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An overview of the applications of LC-MS in characterizing pharmaceutical formulations, and in studying pharmacokinetic and pharmacodynamic parameters

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ABSTRACT

In the last few decades, the hyphenated analytical techniques including liquid chromatography (LC) in combination with a mass spectrometer (MS) i.e., LC/MS, have made a major impact in pharmaceutical drug discovery and development. LC-MS hase been routinely used in pharmaceutical formulation development for drug substance and drug product characterization, molecular weight and fragmentation patterns determination, breakdown studies, and to identify impurities and degradation products. Recent advancements in LC-MS instrumentation, have allowed the technique to be implemented in several indications i.e., cancer, cardiovascular, respiratory, neurological, rare diseases etc. across preclinical (*in vitro and in vivo)* and clinical projects to evaluate pharmacokinetic and pharmacodynamic factors. LC-MS has overcome the sensitivity and dynamic range challenges to successfully identify and characterize drug molecules to help projects that use small molecules, biologics, and gene and cell therapy/editing platforms as drug modalities. This technique may provide several advantages over other analytical approaches including specificity, multiplexing, precision to quantify drug analyte or a biomarker in a variety of matrices like blood (plasma/serum), tissue, cerebrospinal fluid, urine, cells etc. In this review, we have highlighted LC-MS applications to study pharmaceutical formulations, pharmacokinetics and pharmacodynamics involving small molecule modality.

Keywords: LC-MS/MS, mass spectrometry, pharmacokinetics, pharmacodynamics, pharmaceutical formulation

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INTRODUCTION

Recent estimates range from $314 million to $2.8 billion for the mean cost of developing a new drug across all therapeutic areas. The whole process from target identification through approval for marketing, takes over 12 years on average. The goal of the entire process is to find a medication, either as a new molecular entity i.e., small molecule, or a new biological entity i.e., protein, peptide, an antibody, or a cell/gene therapy. At the beginning of the pre-clinical drug discovery process, research teams identify and validate a disease target via assay development, followed by high-throughput screening to identify a hit molecule, before moving to lead optimization. Cellular, genetic, and animal models can be used during this assay development and lead optimization stage. Post hit-to-lead optimization, drug candidate is expected to selectively bind to the target/receptor, reproducibly demonstrate desired functional outcome, and possess adequate bioavailability and bio-distribution profile in animals, with similar levels expected in humans, a time-consuming process that requires analytical techniques like LC-MS.

To select the lead compound as a clinical drug candidate, the molecule must demonstrate the safety profile i.e., it must not be toxic in wild-type animals and/or disease models [1-5].

During the clinical trial process, where the medications can fail due to several reasons, some of the most important factors include clinical dose/exposure, efficacy/potency, and toxicity profile of the drug candidate. Hence, based on the assay/indication/treatment needs, it is essential to determine the amount of drug present in subjects' blood (serum/plasma), tissue, urine, CSF etc. to evaluate the PK-PD relationship and overall efficacy and toxicity profile. In early 20th century (1913), only select analytical techniques were available, like gravimetric analysis methods, to analyse/quantify drugs without proper guidelines. But with recent advances in analytical techniques and emergence of LC and MS, LC-MS has been routinely implemented in pharmaceutical formulation characterization, i.e., determination of analyte's molecular weight, stability/degradation/by-products, impurity etc., and to study bioavailability, PK profile of the molecule and treatment-induced PD/biomarker effect [6]. The toxicological qualities of active components, as well as impurities and degradation products, are the three key issues that determine the drug's safety. Several analytical techniques, such as titrimetric, chromatographic, spectroscopic, and electrochemical approaches, are employed in quality control for qualitative and quantitative analysis of the drug product and component/s [7, 8]. LC-MS/MS has been extensively used in toxicology to investigate antidepressants, antipsychotics, and benzodiazepines (BDZ) including designer benzodiazepines [9]. The 52 compounds of BDZ Tricyclic/tetracyclic antidepressant,

selective serotonin inhibitors, and others typical and atypical neuroleptics were estimated by LC-MS [10].

LC-MS technique combines liquid chromatography, which has the physical separation capability, and mass spectrometry, which performs mass analysis. Combination of LC-MS was first reported in 1967 while the system was later introduced in 1980 [11]. The chromatography technique is commonly used in the pharmaceutical industry. Separation, identification, and quantification are the fundamental principles of this approach, which has been utilized for the assay of bulk pharmaceuticals in the United States and Europe since 1980 [12]. Because of its great resolving power, LC is used to estimate contaminants in bulk medicinal materials and pharmaceutical formulations, as well as for structural determination and quantitation of degradation products. It is crucial that the detector should be able to identify all essential components. UV detector is widely used because it covers a wide range of wavelengths and can monitor many wavelengths simultaneously using multiple wavelengths scanning programs [13].

The separation and detection of analytes by LC distinguishes it from other analytical tools and quickly aids pharmaceutical development, ensuring pharmaceutical quality and safety [14]. In the arena of drug discovery and development, LC-MS/MS plays an important role because of its high performance, selectivity, and sensitivity [15-17]. There are two main factors in the accuracy of LC-MS results, 1) instrument's separation power, and 2) the mass analysis capability of MS. Chemical mixtures can be separated into pure or nearly pure fractions based on the separation power of the instrument. A mass analysis instrument with high molecular specificity and sensitivity can identify specific compounds. Like other hyphenation techniques i.e., GC –MS (Gas chromatography – mass spectrometry) and CE –MS (Capillary electrophoresis – mass spectrometry), LC-MS can be used as a method for solving a wide range of difficulties in the structural characterization of molecules [11]**.**

