



## LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) - AN UPDATE

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**ABSTRACT**

Mass Spectrometry (MS) is an important analytical tool for molecular research and used around the globe to identify, quantify and characterize biomolecules like proteins from any number of biological conditions or sample types. As instrumentation is advanced and with the coupling of Liquid Chromatography (LC) for high-throughput, Liquid Chromatography-Mass Spectrometry (LC-MS), a proteomics experiment measuring hundreds to thousands of proteins/protein groups is now commonplace. Chromatography is a separation technique used to separate the individual compound from a mixture using a stationary and mobile phase. Discovery of chromatography is a milestone event in biomedical research. Chromatographic separation is based on the principles of adsorption, partition, ion exchange, molecular exclusion, affinity and Chirality. There are many types of chromatography available for quantitative and qualitative analysis of pharmaceutical agents, which includes LC-MS. It is now a routine technique with the development of electrospray ionisation (ESI) providing a simple and robust interface. The main objective of this review is to overview the principle, applications and various studies done using of LC-MS.

**KEYWORDS:** Mass Spectrometry, Chromatography, Analytical Technique, Tandem Mass Spectrometry.**INTRODUCTION**

Combination of chromatography with spectrometry is first reported in 1967 and first LC-MS system was introduced in 1980s. LC-MS is an analytical chemistry technique that combines the physical separation capabilities of LC with the mass analysis and MS. Mainly the LC-MS contains liquid chromatography assembly, ion generation unit/ ionization source, mass analyzer and mass spectrometric data acquisition. LC-MS is most commonly used in biomedical sciences for pharmacokinetic analysis, genetic analysis, structural elucidation, etc. It can be applied to a wide range of biological molecules and the use of tandem MS and stable isotope internal standards allows highly sensitive and accurate assays to be developed although some method optimisation is required to minimise ion suppression effects. Fast scanning speeds allow a high degree of multiplexing and many compounds can be measured in a single analytical run. With the development of more affordable and reliable instruments, LC-MS is starting to play an important role in several areas of clinical biochemistry and compete with conventional liquid chromatography and other techniques such as immunoassay.<sup>[1]</sup>

**Principle of LC-MS**

The LC-MS technology involves use of high performance/pressure liquid chromatography (HPLC), wherein the individual components in a mixture are first separated followed by ionization and separation of the ions on the basis of their mass/charge ratio. The separated ions are then directed to a photo or electron multiplier tube detector, which identifies and quantifies each ion. The ion source is an important component in any MS analysis, as this basically aids in efficient generation of ions for analysis. To ionize intact molecules, the ion source could be APCI (Atmospheric Pressure Chemical Ionization), ESI etc. to name a few popular ones. The choice of ion source also depends on the chemical nature of the analyte of interest, i.e., polar or non-polar. The major advantages of this technology include sensitivity, specificity and precision as analysis is done at molecular level. Also, structural details of the analyte can be deciphered, typical LC-MS system is combination of HPLC with MS using interface (ionization source). The sample is separated by LC and the separated sample species are sprayed into atmospheric pressure ion source, where they are converted into ions in the gas phase. The mass analyzer is then used to sort ions according to their mass to charge ratio and detector counts the ions emerging from the mass analyzer and may also amplify the signal generated

from each ion. As a result, mass spectrum (a plot of the ion signal as a function of the mass-to-charge ratio) is created, which is used to determine the elemental or isotopic nature of a sample, the masses of particles and of molecules and to elucidate the chemical structures of molecules.<sup>[2,3,4]</sup>

