the other. The RF voltage is then overlaid with a direct current voltage. Between the rods, ions pass along the quadrupole. For a certain voltage ratio, only ions with a specified mass to-charge ratio (m/z) will reach the detector.

Ion trap analyzer:

The quadrupole ion trap analyser (QIT) is another name for an ion trap analyzer. GC/MS will be the most common application, rather than LC/MS. The ions are stored in a device called an ion trap, which consists of a ring electrode and two end cap electrodes. Applied DC and RF fields are used to control these ions. The analyser may trap ions with a certain mass to charge ratio within the analysing device because of the amplitude of the applied voltages. The electrostatic field gives non-selected ions a route that causes them to leave the trap. It is feasible to fragment chosen ions by filling the trap with inert gas. When structural information is necessary, this is beneficial **[13]**.

DILUTION:

Dilution Prior to LC-MS/MS analysis, dilution or procedures merely require the addition of purified water or the LC mobile phase to the patient sample. Because it is rapid, simple, and affordable, this approach is extensively employed for low-protein matrices (e.g., urine or CSF).

Precipitation of proteins (PPT)

Protein precipitation is similar to dilution procedures but is designed for high-protein matrices such as serum, plasma, or whole blood. The sample, internal standard, and a precipitating agent, such as acetonitrile or methanol/ZnSO4, are combined together and centrifuged to separate out the precipitated proteins before

the supernatant is introduced into the LC-MS/ MS system.

Liquid-liquid extraction (LLE)

For many years, liquid–liquid extraction has been utilised in sample preparation processes, and it entails partitioning analyte(s) from an aqueous biofluid into a water-immiscible organic solvent based on polarity

Phospholipid removal media (PLR)

The post-precipitation supernatant passes over a bed of moieties, such as zirconia-coated silica, that contain phospholipids. This provides better selection while keeping the PPT techniques' simplicity.

Solid phase extraction (SPE)

A selective stationary phase is used in solid-phase extraction to bind or partition the analyte. This phase frequently necessitates pre-treatment for optimum extraction, after which the diluted biofluid passes through the stationary phase, which retains the analyte while letting other matrix components to flow to waste. An elution solvent is used to recover the analyte after many wash processes **[14]**.

METHOD DEVELOPMENT

Literature survey

Gather the data on physiochemical, pharmacokinetic, chromatographic and extraction strategies of the medication/Metabolites from accessible sources, for example Medline, Journals, Analytical conceptual, Physician Desk Reference, Library and so on

Initial conditions

Distinguish the various medications for their reasonableness as interior norm. Select the gear as indicated by the accessibility and required affectability. Select starting chromatographic conditions, with segment, buffer, mobile phase, low rate and detector. Recognize the extraction methodology like liquid phase extraction or solid-phase extraction, or some other strategy thinking about the necessary sensitivity.

Planning of Buffer, Mobile Phase, Reagents and Solutions

Guidelines for preparation of mobile phase

Little changes in pH and acid concentration may have a big influence on separation, it's important to apply the same procedures every time you make a mobile phase to maintain consistency. Before diluting to final volume and mixing any organic solvents, the pH of the mobile phase should be corrected if necessary by adding reagent. To produce ionisation or suppression of the analytes, choose a mobile phase with a final pH at least one pH unit apart from any analyte's pK value while constructing a tough technique. Because the influence of organic solvent type and concentration on either mobile

phase pH or solute pK values is unknown **[15]**.

Solution Preparation

To ensure the tidiness of the space, contraption and crystal prior to setting up any arrangement. Gauge precisely or measure the substance and cautiously move the substance in an appropriate compartment that is prelabeled **[16]**.

Mobile Phase Preparation

To measure the necessary natural, fluid dissolvable stage independently in various estimating chambers and transfer them into a suitable container. Blend the substance well by gentle shaking. Fix the Buchner pipe with flask and associate with vacuum pump. Channel the arrangements utilizing layers whenever required. Fill the dissolvable in the Buchner pipe and switch on the pump.

Chromatography technique

To tune the instrument (LC-MS/MS) with reasonable arrangements. Weaken the analyte and inside norm at the necessary fixation dependent on the sensitivity of the instrument **[17]**.

Product to be assesed	Chemicals used	Instrument	Lc conditions	MS conditions	Sample	Method validation
NC- 8	Acetonitrile, methanol and formic acid	LC-MS/MS and quattro ultima triple quadruple	Reverse phase	Electrospray ionization in positive ion mode	Rat plasma samples	The LC-MS/MS method validated according to guidelines for bioanalytical method validation with respect to selectivity, linearity, precision, accuracy, recovery, matrix effects, stability and dilution. [18]
lusutrombopag	Acetonitrile, methanol, formic acid, ortho phosphoric acid and ammonium acetate	LC-MS/MS	Reverse phase	Electrospray ionization	Rat plasma sample	The LC-MS/MS method validated according to guidelines by USFDA [19]
Alogliptin benzoate	Acetonitrile, ammonium carbonate	Agilent 1200 HPLC system	Reverse phase	Electrospray ionization	Alogliptin tablets	The method validated according to guidelines by ICH and FDA [20]
Cefprozil diastereomers	Formic acid, acetonitrile, methanol	HPLC (SIL-20AC autosampler)	Reverse phase gemini c_{18}	Positive ion mode with multiple	Human plasma	The LC-MS/MS method