Over the years, LC-MS have been implemented across drug discovery (preclinical and clinical), metabolite investigations (*in vitro* and *in vivo*), and contamination/degradation product detection, and PK-PD evaluation [18]. LC-MS has become a routinely used technology for analysis in the pharmaceutical field since the development of electrospray ionization (ESI). The application of tandem MS and use of stable isotope internal standards, make it a highly accurate and sensitive technique. Due to the rapid scanning speed, numerous chemicals are scrutinized in a single analytical run [19]. Since 1990s, automated electrospray tandem mass spectrometry (ESI-MS/MS) has been used for metabolic profiling of amino acids and acylcarnitines from blood spot diagnostic samples for inborn errors of metabolism. This instrument can produce precise and accurate results

in clinical biochemistry laboratories. Due to the increased demand of LC-MS in the analytical field, the cost and use significantly enhanced in the mid-1990s. The dependence on this instrument in clinical analysis and pharmaceutical industry has been very high and hence, today it would be almost impossible to conduct drug discovery and development without the use of this technique [20].

For several years, LC-MS has served as an invaluable tool in drug discovery and development to evaluate absorption, drug metabolism, and excretion of drugs (ADME). From initial compound screening/ranking via *in vitro* DMPK assays (plasma/metabolite stability, solubility, protein binding, CYP inhibition, Caco-2 permeability, reactive metabolite screening, *in vivo* PK etc.) to the late-stage development including quantification of drug/metabolites in plasma or other matrices obtained from clinical trials using validated bio-analytical methods [21]. In addition to PK profiling of small molecules, biologics (proteins, peptide, and antibodies), gene therapy, oligonucleotide and cell therapy products in appropriate matrices, LC-MS has also revolutionized biomarker discovery globally by identifying and comparing quantified proteins in different wild-type and disease matrices such as serum/plasma, tissue/cell lysate etc [22]. Validated LC-MS methods have been globally implemented across the industry, CROs, and academic labs for biomarker detection/quantification to help find treatment options for debilitating diseases.

Along with protein identification and quantification, the combination of high-resolution LC separations with fast and sensitive MS detection provides one of the most powerful analytical tools for organic compound analysis. The key advantages include selectivity i.e., co-eluting peaks isolated by mass selectivity; quantitative and qualitative data with limited instrument optimization; low sample volume requirement; molecular weight and structural information; peak assignment - a chemical fingerprint for the compound of interest in the complex matrices; rapid method development; sample matrix adaptability. LC-MS also encounters a few challenges in terms of method development/validation i.e., cost, relatively low sensitivity, need of an internal standard, moderate throughput efficiency, requirement of an experienced operator and instrument installation/portability issues [23-25, 11]**.**

In this review, we highlight the importance of LC-MS in the field of pharmaceutical drug discovery and development, with respect to pharmaceutical formulation characterization, and pharmacokinetics/pharmacodynamics (PK/PD) evaluation. Additionally, we briefly discuss how LC-MS works and provide a few industrial applications along with recent advancements for pharmaceutical analysis and PK/PD testing.

WORKING OF LC-MS

Ionization:

The characteristics and configuration of the ionization source have a substantial impact on the LC-MS performance. As a method for analysing thermally labile, non-volatile, and polar compounds in pharmaceuticals, electrospray ionization (ESI) is one of the most used technique, followed by atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), for compounds with lower molecular weights or non-polarity [26]. Gaseous analyte are analyzed using mass spectrometry by moving them from their condensed to gaseous state and followed by ionization [27].

Modern LC-MS instruments can change polarity in tens of milliseconds or lower amount of time [28,29]. The introduction of ESI-MS has allowed for the proper investigation of analyte fragmentation. Electron ionization, chemical ionization, and other types of ionization were previously used. During the mid-1980s, it was not possible to quantity the molecular mass of proteins in an accurate and precise manner in biological samples.

The usual ionization method resulted in structural damage of proteins because the proteins were polar, non-volatile, and thermally labile. Fast atom bombardment (FAB) ionization was developed at the time, but it produced mostly single-charged analyte ions, with a restriction of smaller species with masses less than 1000 Da. These issues were resolved in 1989, when a scientist named John B. Fenn developed electrospray ionization (ESI) for mass spectrometry of large biomolecules. Unlike previous techniques, electrospray spectra for large molecules were coherent sequences of peaks in which component ions are multiply charged, the ions of each peak differed by one charge with respect to adjacent neighbours in the sequence [30,31]. The benefit of producing multiple charged ions is that the resulting ions have lower m/z values, allowing them to fall within the mass range of the entire conventional mass analyzers. Since then, ESI has proved to be a highly successful ionization technique used for generating gas-phase ions from bulky bioactive macromolecules, including nucleic acids and proteins, and for rapid identification of molecular species [32]. Furthermore, on average, ESI is 1.7 times more sensitive than APCI, but ESI-APCI shows 2 times more sensitivity than APCI alone [33]. In 2002, John B. Fenn shared half of the Nobel Prize in Chemistry award with Koichi Tanaka for their work in mass spectrometry.

Mass Analyzers:

In MS, the mass analyzer does refer to as the instrument's heart. Similar to a prism, it splits light into wavelength components and detects them via optical receptors in the prism. Similarly, an ion beam's distinct types of ions (m/z) are separated and further separated in a mass analyzer before

being transmitted to the detector. These mass analyzers are classified as magnetic or electric sector mass analyzers, linear quadrupole ion traps (LITs), three-dimensional quadrupole ion traps (QITs), orbitraps, time-of-flight mass analyzers (TOFs), and ion cyclotron resonance mass analyzers (ICRMAs). When picking a mass analyzer, it is essential to consider the image resolution, feed speed, mass exactness, mass variety, sensitivity, and detection limit. The operating principle is based on two basic physical laws: the Lorentz force law and Newton's equation of motion [34-36].