### Applications and studies on LCMS

LC-MS is a routine technique with the development of ESI providing a simple and robust interface. It can be applied to a wide range of biological molecules and the use of tandem MS (MS/MS) and stable isotope internal standards allows highly sensitive and accurate assays to be developed although some method optimisation is required to minimise ion suppression effects. LC-MS is starting to play an important role in several areas of clinical biochemistry and compete with conventional LC and other techniques such as immunoassay.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has seen enormous growth in clinical laboratories during the last 10–15 years. It offers analytical specificity superior to that of immunoassays or conventional HPLC for low molecular weight analytes and has higher throughput than Gas Chromatography-Mass Spectrometry (GC-MS). Drug/Toxicology and Biochemical Genetics/Newborn Screening laboratories were at the vanguard of clinical LC-MS/MS use, but have been eclipsed by Endocrine Laboratories. In USA reference/referral laboratories, most steroids and biogenic amines are now assayed by LC-MS/MS, and the technology has started to penetrate into smaller laboratories. Assays for mineralo- and gluco-corticoids and their precursors, sex steroids, metanephrines and 25-hydroxy vitamin D highlight the advantages of LC-MS/MS. However, several limitations of LC-MS/MS have become apparent, centring on the interacting triangle of sensitivity – specificity – throughput. While sample throughput is higher than for conventional HPLC or GC-MS, it lags behind automated immunoassays. Techniques which improve throughput include direct sample injection, LC-multiplexing and sample multiplexing. Measures to improve specificity and sensitivity include sample clean-up and optimising chromatography to avoid interferences and ion suppression due to sample-matrix components. Next generation instrumentation may offer additional benefits. The next challenge for clinical LC-MS/MS is peptide/protein analysis. The quest for multi-biomarker profiles for various diseases has largely failed, but targeted peptide and protein testing by LC-MS/MS, directed at analytical and clinical questions that need to be answered, is proving highly successful. We anticipate that this will result in similar growth of clinical protein/peptide LC-MS/MS as has been seen for low molecular weight applications.<sup>[5]</sup>

Advances in LC-MS or GC-MS have enabled high-throughput identification and quantitative analysis of biomolecules including proteins, glycans, glycoproteins

and metabolites with onset of cancer. While results from separate analyses of different biomolecules have been reported widely, the mutual information obtained by partly or fully combining them has been relatively unexplored. In an earlier study, it was investigated the integrative analysis of proteins, N-glycans and metabolites to take advantage of complementary information to improve the ability to distinguish cancer cases from controls.<sup>[6]</sup>

Jiaqi Ganxian Granule (JGG) is a famous traditional Chinese medicine, which has been long used in clinical practice for treating liver fibrosis. The mechanism underlying its anti-hepatic fibrosis is studied in this study, an Ultra-Performance Liquid Chromatography-Time-Of-Flight Mass Spectrometry (UPLC-TOF-MS) - based metabolomics strategy was used to profile the metabolic characteristic of serum obtained from a carbon tetrachloride (CCl<sub>4</sub>) - induced hepatic fibrosis model in Sprague-Dawley rats with JGG treatment. Through Principal Component Analysis (PCA) and Partial Least Square Discriminant Analysis (PLS-DA), it was shown that metabolic perturbations induced by CCl<sub>4</sub> were inhibited after treatment of JGG, for 17 different metabolites related to CCl<sub>4</sub>.<sup>[7]</sup>

MS is one of the most frequently employed techniques in performing quantitative analysis. Its specificity, selectivity and typical limit of detection are more than enough to deal with most analytical problems. This is the result of significant effort, either from the scientists working in the field or from the manufacturing industry, devoted to the development of new ionization methods, expanding the application fields of the technique and new analysers capable of increasing the specificity mainly by collisional experiments or by high mass accuracy measurements. Thus, the MS panorama is made up of many instrumental configurations, each of which has specific positive and negative aspects and different cost/benefit ratios. These mass spectrometric approaches are usually employed when linked to suitable chromatographic systems. The synergism obtained allows Chromatography-MS to be used worldwide and is of considerable interest to researchers involved in basic chemistry, environmental and food controls, biochemistry, biology and medicine. It is to be expected that this diffusion will grow in the future, due to the relevance of the information that quantitative MS can provide, in particular in the field of public health.<sup>[8]</sup>

