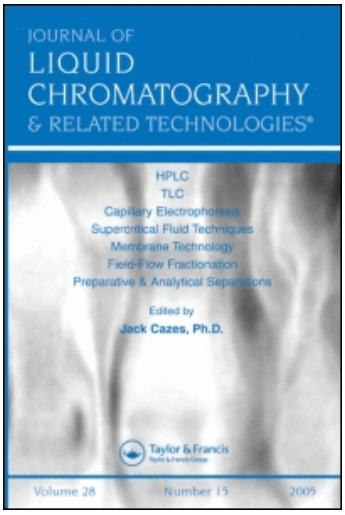

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Pharmaceutical Applications of Liquid Chromatography Coupled with Mass Spectrometry (LC/MS)

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Abstract: High performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) is an extremely powerful and indispensable methodology practiced in virtually every stage of pharmaceutical discovery and development processes, including biological target discovery, biological assay for high throughput screening, characterization of physicochemical properties of drug candidates, and drug metabolism and pharmacokinetics. With continued advances and innovations in the areas of column technologies, LC/MS interfaces, and instrumentation, the field of LC/MS is strengthening and expanding to new disciplines constantly. In this report, the recent developments in the LC/MS interface ionization techniques, such as ESI, APCI, APPI, and MALDI, and novel hardware and software of mass spectrometry devices, are compared and reviewed in the light of drug discovery and development effort. The diverse qualitative and quantitative applications of LC/MS in the pharmaceutical industry are reported in four key fields; synthetic organic chemistry, combinatorial library parallel synthesis, bioanalysis in support of ADME, and proteomics. Other analytical techniques used in conjunction with LC/MS to augment analytical information content are also discussed. As the use of LC/MS is exceedingly widespread and ever increasing, this paper primarily focuses on recent progresses reported in the literature with selected publications.

Keywords: LC/MS, Pharmaceutical applications, HPLC, Mass spectrometry

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INTRODUCTION

Recent articles, which appeared in well-known scientific publications,[1,2] demonstrate that mass spectrometry has evolved from an analytical technique practiced only by specifically trained scientists, to an established essential tool actively employed by colleagues in other fields. The report by Aebersold et al.[1] reviewed successes of mass spectrometry-based proteomics in the area of protein profiling, protein interactions, and protein modification. The areas which mass spectrometry has contributed in proteomics include primary and secondary structures of proteins, quantitative analysis of protein expression using stable isotopes, post-translation modification of proteins, and protein–protein interaction in order to facilitate understanding of the structure and function of proteins. In another article, mass spectrometry was utilized to identify proteins in the culture of coronavirus, which is believed to cause severe acute respiratory syndrome (SARS).[2] SARS has caused pandemic in China, Singapore, Vietnam, Canada, the U.S., Taiwan, and several European countries in the years of 2002 and 2003. The authors prepared the culture of viral samples from SARS patients through SDS-PAGE with Western blot, proteolytic digestion, subsequent HPLC fractionation, and off-line MALDI-TOF experiments (see below for explanation of terms). De-novo sequencing of the viral protein in conjunction with database searching was undertaken through tandem mass spectrometric analysis, and novel nucleocapsid and spike proteins were identified. The discovery of the viral proteins can be used for early diagnosis and intervention of SARS. As can be seen in these examples, mass spectrometry played an indispensable role in the advance of medicinal science.

High performance liquid chromatography coupled with mass spectrometry (HPLC/MS or LC/MS) has been widely accepted in the pharmaceutical industry as an analytical method of choice, and is utilized in virtually every stage of drug development. HPLC provides the separation of components from a complicated mixture and is the primary analytical method accepted throughout the pharmaceutical research and development process.[3,4] Mass spectrometry offers analytical figures of merit, such as sensitivity, selectivity, specificity, and speed of analysis. Recent developments in MS instrumentation has further enhanced the performance expanding its capabilities.[5] By combining these two powerful analytical techniques, LC/MS has proven to be an established and essential analytical method in various research fields. The interface between HPLC and MS, which is discussed in detail below, has been a formidable obstacle until realization of the electrospray process as an ionization method for mass spectrometry. The work on electrospray ionization and laser desorption ionization that enabled investigation of biological macromolecules by mass spectrometry were recognized by the Nobel Prize in 2002.[6]

Areas of LC/MS application in the pharmaceutical industry cover target identification (e.g., proteomics), high throughput screening, combinatorial chemistry, medicinal chemistry and process research, absorption/distribution/metabolism/excretion (ADME), and clinical trial support.[7–10] In this article, recent publications on LC/MS applications in the pharmaceutical field are reviewed, as well as the instrumentation and LC/MS interfaces, with selected references.

CONVENTIONAL MASS SPECTROMETERS AND NEW DEVELOPMENTS

There are five types of commercially available mass spectrometers regarding the manner in which the ions with different m/z value are separated to generate mass spectra, namely, magnetic/electric sector, 3-dimensional quadrupole ion trap (routinely referred as ion trap), quadrupole, time-of-flight, and Fourier transform ion cyclotron resonance (FT-ICR) MS. These five mass spectrometers are schematically depicted in Figure 1, and brief descriptions on how they operate are given below for each type.

In a magnetic/electric sector type mass spectrometer, which is considered to be the classical mass spectrometer, ions travel through a magnetic/electric field region, and the ion trajectory is deflected depending on the field strength (Fig 1(A)). In a typical double focusing sector MS, ion energy is focused by the electric sector and the mass analysis is performed by the magnetic sector. By scanning the magnetic field, the ions are detected according to their m/z ratio generating mass spectra. This type of mass spectrometer typically operates at very high vacuum and can provide high resolution mass spectral data for accurate mass measurement.

The 3-dimensional quadrupole ion trap (QIT) consists of a ring electrode and two end-cap electrodes that form a hyperbolic surface (Fig. 1(B)). Ions can be "trapped" and stored within the hyperbolic cavity of the ion trap, depending on the RF/DC voltages applied to the ring and end-cap electrodes. A trajectory of ions with particular m/z is stable under certain RF/DC combinations, therefore by scanning the RF/DC voltages the user is able to manipulate the ions to be either stored or ejected. This unique trapping feature enables "tandem-in-time" mass spectral capability. Tandem-in-time MS represents the mass analysis and the transition from the parent to fragment ions that occur in the same physical space but at different times. For example, ions with m/z 500 (MS^1) can be selectively trapped within the ion trap cavity and subjected to collision induced dissociation (CID), resulting in fragment ions with m/z 350 (MS^2). This fragment ion can then be subsequently trapped selectively and undergo another stage of fragmentation, to produce ions with m/z 280 (MS^3) and so on. All of the three processes occur in the same ion trap space sequentially (tandem-in-time) in this example.