LC-MS instrumentation:

Liquid chromatography can be improved by adjusting the diameter of the stationary phase particles, which in turn produces fully porous particles (FPPs) as well as superficially porous particles (SPPs). Due to the small particle size, ultra-high pressure (1500 bar) can be delivered with less fluctuation in the extra column [37, 38]. By combining low particle sizes and high pressures, the analysis time can be reduced by 5-9 times, thereby saving method development and validation costs [39]. With these developments, there are still a lot of flaws in the study. For example, mass spectrometry, is incapable for distinguishing isomeric compounds, i.e., molecules having same chemical formula, but distinct molecular structure. Secondly, it is discovered that introducing numerous chemicals into a mass spectrometer at the same time affects the ionization of the compounds, ultimately affecting the quantification of molecules of interest [40].

Miniaturization and Microfluidics:

Hyphenated capillary and nano-scale LC-MS analysis require small volumes to provide highly sensitive information, making it useful for analysing small volumes of scarce materials. Volume can be lowered to 100 mm or less due to the ultra-low column dimension. Furthermore, no nebulizer gas is required if the mobile phase comprises of 15-20% concentration of organic modifier [41]. Since only a small volume of material is needed, capillary and nanoscale LC are highly sensitive and produce a complete PK profile of a single animal when proteomics workflow is applied [42].

Additionally, the developed analytical method appears robust, and capillary scale separation offers extra sensitivity which can be applied to the entire batch to detect metabolites in *in-vivo* pharmacokinetic studies, where only negligible amount of a drug is required. This approach permits the complete study of compounds without resorting to large-scale synthesis [43]. Despite these advantages, there are downsides, such as the small column volume, which makes dispersion-free connections more difficult, as well as the broadening of the extra-column band, which becomes more of a problem than with standard bore separations. Secondly, smaller columns and tubing with smaller IDs are further likely to clog, especially when dealing with "dirty" samples.

Thirdly, larger flow in the range of nL- µL are needed to run MS sources with LC. These issues have been dealt by developing entirely integrated devices. LC columns with reduced column IDs and enhanced spray/tip couplings are typically used with these devices to enhance hyphenation with MS [44].

Matrix Effects:

LC-MS is an excellent tool for drug analysis, but to extract drugs and their metabolites from the matrix, appropriate sample preparation is needed in many cases. There can be several factors that contribute to inadequate separation, such as interaction among sample contaminants and stationary phase, leading to detector noise, interactions between drugs and other matrix components, and lack of resolution, all resulting in increased matrix effects (MEs). These changes are often the consequence of changes in the ionization efficiency of compounds, which limit quantitative analysis and have an impact on reproducibility, linearity, and precision [45].

There are several ways of reducing MEs, for instance, using different sample preparation strategies, using specialized chromatography techniques, using calibrated MS detectors, and selecting the right ionization source. The pharmaceutical industry practices solid phase extraction (SPE) and liquid-liquid extraction (LLE) extensively, therefore, solid-phase micro extraction (SMPE) and supported liquid extraction (SLE) are becoming increasingly popular as pre-treatment procedures. Many sources have reported the contribution of glycerophosphocholine (GPCho) lipids to MEs in biological fluids due to their surfactant-like characteristics [46, 47].

Ion mobility mass spectrometry:

In a drift cell, ions of interest experience drag forces while travelling through an electrical field and collides with stationary buffer containing gas molecules (usually nitrogen or helium gas), which results in ion mobility. On the basis of this idea, many types of ion mobility have been developed, including drift tube ion mobility, travelling wave ion mobility, and trapped ion mobility (TIM), which are previously integrated into commercial LC-MS systems [48]. The combination of electric field acceleration and gas collision deceleration results in particles riding at a steady rate. The electric field applied as well as the structural features of the particles, such as diameter, structure, charge density, and density is used to evaluate the kinetic energy. Ion mobility allows the splitting of enantiomers, isobars, and conformers, as well as the decrease of chemical noise and the quantification of ion sizes in this approach. In addition, ions with similar charge states and patterns can also be classified into ion families based on their mass-mobility correlations [49].

APPLICATIONS OF LC-MS IN DRUG DISCOVERY AND DEVELOPMENT

The combination of advanced liquid chromatography with mass spectrometry (LC-MS) has greatly influenced the process of drug development during the last decade. The continuing improvement of LC-MS interface technologies and the ability to view structure qualitatively and quantitatively, have led to a wider array of applications. As a result, these advances occurred simultaneously with advances in molecular biology and combinatorial chemistry. In recent years, the speed at which new drug modalities like gene/cell therapy (including gene editing), oligonucleotide (RNA/DNA)-based drugs, next-generation biologics like antibody-drug conjugates (ADCs), have been generated has far outpaced the rate at which they could be analyzed with existing analytical tools. Hence, scientific community have started to develop advanced tools to help analyse drugs and related drug substance samples. It has become apparent that retention time and molecule weight is two of the most important analytical properties for LC-MS applications, from target identification to lead optimization.

Surrogate models and freely available systems based on LC-MS have become a vital part of the drug development method. A continual search of the analytical technique to detect/quantify analyte of interest to answer questions likes “what is it?" and "how much is there?" fuelled the rise of LC-MS in the pharmaceutical industry. As a result, LC-MS has gained widespread recognition as an integral part of drug development. An insight into major developments in pharmaceutical analytical strategies is presented as a part of this paper, in which LC-MS techniques are demonstrated to be effective in accelerating drug development. Additionally, future LC-MS applications and emerging industry trends are discussed [50].