LC-MS/MS is becoming the method of choice for clinical steroid analysis. In most instances, it has the advantage of higher sensitivity, better reproducibility and greater specificity than commercial immunoassay techniques. The method requires only minimal sample preparation and a small sample volume. Furthermore, it has the potential to analyze multiple steroids simultaneously. Modern instruments guarantee high throughput, allowing an affordable price for the individual assay. All this makes LC-MS/MS an attractive

method for use in a clinical setting. Reliable reference ranges for the detected analytes are the pre-requisite for their clinical use. If these are available, LC-MS/MS can find application in congenital disorders of steroid metabolism, such as congenital adrenal hyperplasia, disorders of sex development and disorders of salt homeostasis, as well as in acquired disorders of steroid metabolism, such as primary aldosteronism, Cushing's disease, Addison's disease and hyperandrogenemia, as well as in psychiatric disease states such as depression or anxiety disorders.<sup>[9]</sup>

LC-MS/MS is a technique that uses liquid LC or HPLC with the mass spectrometry. LC-MS/MS is commonly used in laboratories for the qualitative and quantitative analysis of drug substances, drug products and biological samples. LC-MS/MS has played a significant role in evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data.<sup>[10]</sup>

A LC-MS/MS method has been developed and validated for the determination of glycine in human cerebrospinal fluid (CSF). The validated method used artificial CSF as a surrogate matrix for calibration standards. Pre-validation experiments were performed to demonstrate parallelism with surrogate matrix and standard addition methods. The mean endogenous glycine concentration in a pooled human CSF determined on three days by using artificial CSF as a surrogate matrix. A percentage difference of -2.6% indicated that artificial CSF could be used as a surrogate calibration matrix for the determination of glycine in human CSF. Quality control (QC) samples, except the lower limit of quantitation (LLOQ) QC and low QC samples, were prepared by spiking glycine into aliquots of pooled human CSF sample. The low QC sample was prepared from a separate pooled human CSF sample containing low endogenous glycine concentrations, while the LLOQ QC sample was prepared in artificial CSF. Standard addition was used extensively to evaluate matrix effects during validation. The validated method was used to determine the endogenous glycine concentrations in human CSF samples. Incurred sample reanalysis demonstrated reproducibility of the method.<sup>[11]</sup>

Interest in the medicinal properties of secondary metabolites of *Boesenbergia rotunda* (fingerroot ginger) has led to investigations into tissue culture of this plant. In this study, we profiled its primary and secondary metabolites, as well as hormones of embryogenic and non-embryogenic (dry and watery) callus and shoot base, UPLC-TOF-MS together with histological characterization. Metabolite profiling showed relatively higher levels of glutamine, arginine and lysine in embryogenic callus than in dry and watery calli, while shoot base tissue showed an intermediate level of primary metabolites. For the five secondary metabolites analyzed (ie. panduratin, pinocembrin, pinostrobin, cardamonin and alpinetin), shoot base had the highest concentrations, followed by watery, dry and

embryogenic calli. Furthermore, intracellular auxin levels were found to decrease from dry to watery calli, followed by shoot base and finally embryogenic calli. The morphological observations showed the presence of fibrils on the cell surface of embryogenic callus while diphenylboric acid 2-aminoethylester staining indicated the presence of flavonoids in both dry and embryogenic calli. Periodic acid-Schiff staining showed that shoot base and dry and embryogenic calli contained starch reserves while none were found in watery callus. An evaluation study with LCMS identified several primary metabolites that could be used as markers of embryogenic cells, while secondary metabolite analysis indicated that biosynthesis pathways of these important metabolites may not be active in callus and embryogenic tissue.<sup>[12]</sup>