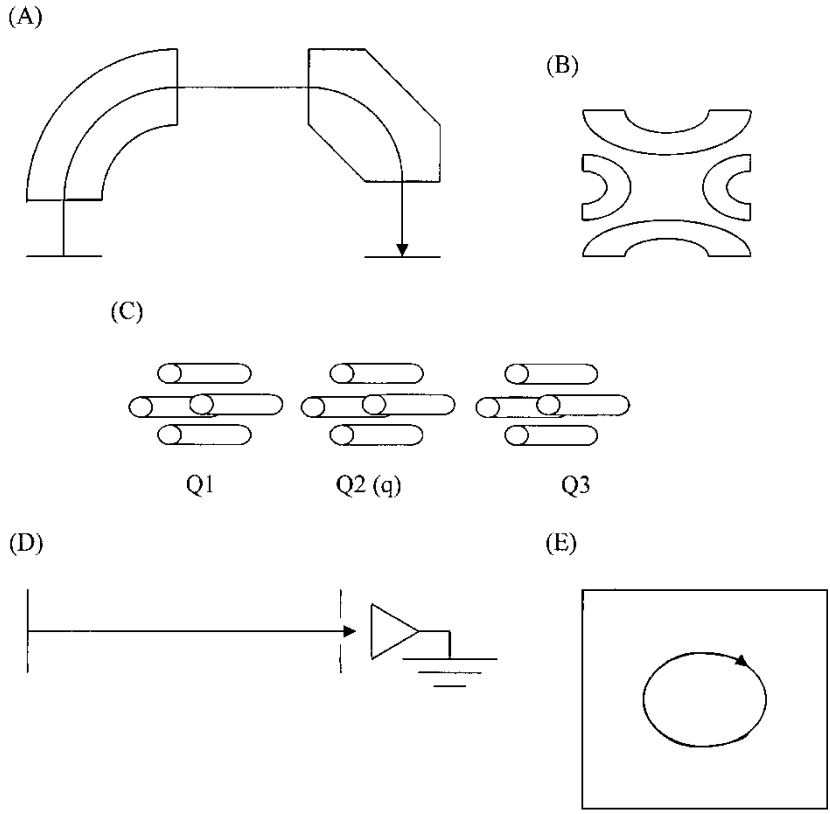

Figure 1. Schematic diagram of the conventional mass analyzers. (A) double focusing magnetic sector MS, (B) ion trap MS, (C) quadrupole MS, (D) time-of-flight MS, (E) Fourier transform ion cyclotron resonance (FT-ICR) MS.

As there is no theoretical limit on how many stages of fragmentation one can perform, it is said that the ion trap MS is capable of providing tandem mass spectra in the order of n (MS^n). The advantages of the ion trap MS are its small size, low cost, and sensitivity due to the ion accumulation capability. However, ion trap MS has a limited ion storage capacity and narrow dynamic range because of its small trapping volume. Loss of mass accuracy would occur if one overfills the trap and causes space charging.

The most commonly used mass spectrometer in the pharmaceutical industry is, indisputably, the quadrupole type MS including single (Q) and triple quadrupole (QqQ) MS. In contrast to the 3-dimensional quadrupole ion trap MS mentioned above, the quadrupole type mass analyzer is 2-dimensional. The surface of quadrupole electrodes should ideally be hyperbolic; however, most instrument manufacturers employ four round rods to

approximate the hyperbolic surface for ease of manufacturing. The ion stability of certain m/z value depends on RF/DC voltage applied to the quadrupole, and masses are analyzed via RF/DC scanning. Single quadrupole MS has only one set ("single") of quadrupole (Q1) and lacks the ability to perform tandem mass spectrometry. Triple quadrupole MS comprises three sets ("triple") of quadrupole (Q1, Q2, and Q3) as illustrated in Figure 1(C). In the case of tandem mass spectral analysis using a triple quadrupole mass spectrometer, Q1 serves as the first mass analyzer (Q), Q2 as a collision cell where CID fragmentation occurs (q), and Q3 as the second mass analyzer (Q). Customarily, the triple quadrupole mass spectrometer is denoted as QqQ for this reason. In comparison with the "tandem-in-time" analyzer as discussed above, the triple quadrupole mass spectrometer is "tandem-in-space" and is capable of executing only to the second order (MS^2) of tandem mass spectrometry.

The most frequently used tandem mass spectral modes are product ion scan and single (multiple) reaction monitoring (SRM or MRM). In the product ion scan mode, Q1 is fixed to pass the parent ion only; the collision energy and pressure are programmed for the collision cell (q); and Q3 serves as a mass analyzer scanning to generate the product ion spectrum. Product ion scan is an important tool for structure identification of drug metabolites, degradates, and unknowns. In SRM (MRM) mode, Q1 is again fixed to pass the parent ion only, the collision conditions are programmed for the collision cell (q), and Q3 is fixed to pass only the target product ion. Therefore, no mass spectrum is generated in this mode. If the method is monitoring only one parent/product pair then it is called SRM, whereas if multiple transitions are monitored then it is called MRM. The sensitivity of SRM (MRM) is typically 100 times higher than the scan mode by devoting the instrument time solely on the ion(s) of interest. Therefore, SRM/MRM is dominantly used in quantitative analysis. When LC/MS/MS is utilized in SRM/MRM mode, the method is extremely selective and specific to the analyte of interest. The criteria to identify the analyte are retention time, the parent ion, fragmentation path, and the product ion. Therefore, samples of very complicated mixtures, such as in biological matrices, can be analyzed by the methods with great confidence. In case of single quadrupole MS, Q1 could be fixed to pass an ion of interest only and this mode is named as single ion monitoring (SIM). The degree of specificity of SIM is clearly not as good as that of SRM/MRM, however, the sensitivity achieved is beneficial using a less expensive single quadrupole mass spectrometer.

The other two less frequently used, but very useful, modes are neutral loss scan and precursor ion scan. In neutral loss scan, Q1 and Q3 are pre-fixed to monitor a specific fragment transition (a particular neutral loss from the parent ion). Contrary to product ion scan, in precursor ion scan mode Q3 is fixed for a certain fragment ion and Q1 scans for the parent ion. Both the neutral loss and precursor ion scan modes are valuable when a specific transition is known for the compound of interest in a complicated sample mixture.[11] Recent

developments[5,12] in MS instrumentation utilized the third quadrupole (Q3) as a linear ion trap (LIT), taking advantage of its trapping efficiency and ion storage capacity (see below).

The next type of mass analyzer to be discussed is time-of-flight (TOF) mass spectrometer shown in Figure 1(D). Ions are extracted into the flight tube with a pulse of preset electric potential. The potential energy is converted into kinetic energy as the ions travel through the flight tube. Lighter ions would travel faster arriving at the detector at the end of the flight tube earlier, while heavier ions would travel slower arriving at the detector later. This "time-of-flight" is correlated with the m/z value of the ions and mass spectra are generated. Unlike other mass analyzers discussed so far, this is not a scanning device. Mass spectral acquisition speed does not depend on the scanning speed, and TOF MS is one of the fastest mass analyzers to acquire a single mass spectrum. In addition, a complete mass spectrum can be acquired for each pulse of ions accelerated into the flight tube. Another characteristic of TOF MS is that there is no theoretical limit on the maximum mass that can be analyzed as it measures time-of-flight. A mass range up to 150,000 was reported by Sobott et al.[13] Because of its pulsed nature TOF MS has been mainly coupled with matrix assisted laser desorption/ionization (MALDI), which also utilizes a pulsed laser for ionization. Recent improvement of TOF-MS rendered high resolution capability for accurate mass determination,[5,] and atmospheric pressure ionization (API), such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), has been interfaced with TOF MS.

Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry requires a magnetic field, and an ion trap cell is positioned in the static magnetic field (Fig. 1(E)). The trapped ions oscillate inside the ICR cell due to the magnetic field and the cyclotron frequency is determined by the magnetic field and m/z of the ions. Upon application of excitation RF sweep to the excitation plate, the image current of ion motion on the detector plate is registered. The image current recorded in time domain is then converted to frequency domain via Fourier transformation, and consequently, the frequency of oscillation is correlated to the ion's m/z value. This type of mass spectrometer requires high vacuum to operate and is capable of high resolution mass spectral analysis.[14,15] Since FT-ICR MS calls for a spatially uniform magnet, the cost is relatively high compared to other types of mass spectrometers. As this is a trapping device, FT-ICR MS could also carry out tandem-in-time experiments.

The trend in recent mass spectrometry instrumentation development is hybridization of the conventional mass spectrometers in order to acquire additional or enhanced functionality.[5] Many different kinds of hybridization have been reported in the literature, including quadrupole ion trap(QIT)-TOF,[16] ion guide-QIT,[17] TOF-TOF,[18] QqTOF,[19,20] LIT-FT-ICR,[21] and QqLIT.[12] The combination of QIT and TOF provided advantages such

as, accumulation of ions for enhanced sensitivity and duty cycle; precursor ion selection, and fragmentation by resonance excitation.[16] Voyksner et al. reported the use of an octopole ion guide in front of an QIT for ion storage and mass filtering.[17,22] By preventing unwanted low-mass chemical noise from being introduced into the QIT, improved sensitivity, mass resolution, and dynamic range were achieved. At the same time, the ion guide served as an ion storage device to accomplish nearly 100% duty cycle. Commercially developed reflectron-based QqTOF exhibited high resolution of up to 20,000 FWHM (full width at half maximum), high sensitivity, and good mass accuracy of less than 5 ppm.[19] Medzihradszky et al. reported CID tandem mass spectral data using a commercial TOF-TOF instrument,[18] where the parent ion selection was achieved by timed ion selector. The CID spectra observed showed both PSD (post source decay) and high energy CID fragmentation processes. High energy CID route exhibiting significant side-chain fragmentation was typically characterized by sector type tandem instruments, previously. Linear octopole ion trap was utilized to improve ion extraction efficiency to FT-ICR,[21] resulting in 10 times enhancement in signal-to-noise ratio.

Triple quadrupole linear ion trap (QqLIT) was a latest commercial development, and it retained all the functions of a regular triple quadrupole (QqQ) device, while allowing ion trap mode of operation[12] for the third quadrupole (Q3 in Fig 1(C)). The use of information-dependent data acquisition could increase throughput by performing on-the-fly determination of necessary MS/MS analysis, based on predefined criteria in the same LC/MS run. The major advantages of LIT over QIT were reported to be larger ion storage capacity and higher trapping efficiency.[12]

The goal of modern mass spectrometer instrumentation development is to obtain as much analytical information as possible with minimum time and resources. The progresses have been made in the areas of sensitivity, selectivity, accuracy, speed, and intelligent software, to meet the demand of drug discovery and development processes as discussed in the next sections.

LC/MS INTERFACE

The clash between HPLC and MS is that HPLC is a liquid phase-based methodology, and MS is inherently a gas phase technique. The lack of channels for smooth transition of a sample from liquid phase to gas phase was a huge barrier interfacing LC and MS, which was compared to a marriage between a fish and a bird. The modes of ion generation for mass spectrometry have been reviewed by Vestal[23] and various separation methods coupled with mass spectrometry were reported by Tomer.[24]

Electrospray (ES) has been known for over a century, however, its importance as a sample ionization method for mass spectrometry was

realized[25] only decades ago. With the advent of electrospray ionization, it was no longer required for the polar and thermally labile biomedical samples to be evaporated prior to the ionization event.[26,27] The first step in electrospray ionization is the formation of charged droplets assisted by pneumatic nebulization when LC flow passes through a needle at a high electric potential. As the solvent evaporates away from the droplets, the size of the droplets reduces, resulting in increase of the charge concentration. When the charge concentration reaches the Rayleigh limit where the Coulombic repulsion between charges exceeds the surface tension of the droplet liquid, droplets undergo Coulomb explosion generating smaller droplets.[28] This process repeats multiple times until analyte ions are "evaporated" from the liquid phase into the gas phase (ion evaporation model), or until a single solute molecule is left in the droplet (charged residue model).[29,30] Therefore, the electrospray ionization process transfers solute analyte ions in the liquid phase to gas phase without requiring evaporation of the neutral molecules into the gas phase prior to ionization. Electrospray ionization works well with organic solvents, such as methanol, ethanol, and acetonitrile, which have a low surface tension, whereas the ES sensitivity drops with water, which has a higher surface tension. The multiple charging event of ES process and its application for biological macromolecules were investigated in depth.[25] Fenn received the 2002 Nobel Prize in Chemistry for his work on electrospray ionization for biological molecules.[6]

Another frequently employed atmospheric pressure ionization (API) method for LC/MS interface is atmospheric pressure chemical ionization (APCI).[26,31] In APCI, the compound solutes must be volatilized first before gas-phase ionization occurs. The compounds need to be non-ionic in the solution for easy vaporization and thermally stable to survive the vaporization step. Consequently, the effects of mobile phase and additives are minor in APCI compared to ESI. The primary ionization source in APCI is a corona discharge needle generating a stream of electrons in order to form reactant ions. The most abundant reactant ions in the LC/MS APCI interface are the charged clusters of water and organic mobile phases, such as methanol and acetonitrile in the case of reversed phase LC. Ion-molecule reactions between charged solvent clusters and gas-phase analyte solutes are mainly responsible for analyte ion production through gas phase acid-base chemistry, depending on the proton affinity of species involved.

Thurman et al.[31] summarized their work on LC/MS analysis of various pesticides by ESI and APCI, using an ionization-continuum diagram in order to facilitate the process of choosing the optimum LC/MS interfaces. It was observed that neutral and basic compounds showed better sensitivity on APCI, and cationic and anionic compounds exhibited enhanced sensitivity with ESI. The report provided a detailed description of ionization processes in ESI and APCI, and included topics of sodium adduct formation and

"wrong-way-around" results. Sodium adducts are commonly observed in ESI and are sometimes the most abundant molecular ion species, especially when a carbonyl group is present in the molecule. It is assumed to be due to the fact that the sodium adducts form in the solution and the ESI brings ions in the solution phase to gas phase. On the other hand, APCI interface, where the ionization takes place in the gas phase, does not form sodium adducts because sodium ions cannot be easily vaporized. The term "wrong-way-around" electrospray process refers to the fact that intense $[M + H]^+$ ions were observed from basic solutions and $[M\text{-}H]^-$ ions from acidic solutions. The current hypothesis to explain the phenomena is that the pH at the surface of the micro-droplets is different from the bulk mobile phase pH. This observation makes the mobile phase selection easier for method development.

ESI and APCI were compared for analysis of naturally occurring amino acids and peptide enantiomers by Desai et al.[32] They studied the sensitivity of the UV and MS detection (both ESI and APCI) in terms of flow rate, mobile phase additives, and mobile phase compositions. It was reported that MS showed much lower limits of detection than UV at 210 nm for various neutral, acidic, and basic amino acids. For the peptides tested in this publication, APCI was more sensitive for peptides with molecular weight less than 200 amu, and ESI exhibited better sensitivity for peptides with molecular weight higher than 300 amu. The peptides with 200–300 amu molecular weights showed similar response for ESI and APCI.