Drug Metabolism Studies:

To address metabolic liabilities during compound optimization, it is imperative to quantitatively identify metabolites during the discovery stage. For new drug discovery and investigational toxicological studies, it is vital to identify and monitor key pharmacologically active metabolites. In early-stage metabolic research, LC-MS is used as sample analyzer, where samples were generated in *in-vitro* or *in-vivo* experiments, allowing metabolites to be structurally characterized and identified in a timely manner [51].

The FDA-approved drug Atazanavir is an anti-HIV drug of the protease inhibitor class and its metabolites have been examined by LC-MS covered in many scientific reports [52,53].

High-Throughput LC-MS for drug discovery:

Using multiple columns concurrently, can easily increase analysis throughput without drastically changing experimental conditions. While the second column is being equilibrated, the first column performs the actual analysis. This allows injecting multiple samples sequentially, permitting for

higher analysis rates per day. This liquid chromatographic method is referred to as "staggered," "multiplexed," or "MISER" (Multiple Injections in a Single Experimental Run). Staggered systems are available from several vendors, including MPX (Sciex), Transcend II (Thermo), and Stream Select (Agilent). To increase the speediness of high-throughput LC-MS analysis, fast auto samplers are needed to execute chromatographic separations in the sub-five-second range. Zawatzky along with his co-workers, created an auto-sampler containing duple needle with 10 s injection cycle time that uses UV-ESI-QMS for increasing MISER analysis throughput. This auto-sampler is compatible with any standard HPLC system [54].

The analysis of plasma samples from subjects with serotonin syndrome [55] and the characterization of pyrophosphate-dependent phosphofructokinase in vitro samples [56]. The use of this online approach reduced analysis times to as low as 10 s per sample, enabling the analysis of a bio fluid sample in approximately 1 min (allowing for polar and non-polar stationary phases and +ve/-ve ion MS acquisition) and loss of biological information. All of these limitations of DIMS have meant that LC-MS has remained the dominant approach for comprehensive metabolic and lipid profiling [57].

Impurity and degradation product analysis and recognition:

Impurities are ingredients in pharmaceutical goods that cause differences in quality in terms of both safety and effectiveness. Impurities can be classified based on their source, contents, and biological safety. Contaminants are found in raw materials, catalysts, intermediates, solvents, reagents and degradation products produced throughout the drug storage process [58]. Advanced analytical tools are indispensable for the characterization of various oligonucleotide impurities and degradation products, some of which are present at a very low level. One popular method for oligonucleotide analysis is ion-pair reversed-phase liquid chromatography coupled with mass spectrometry (IP-RP LC-MS) [59, 60].

Analysis of chiral impurities:

Trace elements found in drugs that have a similar structure, but are oriented differently, typically centred on a chiral carbon atom, are referred to as enantiomer defects. Because so many prescription drugs are chiral, pharmaceutical companies are focusing on enantioselective manufacturing techniques that can be improved and controlled. There is a high probability of chiral impurities in drug compounds with optical isomers. These impurities possess different therapeutic and pharmacological profiles than the drug component and can pose health-related risks, for example, the s-isomer of thalidomide is a teratogenic impurity that can cause birth abnormalities [61]. So, there is a need to identify and quantify such impurities. It was performed in packed

columns that were fully porous having 3 or 5 cm length, and taking approximately 30 minutes to separate enantiomeric combinations, as the efficiency of chiral columns is a major determinant of chiral LC-MS analytical throughput [62].

The rapid chiral method was developed for enatioseperations and determination of cabotegravir and its enantiomeric impurities by using LC-MS [63].

Pharmacokinetics and pharmacodynamics (PK/PD) research:

During the drug discovery, lead optimization, and preclinical development, researchers conduct ADME-TOX research to predict the results of the substance in the human body. During the initial stages of the drug discovery process, it is important to discard compounds with poor ADME properties, rather than keeping toxic compounds until later phases of drug development. The entire procedure of selecting the finest candidate for the clinical trials is lengthy and complex and requires extensive research and continual reconsideration of chemical entities under development. During the last decade, the approach to drug discovery and development, pharmacokinetics, pharmacodynamic and toxicity studies have changed significantly because of a better understanding of cellular and molecular activities, and the introduction of highly sensitive, selective, reproducible and robust analytical tools. Nowadays, LC-MS has become the utmost reliable and powerful analytical tool in the pharmacokinetics/pharmacodynamics (PK/PD) research as well as for therapeutic drug monitoring (TDM) analyses [64].

Due to good selectivity, minimal matrix interference and low volume requirements, LC-MS can quantitatively determine drugs in human biological matrices and has widespread use in clinical pharmacology, an essential area in in today's medicine. Additionally, LC-MS, is capable of analysing pharmacokinetics and pharmacodynamics parameters of different class of drugs including anticancer, anti-dementia, antidepressant, antiepileptic, antifungal, antimicrobial, antipsychotic, antiretroviral, anxiolytic/hypnotic, and cardiovascular drugs etc. [65]. Some of the studies are discussed below.

Quantification of Oncology Drugs

LC-MS/MS, which is also known as rapid liquid chromatography-tandem mass spectrometry, has been widely implemented in bioavailability/PK profiling of oncology drugs as mentioned in several preclinical and clinical studies. The technique also supports determination of potential drug–drug interactions during early phase of drug development. LC-MS/MS has emerged as a powerful tool for clinical therapeutic monitoring of oncology drugs as it has the required sensitivity, specificity and reproducibility [66]. Few examples of PK assessment by LC-MS are provided in Table 1.

Table 1: Formulation studies using LC-MS/MS.