In a study a robust and sensitive method based on a proposed sample purification procedure, using zirconia-coated Phree columns and analysis by UPLC with triple quadrupole tandem mass spectrometry are presented for the assessment of multi-class antibiotics in farmed fish species. The sample preparation procedure benefited from combined precipitation of proteins and selective removal of phospholipids by Phree columns resulting in a high sensitivity of the method. The in-house validation results (precision, repeatability, decision limit  $CC\alpha$ , detection capability  $CC\beta$ , etc.) indicate that the elaborated method is fully suitable for the analysis of the main classes of antibiotics in accordance with the European Union Commission Decision. The method was applied to the analysis of antibiotics in trout and sturgeon samples obtained from the local inland aquacultures in Latvia. The results revealed the presence of two antibiotics (enrofloxacin and trimethoprim) in 12 out of the 20 analysed fish samples at concentrations below the MRLs, thus causing no acute risks to consumers.<sup>[13]</sup>

Ovarian steroid hormones are major regulators of the physiology of the oviduct and reproductive events occurring within the oviduct. To establish a whole steroid profiling of the bovine oviductal fluid during the estrous cycle, contralateral and ipsilateral (to the corpus luteum or preovulatory follicle) oviducts were classified into four stages of the estrous cycle: postovulatory, mid-luteal, late luteal and preovulatory on the basis of the ovarian morphology and intrafollicular steroid concentrations. Steroids can be extracted from pools purified, fractioned by HPLC and analyzed by GC-MS/MS.<sup>[14]</sup>

The liquid chromatography mass spectrometry confirmatory techniques are applied to urine, serum, whole blood, oral fluid, hair and tissues.<sup>[15]</sup> Although immunoassays in measuring 25-hydroxyvitamin D [25(OH)D] have been improved recently, relatively large differences are still seen between results of 25(OH)D measured by immunoassays and by LC-MS/MS. Concentrations of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> in serum samples from 59 healthy subjects

were measured by two immunoassays including Siemens ADVIA Centaur Vitamin D Total (Centaur) and Roche Elecsys Vitamin D Total (Elecsys) and LC-MS/MS. To determine the cross reactivity of Elecsys and Centaur toward 25(OH)D<sub>2</sub>, a dosage of 200,000 IU vitamin D<sub>2</sub> was given after first sampling. Serum samples were obtained 30 days later and concentrations of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> were measured again. The results showed poor agreement between the immunoassays and LC-MS/MS in 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> measurements. The clinicians need to be aware of the underestimation of vitamin D status when using these immunoassays for measuring individuals supplemented with vitamin D<sub>2</sub>.<sup>[16]</sup>

An UHPLC-MS/MS method was developed for known and tentatively identified compounds characterized via UHPLC-quadrupole time-of-flight (QTOF) screening of fungal culture extracts, wall scrapings and reference standards.<sup>[17]</sup> A sensitive and specific LC-MS/MS method was developed for the measurement of isomangiferin in rat plasma. Chloramphenicol was selected as the internal standard. Sample preparation was carried out through a simple one-step protein precipitation procedure with methanol. The validated method has been successfully applied to a preclinical pharmacokinetic study of isomangiferin in rats for the first time. LC-MS/LC-MSMS are most widely used in food industries, pharmaceutical and chemical industries for quantitative and qualitative analysis. Applications of LC-MS/MS are as follows, to determine the molecule weight of chemical substance, pharmaceutical substances, proteins, etc; MS/MS used to determine structural information using mass spectral fragmentations; to determine the pharmacokinetic profile of the pharmaceuticals like drug, drug metabolites/degradation product, impurities and chiral impurities. The separation and detection of chiral impurities in pharmaceuticals are of great importance because the D-isomer of a drug can have different pharmacological, metabolic and toxicological activity from the L-isomer; used in SNP genotyping, quantification of DNA, gene expression analysis, DNA and RNA sequencing; used to identify aflatoxins (toxic metabolic product in certain fungi), determine the vitamin D<sub>3</sub> in poultry fed supplements, etc; used for analysis of peptides.<sup>[18]</sup>

## CONCLUSION

The advancement of hyphenated techniques, high resolution mass analyzers as well as high throughput separation approaches, quantitative and qualitative analysis of pharmaceutical drugs and metabolites can be achieved with good sensitivity. With the development of more affordable and reliable instruments, LC-MS is starting to play an important role in several areas of clinical biochemistry.

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