APCI is, in general, useful for small, volatile, and thermally stable compounds; whereas, ESI shows high sensitivity for polar, thermally labile, and high molecular weight compounds. It is also demonstrated that because MS provides another dimension of specificity to the chromatographic analysis, it is not necessary to resolve all achiral components as long as they have different molecular weights, effectively reducing the analysis time. In addition, MS detection does not require derivatization of compounds (such as dansyl chloride derivatization) that lack strong chromophores for UV detection, thus simplifying the sample preparation step.

Recently the combination of the two API sources, ESI and APCI, were developed commercially (named ESCi source) and was evaluated by Gallagher et al.[33] The researchers reported that 80% of their compound collection for drug candidates was ionized easily by ESI. For the additional 10% of the samples, the LC mobile phase may need to be modified to promote ion formation, or a different ionization technique could be used. Intermediates of pharmaceutical drug synthesis are usually less polar than the target drug candidates, making the ESI technique less amenable for the LC/MS analysis. The ESCi source combines the regular ESI hardware with the addition of an APCI discharge needle. The high voltage power supply can switch between the electrospray capillary and the APCI discharge needle (polarity switch) within a 100 ms interscan delay time. The qualitative

performance of the ESCi source was comparable to the regular ESI and APCI mode, while reducing method development and sample analysis times.

For efficient ionization of nonpolar compounds, atmospheric pressure photoionization (APPI) has been developed as an alternative ionization source for LC/MS interface.[34,35] Three key steps in photoionization are eluant evaporation, production of photoions via UV source and analytes, and detection (in this case by MS). Evaluation of ionization energies (IE) of common compounds, and the energies of available discharge lamps, suggested that the Kr-filled UV lamp with 10 eV energy is mostly suitable for pharmaceutical applications.[35] With a Kr-filled UV lamp, the photon energy is lower than the major component gases in the air and commonly used solvent, whereas IEs of molecules of interest are below 10 eV. A dopant, a substance that is photo-ionizable and functions as an intermediate between the photons and the analytes via charge exchange or proton transfer mechanism, could be used to significantly increase the number of ions. Applications of APPI for carbamazepine, idoxifene, and steroidal compounds were reported with comparable or better sensitivity than ESI and APCI.[35] Kauppila et al.[36] reported the use of anisole as a new dopant for APPI in order to increase the ionization efficiency of analytes with low proton affinities in reversed phase conditions where the solvents typically have high proton affinities. The performance of APCI and APPI was compared for the detection of ubiquinone-6 and menaquinone-4 by Geyer and colleagues,[37] and they reported that APPI exhibited three times lower LLOD (lower limit of detection) for these compounds. General applicability of the three API methods is represented in Fig. 2, with reference to the size and polarity of the molecules.

Karas and Hillenkamp[38] discovered laser ionization of large bioorganic molecules in 1987 with the assist of matrix compounds. In this ionization mode, the analyte is mixed with a solution of matrix which strongly absorbs

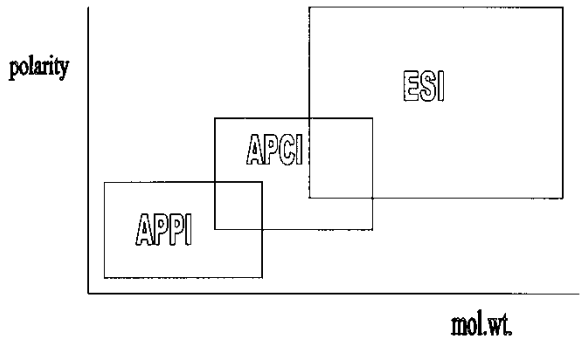

Figure 2. General applicability of the three API techniques with reference to molecular weight and polarity of molecules.

a UV light, and allowed to dry and crystallize. The matrix compound is excited via absorption of a pulsed UV laser and imparts the energy to the analyte, resulting in desorption and ionization,[23,38] called matrix assisted laser desorption/ionization (MALDI). As the MALDI sample preparation involves drying and crystallization, coupling MALDI with the continuous LC flow is not straightforward. MALDI has been primarily used for peptide mixture analysis without LC separation. However, Stapels et al.[39] recently reported the use of an automated robotic device to fractionate the HPLC eluant into discrete spots on a MALDI sample plate. By comparing DNA-binding protein digests directly by LC-ESI-MS and LC-MALDI-MS, they concluded that the two ionization methods provided complementary results regarding peptide identification. On the other hand, Zhang et al.[40] described a new LC-MALDI interface using a transfer tube to form hanging droplets dislodged onto a MALDI plate. LC flow rate up to 200 uL/min was accommodated using this interface, and the sensitivity was in low femtomoles of peptides.

The most widely adopted LC/MS interfaces to date are undoubtedly ESI and APCI. Along with APPI and MALDI as alternative ionization methods for LC/MS, the horizon of possible LC/MS applications is expanding continuously.

SYNTHETIC CHEMISTRY AND OPEN ACCESS LC/MS SYSTEMS

In the drug discovery programs, an enormous numbers of unique novel chemical entities are generated to feed biological high throughput screening. In order to confirm the structures of new synthetic products before submission to the screening, synthetic chemists routinely use NMR systems that are usually available as open-access. On the other hand, unit molecular mass measurement is the most easily interpreted analytical technique that can either support or disprove the successful synthesis of desired structures. Until the arrival of the rugged LC/MS interfaces, mass spectral data acquisition was performed by specially trained mass spectrometrists and the data were reported back to synthetic chemists. The recent development of robust and user-friendly LC/MS interfaces, as well as intelligent software, enabled open access use of MS and LC/MS.[41–43]

In the frame of open access LC/MS, the key parameters to consider for instrument hardware and software are minimum instrument down time, ease of use, robustness, reliability, fast turn around time, and high quality data. Typically, the user prepares the samples in autosampler vials according to the recommended concentration range and solvents, then submits the samples in through open access login software by entering sample ID and target molecular weight using his/her account. The user may choose from

several predefined methods available, including ionization polarity, mass range, varying LC gradient, and analysis time. Upon sample submission the LC/MS system runs the sample based on the user selection and processes the data file to generate LC/MS report. Subsequently, the software either automatically prints the report or e-mails the report to the user. The LC/MS open access software is capable of accepting samples to the queue regardless of the data acquisition status. It has the ability to start up on sample submission and shutdown automatically after a predefined idling period. Coddington et al.[43] discussed critical parameters considered during their open access LC/MS system development and implementation stages, such as column selection, mass spectral setup, software interface, user training, and system maintenance.

Open-access LC/MS instruments in the drug discovery environment providing rapid unit molecular mass information to synthetic chemists,[41–45] became a landmark in the pharmaceutical companies including Boehringer Ingelheim Pharmaceuticals, Inc., where open access (OA) LC/MS systems are available in Medicinal Chemistry and Chemical Development departments. The systems in the Medicinal Chemistry department provide flow injection analysis MS (FIA-MS) and short (4.6 mm i.d. × 30 mm long column) generic 3-minute LC/MS, mostly to provide quick confirmation of the starting material and product of the synthesis step. The mass spectrometers equipped with the open access systems are single quadrupole type, and OpenLynx (Micromass, Manchester, UK) software is used for user sample submission. For medicinal chemists, rapid delivery of new chemical entities is important to expedite lead identification (LI) and lead optimization (LO) processes. In contrast, the chemists in Chemical Development departments are concerned about reaction yield and impurity profiles for reaction optimization. Therefore, the open access LC/MS system in Chemical Development departments is set up with project-specific LC/MS methods, with run time ranging 15 to 40 minutes in addition to generic 5- and 10-minute methods, and employing a column switching valve to accommodate different columns. This system acquires three analytical signals from a single injection, namely UV, MS, and evaporative light scattering detection (ELSD). Figure 3 shows LC/UV/ELSD/MS data for a test mixture obtained, using one of the generic 5-minute methods programmed in the Chemical Development department's OA LC/MS system. A single quadrupole mass spectrometer is utilized for this system with EasyAccess (Agilent, Palo Alto, CA, USA) as sample login software. The OA LC/MS systems in both departments are heavily utilized and the technique is replacing thin layer chromatography (TLC) in synthetic laboratories.