S. No.	Method	Outcomes	Ref.
1.	The development of LC-MS/MS technique for quantification of doxorubicin and its metabolites, and pharmacokinetic investigation of 13-hydroxy doxorubicin in mouse biological matrices.	The study was conducted using mouse plasma, urine, and tissue samples for analyzing doxorubicin and its primary metabolite, doxorubicinol. The medication and its metabolite were successfully estimated in a specific sample. The new method was authenticated in compliance with USFDA criteria and was used to evaluate ferritin nanoparticles or liposomes that were free or nano-formulated. This research could be applied more efficiently to oncological treatment, as well as pharmaceutical research and therapeutic medication monitoring.	[85]
2.	Preclinical pharmacokinetic study of an experimental PTX-LAN-PLGA nano-formulation with LC-MS/MS analysis of paclitaxel and lansoprazole in rat plasma.	An evaluation of the pharmacokinetics of paclitaxel-lansoprazole, loaded PLGA nanoparticles in optimized chromatographic conditions was performed using LC-MS and esomeprazole as an internal standard. In the concentration range of 10-320 ng/mL and 100-3200 ng/mL of PTX and LAN, respectively, substantial peak intensities were observed for transition pairs as 854.4, 286.1, 370.1, 251.9, 198, and 346.	[86]
3.	LC-MS was used in a bio-analytical study using mouse plasma for analyzing doxorubicin, and its main metabolites i.e., doxorubicinol, doxorubicinone, doxorubicinolone, and 7-deoxydoxorubicinone.	The sample was prepared for simultaneous quantification of doxorubicin and its main metabolites, doxorubicinol, doxorubicinone, and 7-deoxydoxorubicinone, using a liquid-liquid extraction procedure. The results showed fewer matrix effects and increased sensitivity. The quantification limits of the method were reported for doxorubicin (0.5 ng/mL), doxorubicinol (0.1ng/mL), doxorubicin one, doxorubicinolone, and 7-deoxydoxorubicinone (0.01 ng/mL).	[87]
4.	In a preclinical pharmacokinetic study, liquid chromatography/electrospray tandem mass spectrometry was used to quantify pegylated liposomal doxorubicin and doxorubicinol in rat plasma.	A single CH3OH: CH3COCH3 protein precipitation step was performed, followed by addition of 50 mL of 70% (w/v) zinc sulfate to the samples before analyzing them with the positive turbo-ion spray ionization and multiple reactions monitoring mode. Daunorubicin was used as internal control. Doxorubicin and doxorubicinol concentration ranges were 20 to 8000 ng/mL and 0.05 to 20.0ng/mL, respectively. This method is particularly effective for detecting doxorubicinol in low concentrations. This method is used in the study of doxorubicin and doxorubicinol pharmacokinetics.	[88]
5	A quick and sensitive liquid chromatography-tandem mass spectrometric method for determining timosaponin B-II in blood plasma, as well as a study of saponin pharmacokinetics in rats.	Timosaponin B-II concentration was determined using liquid chromatography-tandem mass spectrometric (LC-MS/MS) technology. The pharmacokinetics and bioavailability of the medication was determined in the plasma using ginsenoside as an internal standard by using simple, accurate, and sensitive approach which was developed and validated according to ICH recommendations. As the part of the analysis, an ODS column of 150 mm x 2.1 mm i.d. was used with acetonitrile-water (35:65, v/v) mobile phase containing 0.05 percent formic acid, with a run time of 3.0 minutes for negative multiple reaction monitoring (MRM). The	[89]

		calibration curve concentration range was 5-15,000 ng/ml, which is useful for pharmacokinetics research because it allows for low concentration determination in rat plasma.	
6	The study of Cajaninstilbene acid, its plasma pharmacokinetics, and tissue distribution in rats by employing LC-MS/MS.	The cajaninstilbene concentrations in tissues and rat plasma were analyzed using LC-MS/MS. A sensitive and quick technique was designed and validated in chromatographic conditions using column HIQ Sil C (18) with a mobile phase of water and methanol (9:91, v/v) containing 0.1 percent formic acid, resulting in a total run time of 10 minutes and isoliquiritigenin as an internal standard. The calibration curve concentration range for both plasma and tissue samples was 10 to 6000 ng/mL. Plasma and tissue recovery rates range from 95.0 to 106.8 percent. This method was used for pharmacokinetic research, and the results revealed that absorption and elimination T_{max} were 10.7±0.31 min and 51.40±6.54 min, respectively.	[90]
7	A pharmacokinetic study using liquid chromatography-tandem mass spectrometry measured xanthone glycosides and timosaponins in rat plasma following oral administration of Zi-Shen Pill extract.	The levels of xanthone glycosides (neomangiferin and mangiferin), alkaloids (palmatine and berberine), and timosaponins (timosaponin E1, timosaponin B-II, and timosaponin E3) were measured using simultaneous LC-ESI-MS/MS. Having mobile phase of acetonitrile and formic acid (0.1%) in water and gradient flow at 0.25mL/min, a Zorbax SB-C (18) column (150mm x 2.1 mm I.D. 3.5 microns) was used. The triple-quadrupole tandem mass spectrometer was used as a detector to measure multiple reactions (MRM). The overall recovery rate ranged from 64.7 to 93.8 %. The new method was thoroughly validated and used in the pharmacokinetic study.	[91]
8	In a pharmacokinetic study using the standardized extract SK-PC-B70M, hederacolchiside E, a neuroactive saponin from *Pulsatilla koreana* extract, was determined in rat plasma using the LC-MS/MS method.	After administering the standardized extract SK-PC-B70M of Pulsatilla koreana to a rat and analyzing the plasma, the pharmacokinetic parameters of neuroactive oleanolic-glycoside saponin and hederacolchiside E were reported. Before running plasma samples through column C_{18}, they were pre-treated with acetonitrile. ESI-MS/MS with negative ion mode was used to identify the eluted samples, with Digoxin serving as the internal standard. The limits of detection (LOD) for hederacolchiside E in 20 L plasma samples were set at 0.5 and 2ng/mL, respectively. The curve was linear (r> 0.997) over the concentration ranges of 2-500ng/ml. The pharmacokinetics study was completed successfully using a validated method.	[92]
9	An oral administration of *Verbena Officinalis L* extract was analyzed in rat plasma using LC-MS/MS and a pharmacokinetic analysis of five flavone components.	A single dose of 8ml/kg *Verbena officinalis* was used for pharmacokinetic studies, and LC-MS/MS was employed to measure apigenin, kaempferol, quercetol, luteolin, and isorhamnetin in rat plasma. The developed method yielded maximal plasma concentrations of 4.33 ±0.58 h, 4.05 ±0.46 h, 2.99 ±0.48 h,, 3.97± 1.48 h, and 4.02 ±0.34 h respectively.	[93]
10.	An LC-MS method to study Clonidine	The concentrations of clonidine hydrochloride in human plasma were determined using	[94]