			column	**reaction monitoring (MRM)**		**validated according to guidelines for bioanalytical method validation [21]**
Nifedipine	**Tolterodine, Formic acid, acetonitrile, methanol**	**LC-MS/MS**	**API 4500 triple quadrupole**	**Positive ion mode with multiple reaction monitoring (MRM)**	**Human plasma**	**A full validation according to the ICH guidelines was performed for the assay in K2EDTA human plasma [22]**
Hydroxychloroquine, azithromycin	**Desethyl-hydroxychloroquine, bisdesethyl-chloroquine, the internal standards azithromycin-d5, hydroxychloroquine-d4, Desethyl-hydroxychloroquine-d4, and bisdesethylchloroquine-d4**	**LC-MS/MS**	**LC column was Pursuit pentafluoro phenyl (PFP)**	**Sciex API5000 tandem mass spectrometer**	**Plasma samples**	**The method was validated in accordance guidelines outlined by both the NIH-sponsored Clinical Pharmacology Quality Assurance Program (CPQA) and the FDA. [23]**
Felodipine	**Pantaprazole, ammonium acetate, acetonitrile,**	**LC-MS**	**LC column was Pursuit pentafluoro phenyl (PFP)**	**Sciex API5000 tandem mass spectrometer**	**Human plasma**	**Validation of the method was carried out after the development of the HPLC method. [24]**
ACE inhibitors	**Enalapril, perindopril, ramipril, atorvastatin**	**mass spectrometer was an AB SCIEX Model API 4000 equipped with turbo Ion spray ionization (ESI)**	**carried out on X-terra C8 (3.5 µm, 4.6 × 50 mm) column.**	**positive ion MRM mode**	**Human plasma**	**Validation of the developed method was carried according to the FDA and EMA guidelines. [25]**
Dextromethorphan hydrobromide	**acetonitrile, methanol, ammonium bicarbonate, HCl, N-Formyl octabase, Dextromethorphan N-oxide**	**UPLC system equipped with a binary solvent manager pump, an auto sampler and PDA detector with empower-3 software**	**Ultra performance liquid chromatography coupled to Single Quadrupole (SQD)**	**mass spectrometer equipped with an ESI**	**Active pharmaceutical ingredient**	**Method validation of the UPLC method was carried out for the determination of related substances and assay of DHB as per ICH guidelines.[26]**

Analytical Validation

Linearity

Authentic standards (glutamicacid, citricacid, isocitricacid, malicacid, succinicacid, fumaricacid, and lacticacid) are spiked in a matrix using deuterated D4-citric acid as an internal standard to produce calibration curves. The calibration curve will be obtained by plotting the ratio of analyte and internal standard peak area against the corresponding concentration.

Sensitivity

LOD and LOQ were determined by injecting diluted compound intermediate solutions into each matrix. LOD was calculated as the concentration of drug intermediate test that created a peak at least three times larger than the baseline noise. The signal-to-noise ratio used to compute LOQ was ten times greater than the baseline noise of these chemicals.

Precision and Accuracy

Plasma samples analysed and the precision of retention duration and concentration will be measured to assess with in run precision. Bioanalytical precision and accuracy will be determined by analysing the same samples over a 5-day period for interday precision **[26]**.

DEVELOPMENT AND VALIDATION USING LCMS:

LITERATURE SURVEY:

APPLICATIONS OF LC-MS

- DOPING AGENTS AND DRUGS OF ABUSE

Steroids, anabolic steroids, diuretic medications, carbamate doping agents, nonsteroidal anti-inflammatory drugs (NSAIDs), opiates, and analgesics are all included in this section. Applications involving doping substances in animal biological fluids were included since the extraction processes are consistent across species and may readily be modified for the evaluation of these chemicals in human biological fluids.

- PESTICIDES AND TOXINS IN NON HUMAN SAMPLES

This section focuses on determining the presence of mycotoxins, marine toxins, and pesticides in animal and vegetable samples. MB, PB, CF- FAB, ES, and TS were the interfaces utilised. Three mycotoxins and several of their metabolites, including a glucuronide conjugate, were detected in excretion samples from rats, chickens, and cows using a TS technique. Because of the more numerous fragmentation pattern, positive filament-on ionisation was favoured over negative ionization **[27]**.

- IDENTIFICATION OF MICRO ORGANISMS

In the last several years, significant progress has been made in the application of mass spectrometry in

microbiology. Despite the fact that MALDI-TOF MS has revolutionised clinical microbiology, significant progress has also been made in the development of LC-MS for microbiological applications **[28]**.

- PROTEOMICS
- PHARMACOKINETIC

LC-MS is utilized in the investigation of assimilation, digestion, and discharge of medications. Bio logical strategies are utilized for quantitative and primary clarification of medications and its metabolites

- BIO ANALYSIS
- FORENSIC SCIENCE

LC-MS is utilized for assurance of poisonousness examines, drug investigation and furthermore in follow examination. By utilizing limited quantity of test the toxic in various material not really settled with LC-MS. Any poisonous metabolites in food or drinks can be dictated by utilizing LC-MS. For example detergent added into orange juice can be dictated by investigating by the juice and detergent sample test. The standard surfactant alkyl diphenylether sulphonic corrosive is utilized. Both juice and cleanser tests are dissected in same chromatographic conditions. The mass chromatograms and mass spectra acquired from the juice and cleanser tests are indistinguishable with the reference spectra

- AGROCHEMISTRY

It is used to detect different components present in fertilizers and pesticides

- PETROCHEMISTRY
- COSMETICS **[10, 29]**

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Conflict of Interest

The authors declare that No conflict of interest for this study.

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