Molecular mass measurement at unit resolution, typically acquired using quadrupole type mass spectrometers, provides useful molecular weight confirmation but gives no information on molecular formula. Accurate mass measurement with sufficient mass accuracy and precision, however, can

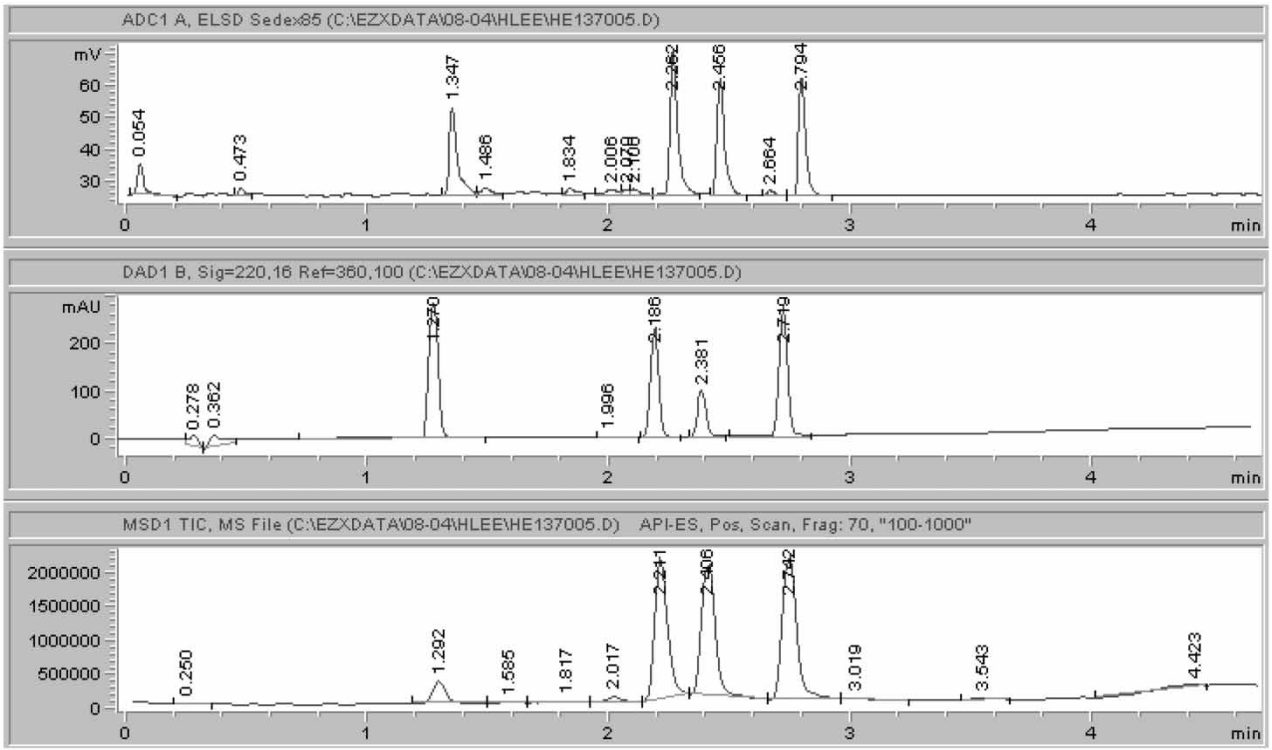

Figure 3. Separation achieved by a generic 5-minute method using an open access LC/UV/ELSD/MS system for a test mixture. The column used was Zorbax XDB-C8 4.6 × 50 mm with 3.5 micron particle size. The test mixture contained caffeine, diltiazem, verapamil, and flavone. The chromatographic traces were acquired by evaporative light scattering detector (ELSD), UV, and MS (TIC) from top to bottom.

offer unequivocal determination of elemental formulas of small molecules. Several groups[44–46] reported either automated or open-access high resolution mass spectrometry in early drug discovery environments. Perkins et al.[45] used a double focusing magnetic sector mass spectrometer with electrospray positive ionization to analyze samples in an unattended batch mode during night time. For mass calibration reference peaks, sodiated polyethylene glycol was used that was present in the mobile phase. The results obtained from the instrument satisfied the 5 ppm error limit in the mass range of 250–800 amu for therapeutic compounds. In recent years, relatively less expensive time-of-flight (TOF) mass spectrometers were introduced in the market that are capable of providing accurate mass measurement. Colombo et al.[46] employed a quadrupole orthogonal acceleration time-of-flight mass (Q-oaTOF) spectrometer to acquire automated accurate mass spectra for medicinal chemistry compounds. An electrospray positive ionization mode was utilized with a single standard reference compound for TOF mass correction via lock spray. Instrument precision, accuracy, and reproducibility were evaluated under the automated conditions. The method was validated with 550 compounds and it provided mass accuracy within 5 ppm for 70% of the compounds tested with automated data processing. Manual data processing of the remaining 30% improved the figure to 91%. While the two groups used automated accurate mass measurement, the samples were prepared, analyzed, and processed primarily by mass spectrometrists. Open access operation of high resolution mass spectrometry for synthetic chemists was reported by Thomas et al.[44] A Q-TOF mass spectrometer with a lock spray probe was operated in FIA-MS and LC/MS modes via OpenLynx software for user sample submission. With routine weekly source cleaning and calibration, accuracy of less than 5 ppm was achieved. The instrument was used by medicinal chemists for product structure confirmation and by-product identification.

Along with open access NMR, open access LC/MS system is becoming ubiquitous in the pharmaceutical industry, and its capability will expand unquestionably as new instrumentation and software develops.

PARALLEL SYNTHESIS AND COMBINATORIAL CHEMISTRY

The advent of combinatorial chemistry of the last two decades changed the drug discovery process in pharmaceutical companies radically.[47] Having the methodology available, the underlying principle of combinatorial chemistry is that screening a compound collection of all possible combinations of diverse components would increase the probability of finding hits and leads proportionally. Mass spectrometry has been applied at different

stages of combinatorial library synthesis,[9] but mainly for library composition assessment.