	Hydrochloride Pharmacokinetics and Bioequivalence in Healthy Male Volunteers..	high-performance liquid chromatography-electrospray ionization mass spectrometry. A ZORBAX-XDB-ODS C_{18} column with acetonitrile: water (60:40) mobile phase and formic acid were used for chromatography (0.2%). The procedure was precise. The limit of quantification (LOQ) was 0.01 ng/ml. The method was validated and used in a pharmacokinetic study.	

Pharmacokinetic study of columbianadin

The pharmacokinetics of columbianadin by LC-MS/MS has been previously evaluated using rat plasma post-intravenous administration of columbianadin. The method provides high sensitivity with a lower limit of quantification, an advantage over other techniques. PK profiling of intravenously administered columbianadin at 10min compared to baseline, and the elimination half-life, confirmed shorter retention time in the body [67].

Mechanism of plant-based active constituents in the treatment of pneumonia

The authors of this study have evaluated the pharmacokinetic parameter with the help of LC-MS/MS method which was developed for the estimation and quantification of sesamoside, shanzhiside methyl ester, and barlerin. These three active constituents were estimated in rat plasma after oral administration. Anti-pneumonia mechanism of this rare Tibetan medicine was assessed using this method precisely. These three compounds which were isolated from the plant Phlomis brevidentata, exhibited extremely similar pharmacokinetic characteristics, possibly due to their highly analogous chemical structure [68].

Quantification of Acipimox in Plasma and Tissues

Pharmacokinetics of acipimox was evaluated in a simulated high altitude hypoxia rat model. Acipimox in rat plasma and tissue homogenates was quantified with an established and validated LC–MS/MS method. Investigation of the expression of free fatty acid (FFA) levels and lipid metabolism-related proteins was estimated through western blotting and enzyme linked immunosorbent assay (ELISA). The pharmacokinetic comparison was established between hypoxic and normoxic rats. The pharmacokinetics parameters of acipimox between normoxic and hypoxic rats were found to be significant different in observed results. Collected information demonstrated the safety and efficacy characteristics of acipimox [69].

Drug-metabolizing Enzymes and its transporters quantification

In vitro to *in vivo* extrapolation (IVIVE) using *in vitro* and *in vivo* models can be done with the help of protein abundance data of drug-metabolizing enzymes and transporters (DMETs). DMET quantification can be done using limited sample volume or protein concentration in which microflow-based LC-MS (µLC-MS/MS) method was applied to quantify DMETs in differential tissue. Liver, intestine, kidney, lung, and heart were evaluated during the study [70].

Pharmacokinetic evaluation of Empagliflozin in healthy Egyptian volunteers

A new LC-MS method was developed and validated to allow the use of an internal standard (IS) dapagliflozin, for a highly specific and precise estimation of empagliflozin, in human plasma collected from the individuals dosed between 25–600 ng mL^{-1}. The pharmacokinetic study was

established with liquid-liquid extraction for enhanced sample preparation to facilitate estimation of the main pharmacokinetic parameters like $C_{max,}$ T_{max}, $T_{1/2}$, elimination rate constant, AUC0-t and AUC0-inf [71].

Pharmacokinetics of aminoglycoside antibiotic Kanamycin using LC-MS

A validated LC-MS assay with high selectivity and sensitivity was used to evaluate the pharmacokinetics of Kanamycin in plasma samples from patients with multi-drug resistant tuberculosis. The accuracy and precision, linearity, limit of detection and quantitation, specificity, and recovery were conducted to validate the assay. Due to the small patient number (cohort size), the authors of the study cannot initially rule out a possible effect of renal impairment, HIV-infection, and antiretroviral drugs on kanamycin pharmacokinetics. Based on the obtained data, authors established that HIV-infection and renal function did not influence kanamycin PK in patients' plasma [72].

LC-MS in Pharmacodynamic/biomarker studies

The use of appropriate critical reagents and robust laboratory techniques are essential elements to detect biomarkers quantitatively and reproducibly, across multiple disease indications. One of the most routinely performed techniques in the clinical laboratory is the mass spectrometry, that is primarily used for toxicological testing and to monitor therapeutic drug, and its metabolites. In the last decade, LC-MS have also been implemented to develop and validate several biomarker assays across multiple disease indications like cancer, cardiovascular, neurological disorders, rare diseases etc. using correct biologically relevant matrices i.e., serum/plasma/tissue. Additionally, LC-MS technique has also replaced or complemented ligand binding assays for biomarker quantitation. For example, an automated binding immunoassay platform, does not discriminate between vitamin D2 and vitamin D3, while mass spectrometry-based methods discriminated between the two isoforms [73].