Using split-and-pool strategy, a large multi-dimensional combinatorial library could be assembled from a small set of reagents. Split-and-pool synthesis of peptides on solid support demonstrated generation of all possible derivatives at each position.[48] This strategy produced hundreds and thousands of analogues while ensuring that each resin bead contained only one chemical component. The peptide sequence of the hit would be characterized ("decoded") after releasing of the peptide from the resin. Biederman et al.[49] described the use of static nanoelectrospray ionization mass spectrometry to elucidate a peptide sequence on a single bead. The bead that had the peptide of interest was placed manually in a nanoelectrospray probe tip, and then the peptide was subjected to cleavage from the bead using ethylamine gas flow. The probe tip held about 10 μL of solution while the typical flow rate obtained with this setup was 100 nL/min. Thus, the analysis time available was about 100 minutes, which was more than enough to acquire data for sequence analysis. The peptide sequence was identified through fragmentation pattern on CID using a triple quadrupole mass spectrometer. Bead-bound split-and-pool polyamine libraries were decoded by HPLC/MS via electrospray ionization by another group.[50] The polystyrene beads were picked up manually under a microscope and subject to TFA cleavage. After TFA evaporation, the sample was dissolved in methanol and injected into an LC/MS system. By partially terminating the polyamine sequence and calculating the mass differences between the truncated oligomers, the polyamine sequence at a single-bead level was identified with a single quadrupole mass spectrometer. Recently characterization of library compounds supported by soluble polymer was performed by LC/MS.[51] As the compounds were synthesized on a soluble polymer, cleavage or a releasing step was not necessary for analysis, enabling automation of the synthesis, analytical control, and biological screening.

The mixture of combinatorial analogues synthesized primarily in the beginning of the combinatorial chemistry era presented a major challenge of identifying the individual compound that showed biological activity in the screening. In addition, the interference from other components and synergistic response hindered straightforward establishment of the structure-activity relationship (SAR). Upon realization of these predicaments, the focus of pharmaceutical industry has shifted from production of library mixtures to parallel synthesis of discrete compounds. Advances in automation of high throughput parallel synthesis accelerated the generation of individual compounds in drug discovery. These compounds should be characterized for identity and purity in order to obtain correct and useful SAR. Due to the inherent disparity between the parallel nature of synthesis and serial nature of analysis, in order to satisfy the increased demand from parallel synthesis, a high throughput analytical method to identify the product and assess its

purity became crucial. The need for high throughput analysis has also emerged in the area of bioanalysis fields for pharmaceutical metabolite and toxicological studies (see bioanalysis section below). The discussion in this section focuses on publications reporting high throughput LC/MS applications for the analysis of combinatorial library and parallel synthesis.

High throughput LC/MS methods have been reviewed in the literature by several authors.[24,52–54] Increase in sample throughput can be achieved either by reducing analysis time or by employing a parallel approach. The goal is to obtain as much analytical information possible in the shortest time period. Samples to be analyzed for combinatorial chemistry and parallel synthesis are typically simple mixtures of a few components, including target product, starting material, and possible by-products. Thus, generic LC methods have been routinely used for compound characterization, instead of spending time to develop a method for each sample. In order to reduce analysis time (speed) without sacrificing separation efficiency (performance) too much, shorter columns with high flow rates were employed.

Dulery et al.[55] reported the analysis of model and production libraries in a lead discovery environment. The model library was analyzed 100% by NMR and LC/UV/MS, using generic reversed phase gradient LC methods with run times ranging 7-12 minutes. MS provided structural identification and UV (diode array detection) supplied purity information. Once the model library was validated, 25% of the production libraries were analyzed by LC/UV/MS and 10–15% of the compounds were analyzed by NMR to serve as a quality control. Use of automation and streamlined data processing also played a major role to accomplish the sample throughput required. Kiplinger et al.[56] performed library characterization and purification, simultaneously, by exploiting mass spectrometry triggered fraction collection. In this report, they used the dual column system to compensate for the column re-equilibration time, where a gradient method was operating on one column, while the other column underwent a regeneration step. The run time of this semi-preparative system was 10 minutes using reversed phase columns with a dimension of 10 mm i.d. $\times$ 50 mm length.

In an effort to further increase the throughput, HPLC gradient methods less than 5 minutes and down to 1 minute were described.[57] A one minute full gradient LC/UV/ELSD/MS analysis to support parallel combinatorial library synthesis was reported in a recent publication.[58] By minimizing the system dead volume and using a high pressure mixing gradient system, the generic reversed phase full gradient method was capable of analyzing library samples in one minute. The performance of the one minute method was compared to existing 2.5 minute and 5 minute methods. Using a short column (4.6 mm i.d. $\times$ 30 mm length) packed with high performance small particles (3.5 μm) with high flow rate (4 mL/min), the observed chromatographic peak width was compressed down to one second. Even though mass spectral data were not used to assess purity, the chromatographic peak had

to be reasonably well represented by a sufficient number of data points across the chromatographic peak for proper data processing. A common quadrupole based mass spectrometer could not provide a scan speed fast enough to acquire necessary data points. A time-of-flight mass spectrometer was employed for this reason. Both UV and evaporative light scattering detection (ELSD) data were used to assess purity of the sample, while mass spectral data provided structural identification. A 400-member library was analyzed less than 8 hours with this method. The data processing and interpretation could easily be the next bottleneck upon improved throughput capability of instrumentation. OpenLynx software was used for automated data processing and reporting. Gerber et al.[59] presented the use of monolithic columns for a fast LC method in a current good manufacturing practice (cGMP) environment, and compared the performance to columns packed with 3 μm particles. Monolithic columns are made of a single piece of porous silica, which has both macropores and mesopores. Owing to its porous structure, the monolithic columns allow much higher flow rates without the back-pressure compared to packed columns. It was reported that by using monolithic columns, the analysis time could be reduced by a factor of up to 6.

Another scheme to amplify analytical throughput is a parallel approach. Zeng et al.[60] described an automated parallel HPLC/MS with two columns operated in parallel for both analytical and preparative modes, effectively doubling the sample throughput with a run time of 4.2 minutes. The ionization source of the mass spectrometer was modified to accommodate two column effluents simultaneously, and it was named a "dual sprayer." They demonstrated that 200 compounds were characterized per instrument per day, and 200 compounds were purified per instrument per night with this parallel system.

Because the cost of mass spectrometer is relatively high compared to that of LC instruments, multiple LC systems or columns were coupled with one mass spectrometer in order to maximize the use of mass spectrometer time via commercially available instrumentation.[61,62] The MUX electrospray interface enables introduction of 4 or 8 separate column flows into a time-of-flight mass spectrometer through a rotating flow selection device. The mass spectrometer registers one column flow at a time and proceeds to the next column flow. The columns involved are essentially connected to the mass spectrometer on a time-sharing basis. As the mass spectrometer must be able to handle all 4 or 8 column effluents without compromising performance, it should have a very high data acquisition speed. Therefore, time-of-flight device is a natural choice for this interface. Eldridge et al.[61] reported high throughput analysis and purification of natural product libraries using 4-channel and 8-channel MUX systems. In this case, the library was produced by fractionation of Taxus brevifolia extract, instead of preparing highly pure discrete compounds. The fractions were purified in a manner so that each well contained one to five compounds

using a 4-channel preparative HPLC. This library, then was analyzed using parallel 8-chanel LC/ELSD/MS and screened against cancer cell lines. The constituents of the active library fraction were purified and characterized by LC/ELSD/MS and NMR. The system throughput was 10 minutes per run (8 samples/run) and one 96-well plates per every 2.5 hours. ELSD signal was incorporated to determine quantity of compounds.