For early detection, prognosis and treatment of cancer, and other diseases, biomarker discovery and verification are required, that can be performed by a robust analytical technique like LC-MS. LC-MS-based global and targeted proteomic analysis can be used for protein biomarker discovery, and accurate and reproducible detection of verified biomarkers [22]. A few examples of the use of LC-MS in clinical diagnostics/biomarker are provided in Table 2. A validated and multiplexed LC-MS assay was used to quantify fasting levels of insulin and C-peptide, both analytes' levels helped predict development of diabetes mellitus [74]. Plasma renin activity (PRA) assay was developed using LC-MS/MS which reduced sample handling compared with an RIA approach; PRA is considered as an essential diagnostic tool for management/treatment of secondary hypertension

[75]. Parathyroid hormone-related peptide (PTHrP)-specific tryptic peptide was analyzed with 2-dimensional LC-MS/MS for the diagnosis and clinical management of patients suspected of hypercalcemia of malignancy [76]. The group developed an antibody-independent candidate reference measurement procedure (RMP) for amyloid-β (Aβ42), based on solid-phase extraction and isotope-dilution LC-MS/MS, to detect/quantify Aβ42 in cerebrospinal fluid (CSF), a well-established biomarker for Alzheimer's disease [77]. HPLC and electrospray ionization mass spectrometry technique was developed to measure/quantitate HbA1c in human blood which is important for the long-term control of the glycaemic state in diabetic patients [78, 79].

Moreover, few more study examples are shown in the Table 2 demonstrating the importance of LC-MS in biomarker-related pharmaceutical/academic research. A mixed-mode solid-phase extraction method and an ultra-performance liquid chromatography tandem mass spectrometry (SPE UPLC-MS/MS) was developed to quantify amyloid beta (Aβ) peptide levels in the brain; it is critical to evaluate and monitor an imbalance between the production and clearance of amyloid beta (Aβ) peptides in the brain that is involved in Alzheimer's disease [80]. LC-MS/MS-based targeted proteomics assay coupled with peptide immunoaffinity enrichment method quantified soluble transferrin receptor in serum- an indicator of bone marrow failure in breast cancer patients [81]. A sensitive and selective immunoaffinity liquid chromatography-tandem mass spectrometry (LC-MS/MS) (IA-MS) assay evaluated CSF tau levels- a common biomarker for Alzheimer's disease [82]. A two-dimensional nano-ultra-high-performance liquid chromatography parallel reaction monitoring/high-resolution mass spectrometry method was developed to monitor high mobility group box-1, a pro-inflammatory cytokine/marker [83]. A sequential protein and tryptic peptide immunoaffinity LC-MS/MS assay was developed for the quantification of human and cynomolgus monkey IL-21. IL-21 was not detected in serum from normal wild-type human, but IL-21 was detected in inflammatory bowel disease (IBD) human colon tissue and hyperplasia human tonsils [84, 79].

Table 2: Examples of quantitative LC-MS assays to support clinical diagnostics and pharmaceutical/academic research.

Category	Analyte(s)	Extraction	Ref.
Clinical diagnostics	C-peptide, insulin	IA-protein	74
	Angiotensin-1 (renin activity)	SPE	73
	Parathyroid hormone-related peptide (PTHrP)	IA-protein	76
	β Amyloid (Aβ-42)	SPE	77
	Hemoglobin A1c	None	78
Pharmaceutical/academic research	β-Amyloid peptides (Aβ)	SPE	72
	Serum transferrin receptor	IA-protein	73

	Tau-protein	IA-protein	74
	High-mobility group box 1 (HMGB1)	IA-protein	75
	Interleukin 21 (IL-21)	IA-sequential	95

LC-MS in pharmacokinetic studies:

The detailed descriptions of pharmacokinetic studies have been discussed in Table 1 [85-94]; the table was modified from reference number seventy-seven. ESI was used as the ionization technique in all the LC-MS assays. Triple-quadrupole mass spectrometer (QqQ) detection was used for all analytes except Aβ-4 and HMGB1, which used orbitrap detection. SPE: solid phase extraction.

Identification of Impurities by using LC-MS

The two most significant components of drug therapy are the safety and effectiveness of medicinal substances. Several factors affect the safety and efficacy of the medicine, including its toxicological and pharmacological profile as well as adverse effects generated by contaminants in bulk and dose forms. A medicine is stated to be safe when it has a low risk of side effects at the recommended therapeutic dose. Because contaminants in drugs frequently have negative pharmacological or toxicological effects, we can conclude that medication quality and safety are inexorably related. Using the right analytical techniques to efficiently manage contaminants can often ensure the purity and safety of medicine [96].

Impurities, according to ICH guidelines, are "any component of the medicinal product that is not the chemical entity identified as the active agent or an excipient in the product". Impurity profiling is thus defined as analytical processes that try to detect, identify, or comprehend the structure of impurities in bulk medicines and pharmaceutical formulations [97].

LC-MS/MS is now the most widely used technology for identifying and characterizing contaminants. An YMC-Triart C-18 column in gradient elution mode was used for testing of potential genotoxic contaminants in certinib API, by using formic acid (0.1%) and acetonitrile as mobile phase at 0.5 ml/min flow rate. Furthermore, using triple quadrupole mass detection combined with electron spray ionization, impurities were quantified in multiple reaction monitoring modes [98].

Identification of Degradation product by using LC-MS

The goal of degradation testing is for the medicine to deteriorate between 10% and 15%. Photolytic, thermal, oxidative, and hydrolytic stresses are applied to the medication. The outcomes are compared to untreated blank samples, untreated standard drug samples, stressed blank samples

and stressed drug sample solutions. Only two samples are obtained under photolytic and thermal stress conditions: a stressed sample and a control sample [99-103]

Hydrolytic decomposition:

In neutral, alkaline, and acidic conditions, the hydrolytic breakdown was carried out. In a 10 mL volumetric flask, 1 mL of drug stock solution (1000 µg/mL) and 1 mL of hydrolytic agent (1M HCl, 1M NaOH, and water) was added. The samples were heated in a water bath at 80°C for specific time intervals if necessary. Before being administrated into the HPLC apparatus, the samples were neutralized with an acid or alkali of equal strength.