Lee et al.[62] described the analysis of parallel library synthesized using a split-and-pool Irori MicroKan method, where 4-channel MUX interface was used to accomplish high throughput. By integrating fast separation and parallel approach, the throughput of 1600 samples/day (over sixteen 96-well plates/day) was demonstrated by LC/UV/ELSD/MS. The system was composed of a single HPLC system, four columns, four UV detectors, four ELSDs, and a time-of-flight MS. The flow from the HPLC system was evenly split into four channels and the injection-to-injection run time was 3.5 minutes. Several ways of visualizing the analytical data were also discussed regarding target compound identification and purity measurement.

Other detection methods employed in conjunction with LC/MS, including ELSD and chemiluminescence nitrogen detection (CLND), have been reported.[63,64] ELSD on-line with LC/MS has been used for purity assessment and quantitative analysis of compounds, in addition to low wavelength UV detection as discussed above. As the column effluent enters into ELSD, the liquid stream is forced to form spraying mist with help of a nebulizing gas (typically nitrogen). Subsequently, the small particles travel down through the drift tube under the nebulizing gas at a controlled temperature, in order to promote evaporation of solvents. At the end of the drift tube, the sample stream passes through a path of a light source and a photomultiplier tube. If the LC effluent only contained solvents and the solvents were evaporated completely in the drift tube, then there would not be any scattering event in the detector. However, if the LC flow had a compound that remained as particles through the drift tube, then light would be scattered by these particles and the scattering would be registered by the photomultiplier tube. If the compound is volatile under the experimental conditions it would not be detected by ELSD. While the UV signal response depends on the extinction coefficient of the chromophore, ELSD is generally contingent on the boiling point of the compound. One point to keep in mind is that ELSD is a non-linear detector, in contrast to the linearity of UV signal on sample concentration according to the Beer's law.

Chemiluminescence nitrogen detection (CLND) has been introduced as not only an alternative detector for compounds with no chromophore but also a means to perform quantitative study without an established standard or a calibration curve. Nitrogen containing compounds introduced to a CLND are converted to nitric oxide through pyrolysis in the presence of oxygen. As the excited species of nitric oxide relaxes down to the ground state, the process emits light which is detected by a photomultiplier. An

obvious limitation is that the solvent should not contain nitrogen excluding acetonitrile as the HPLC mobile phase. The CLND signal is proportional to the number of nitrogens in the compound, therefore, quantification of the compound is possible provided that the number of nitrogens in the chemical structure is known.[64–66] A calibration curve specific to the compound of interest is not required under this experimental approach. For example, a library sample with an impurity can be quantified not only for the target product, but also for the impurity, as long as it is known how many nitrogens are present in the impurity. It is usually possible to deduce the number of nitrogens in the impurity from mass spectral data. Thus, on-line CLND detection with LC/MS could be used for quantitative analysis of novel chemical entities where purified standards are not available.

Taylor et al.[64] evaluated CLND as a universal quantitation technique for nitrogen-containing combinatorial library compounds. Methanol, isopropanol, and water were employed as nitrogen-free mobile phases with trifluoroacetic acid as a modifier. The CLND was shown to be compatible with gradient elution reversed phase HPLC, linear for more than two orders of magnitude, and have detection limits down to 12 picomoles of nitrogen. The response from CLND was equivalent for diverse sets of calibration compounds when normalized for the nitrogen content. Another feature of CLND response was that it only depended on the total mass injected regardless of flow rate or sample concentration unlike UV detection. By employing a generic LC/UV/MS/CLND method, information on the identity, quantity, and purity of the compounds were obtained from a single HPLC injection. The combined methodology was utilized to quantify impurities and metabolites related to the target product without the need for purification, facilitating generation of pharmacological potency values and structure-activity relationships. This proved to be very useful when the samples to be analyzed are available in a limited quantity or impure, which is typically true in the drug discovery environment.

The ease of automation, speed of analysis, sensitivity, and ease of use of the LC/MS technique made it an ideal detection system to handle enormous numbers of samples for high throughput analysis, necessitated by parallel synthesis and high throughput screening.

ADME/DMPK SUPPORT AND BIOANALYSIS

The observation that up to 40% of new chemical entities failed to commercialize due to unfavorable pharmacokinetic properties, has changed the role of drug metabolism pharmacokinetic (DMPK) activities from a study limited to a late development stage, to assessment in early discovery screening.[67,68] The pharmacokinetic evaluation in the late development stage deals with a small set of compounds though the number of samples

from clinical study may be huge; in contrast pharmacokinetic screening in the drug discovery process has to evaluate a lot of compounds with diverse physicochemical properties. To successfully develop an orally administered drug, the drug candidate has to be absorbed through the gastrointestinal (GI) tract, endure the first pass metabolism in the liver, and sustain enough systemic concentration to exhibit efficacy with an acceptable clearance. In vivo study of pharmacokinetic (PK) profile involves all of the absorption, distribution, metabolism, and excretion (ADME) aspects; however, it is time-consuming, labor-intensive, and not amenable to high throughput automation. In this section the use of LC/MS in support of ADME activities, of which drug metabolism and pharmacokinetics (DMPK) plays a key role, is discussed employing a range of liquid chromatographic and mass spectrometric methodologies.[8,53,65–67,69–71]

In order to test intestinal permeability of drug candidates, the Caco-2 in vitro model has been widely used in pharmaceutical development assessing absorption attributes of compounds in the GI tract. A major concern of the Caco-2 model is the reproducibility and control of cell culture maturation. Larger et al.[72] reported the use of LC/MS/MS to characterize the transport activity in the Caco-2 model with reference compounds. The apparent permeability coefficients (P_{app}) were measured for absorption (apical to basolateral direction) and for secretion (basolateral to apical direction) transport. Compared to alternative detection methods employing fluorescent or radioactive chemicals, the LC/MS method is general, so that it is not necessary to have compound-specific methods. Also, LC/MS is highly amenable to automation to achieve high throughput capacity in the characterization of drug absorption properties.

Tandem mass spectrometric techniques such as constant neutral loss scan, parent ion scan, and product ion spectra are very powerful in detecting drugs and metabolites in biological matrices. However, these scanning techniques alone cannot uncover all metabolites present in the samples due to difficulties in transformation prediction. Biotransformation screening of drug candidates was reported[68] using LC/MS and product ion spectra by computer-based cross-correlation of the data, in order to identify drug metabolites formed in vivo or in vitro. After incubation in human liver microsomes (HLM) of drug candidates, the scanning LC/MS data of the sample were searched for molecular ions, and product ion spectra were acquired for the possible metabolites. In order to assign metabolites to the parent drug, the product ion spectra were analyzed by correlation on the basis that the more similar two chemical structures are, the more similar their product ion spectra should be. In addition, a directed mass search was performed for possible phase I metabolites (+14, +16, +18, +30, −2, −14, −18 Da). The chromatographic column and mobile phase conditions were "generic" in order to minimize the method development time. This computer-based approach significantly increased the throughput of metabolic transformation elucidation for drug candidates.

Samuel and colleagues investigated the metabolic activation potential of drug candidates[73] in the drug discovery stage. A detoxification process of drugs in the body, could convert the compounds to reactive electrophilic metabolites that potentially bind to and irreversibly modify nucleophilic macromolecules, such as proteins and DNA, causing idiosyncratic drug reactions. In the report, the reactive intermediates formed during liver microsomal incubation of compounds were trapped as glutathione conjugates, and these thiol adducts were analyzed by MS^n using ion trap mass spectrometry. Based on the tandem mass spectrometric data, the potential sites of bioactivation were identified and the structures of the drug candidates were iteratively modified to block the bioactivation process. The progress of the structural modification to reduce metabolic activation was checked by measuring irreversible binding of tritium labeled analogs to liver microsomal proteins. Intervention of metabolic activation in the early drug discovery stage could reduce costly failures of drug development in later stages.