Thermal decomposition:

Each volumetric flask is sealed and filled with 10 mg drug sample. One flask is heated to a specific temperature and time interval in a hot air oven, while the other is kept in control. As soon as the visibility is acceptable, two distinct solutions are prepared by weighing an appropriate amount of each thermally stressed sample and control, resulting in a concentration of 100 µg/mL. Before injecting the samples into HPLC, a diluent is used to dilute them to the required concentration.

Oxidative decomposition:

To conduct oxidative degradation, hydrogen peroxide is utilized. To make samples, 1 mL of drug stock solution (1000 µg/mL) and 1 mL of 30% H_2O_2 are combined in 10 mL volumetric flask. If necessary, the samples are heated for prescribed periods in a water bath at a constant temperature of 80°C. Following the necessary exposure, samples are diluted to the desired concentration with diluents before being submitted to HPLC analysis.

Photolytic decomposition

Drug layers (1 mm thick) are formed in petri dish and subjected to ICH photo stability conditions. These conditions include a minimum of 1.2 million l x h of overall irradiation and a maximum of 200 W/m^2 of near UV energy integrated. A control petri dish containing the drug is sealed with aluminum foil. Two separate solutions are prepared using suitable amounts of control and stressed samples weighed to produce a concentration of 100g/mL. The samples are further diluted with diluents to the required concentration before being separated by HPLC [104].

LC-MS IN THE DETECTION OF ADULTERATION IN HERBAL PRODUCTS

As defined by the FDA, adulteration for profit involves replacing an original crude drug entirely or partially with a substance that is either devoid of the original substance or has inadequate chemical and medicinal properties. A drug can also be completely replaced with a similar drug that is either of high quality or spoiled, or by adding low-grade or spoiled drugs. Herbal remedies are widely regarded as highly adulterated in the commercial industry, and adulteration can occur either

directly/intentional or indirectly/unintentional. Several molecules are adulterated by their counterparts, for example, beeswax with coloured paraffin wax, papaya seed with black pepper seed. LC-MS can be used to detect/quantify such adulterants [105-110].

In recent years, few publications regarding the investigation of herbal drugs using LC/MS have been published. This breakthrough demonstrates the value of LC/MS when trying to resolve tough problems with herbal products. The three fundamental ionization processes are atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and atmospheric pressure photoionization (APPI). The ESI method is used to analyze charged species, whereas the APCI method is used to evaluate inert or difficult-to-charge species. Both soft ionization methods are suitable for the bulk of chromatographic separations. The chromatographic separation and detection advantages of DAD and MS are combined in the LC-DAD-MS method. Using hyphenated techniques, chromatographic peaks can be quickly located to perform an online comparison analysis.

To identify and test known and unknown therapeutic additives in herbal remedies, a variety of LC-MS methods have been implemented. Quadruple mass spectrometers are used to perform the analysis selectively using the acquisition mode known as chosen ion monitoring. By utilizing selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) modes, it is possible to detect the separate product ions formed by collision-induced dissociation (CID) with a higher selectivity and sensitivity. There are several LC-MS techniques and approaches widely used to screen for contaminants in herbal products and supplements, including triple quadrupole instruments running with MRM mode, which record one or more transitions per target component.

The LC-MS method is also used as an important qualitative device for the detection and separation of a wide range of structurally related compounds and unidentified adulterants. It is also possible to screen and characterize new analogues as adulterants using cutting-edge LC-MS technologies. As a result, LC-MS has emerged as the most precise and dependable method for standardizing and verifying the quality of herbal products.

CONCLUSION

This review highlights the importance of LC-MS technique in the drug discovery and development, and to study PK and PD parameters. The accurate quantification of pharmaceuticals is critical for determining dose and therapeutic effects in cellular/animal/human systems. Hyphenated procedures are essential analytical techniques capable of performing separation, identification, and quantification, all at the same time. The review article discusses how the instrument works, how it is currently used, and provide an insight into future applications along

with few limitations/challenges. Sensitivity of the LC-MS assay has been a major challenge in the past, although, protein- or peptide-level enrichment techniques and ionization methods have further enhanced sensitivity of the platform. Additionally, sample throughput has been relatively poor, and sensitivity in combination with throughput has been a major limitation for widespread implementation of LC-MS in drug discovery and development. However, increased use of multiplexing has improved efficiency and throughput. Insufficient availability of full-length protein reference standards and well characterized capture reagents, also poses an issue for assay availability/reproducibility. Current technological changes have increased LC-MS assay sensitivity and throughput significantly, to detect/quantify protein concentrations in samples from wild-type and patient population, as measured by global and targeted proteomics approaches using LC-MS. In the next 5-10 years, additional LC-MS-based platforms will further help characterize pharmaceutical formulations and evaluate drug concentrations in cellular/murine/NHP/human samples for pharmacokinetics analysis and improve the quality of biomarker assays to help find treatment options for multiple indications including rare diseases. Next-generation LC-MS tools can further revolutionize the field of pharmaceutical analysis and clinical chemistry, that will help enhance our understanding of drug product/substance stability, degradation, impurity, and PK/PD relationship, by providing sensitivity, accuracy, and throughput necessary for broader implementation of LC-MS in clinical laboratories. [21, 77].

DISCLOSURE STATEMENT:

None of the work being submitted/presented or discussed in the publication has anything to do with AbbVie. Similarly, the scope of this review is outside of the work performed at/by PepGen.

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