Hopfgartner et al.[69] evaluated the use of the quadrupole linear ion trap mass spectrometer (Q-LIT) technology for high throughput screening and characterization of drug metabolites. The product ion spectra obtained from Q-LIT MS were similar to that of triple quadrupole instruments, while the sensitivity was much higher due to the trapping capability. Additionally, the system did not suffer low-mass cutoff of a 3D ion trap device. Constant neutral loss scan, combined with enhanced product ion spectra acquisition, was employed effectively for screening of phase II metabolites such as glucuronides.

Quantitative and semi-quantitative information on metabolites in the early drug discovery stage is important to identify potential development liabilities; however, pure metabolite standards are often unavailable at the early stage of discovery. The use of CLND to quantify drug metabolites in the absence of pure metabolite standards was reported by Deng et al.[65] Because CLND provides equimolar responses for nitrogen containing compounds, the LC/MS/MS response factor was "calibrated" against the CLND signal intensity for the pair of parent drugs and the metabolite. The quantification of metabolite was performed using the calibration curve of the parent drug based on the relative LC/MS/MS response ratio of parent compound and the metabolite, avoiding the direct use of CLND for trace level analysis as CLND is less sensitive than MS in general. Use of an accurate radioisotope counting device, another quantitative technique, on-line with LC/MS was reported by Nassar et al.[74] for metabolite identification, exhibiting sensitivity down to 6 cpm. Radioactive labeling allows quantitative detection of unknown metabolites and biological tracing.

The summary of experimental procedures of selected publications reporting bioanalysis in support of ADME is shown in Table 1. The majority of quantitative analyses for metabolism study were performed using a triple quadrupole mass spectrometer in the MRM mode. The analyte

Table 1. Summary of experimental procedure of LC/MS applications on bioanalysis in support of ADME

Ref. no.	Activity	Analyte of interest	Sample prep	LC	MS ionization	MS instruments	Comment
[69]	Metabolite screening and characterization	Drugs in urine, hepatocytes; plasma	Direct injection; dilution; protein precipitation;	C18, 2.1 × 100 mm, 5 μm particle, 250 μL/min	ESI	Information-dependent data acquisition (IDA); QqQ, QIT, QqTOF	Q-linear ion trap
[80]	Genetic method using turbulent flow columns	Drugs in plasma, urine, brain homogenate, liver homogenate, intestinal perfusate, cerebral spinal fluid	Acetonitrile protein precipitation and centrifugation	Clean up, Cyclone HTLC; analytical, C18, 4.6 mm × 15 mm, 3 μm particle, 5 min gradient	ESI	Triple quadrupole, MRM	High throughput
[81]	Rapid sample turn around time using robotic sequential injection	Idoxifen and pyrrolidinone metabolites in human plasma	96-Well plate LLE, acetonitrile protein precipitation and reconstitution	C18, 1 mm × 30 mm, 3 μm particle, 70 C temp, isocratic, 23 sec cycle time	ESI	Triple quadrupole, SRM	Validation for human clinical trial samples, high throughput
[82]	Enantioselective LC/MS/MS	Chiral phenprocoumon in human plasma	SPE	Chiral-Grom-2, 1 mm × 250 mm, isocratic, 13 min run time	ESI	Triple quadrupole, MRM	Validation
[83]	Polarity switching LC/MS/MS	Abacavir in plasma, amniotic fluid, fetal and placental tissues	Homogenization, acetonitrile protein precipitation and dilution	C8, 2.1 mm × 150 mm, 5 μm particle, 15 min gradient	ESI	Triple quadrupole, MRM	Validation, polarity switching between internal standard and analyte
[68]	Biotransformation screening of drug candidates	Drug candidates in human liver microsomes (HLM)	Acetonitrile protein precipitation and reconstitution	C8, 2.1 mm × 150 mm, 20 min gradient	ESI	Triple quadrupole, MS/MS	Production spectra generated for candidate metabolites

[79]	High throughput parallel on-line extraction LC/MS/MS	Drug candidate in human serum	Acetonitrile protein precipitation	On-line extraction, turbulent flow C18, 1 × 50 mm, 50 μm particle; analytical C18, 2 × 30 mm, 2.5 μm particle; 0.8 min cycle time	ESI	Triple quadrupole, SRM	GLP validation for human clinical trial
[84]	Identification of metabolites	Ethoxidine in rat liver microsomes and CYP cell culture	Acetonitrile/methanol protein precipitation and reconstitution	C18, 1 mm × 150 mm, isocractic, 20 min run time	ESI	Triple quadrupole, TOF	Production spectra, accurate mass measurement
[85]	Ultra-performance LC/MS	Midazolam in bile	Dilution	C18, 2.1 mm × 100 mm, 1.7 μm particle, 6 min or 30 min gradient	ESI	Q-TOF	Increase in chromatographic peak resolution; accurate mass measurement
[86]	Trace level determination	Gemcitabine and metabolite in human urine	SPE and reconstitution	C8, 4.6 mm × 150 mm, 5 μm particle, 10 min run time	ESI	Triple quadrupole, MRM	Validation
[87]	Urinary excretion determination	MK-0767 and 7 metabolites in rat urine	Dilution and centrifugation	C8, 2 mm × 50 mm, 5 μm particle, 5 min run time	ESI (APCI)	Triple quadrupole, MRM	Validation
[73]	Assessment of metabolic activation potential	Drug candidates in liver microsomes	Acetonitrile protein precipitation and concentration	RP, 4.6 mm × 250 mm, 4 μm particle, 60 min run time	ESI	Ion trap, data-dependent MS/MS	Iterative structure modification to minimize bioactivation, tritium-labeled analogs to check irreversible binding to proteins

(*continued*)

Table 1. Continued

Ref. no.	Activity	Analyte of interest	Sample prep	LC	MS ionization	MS instruments	Comment
[88]	Screening, identification and quantification	Benzodiazepines in plasma	LLE and reconstitution	RP, 2 mm × 125 mm, 10 min run time	APCI	Single quadrupole, SIM	Validation
[89]	Characterization of metabolites	Pyronaridine in human liver microsomes, urine and feces	Acetonitrile protein precipitation, acetonitrile extraction	C18, 2 mm × 150 mm, 5 μm particle, 23 min gradient	ESI	Ion trap, MS/MS	In vivo and in vitro metabolic profiling
[74]	Metabolite Identification	Propranolol metabolites in liver microsomes	Acetonitrile protein precipitation	RP, 2 mm × 150 mm, 4 μm particle, 30 min gradient	ESI	Ion trap, MS/MS	Use of LC-accurate radioisotope counting (ARC)
[65]	Quantitation of drug metabolites	Oxazepam and temazepam in urine	Methanol extraction	RP, 2 mm × 100 mm, 40 min gradient	ESI	Triple quadrupole, MRM	Use of CLND for quantitation without standards
[72]	Characterization of Caco-2 absorption properties	Phenylalanine, atenolol, propranolol	None	RP, 2.1 mm × 50 mm, 5 μm particle, 4 min run time	APCI	Ion trap, MS/MS	Production spectra for quantitation
[90]	Identification and quantification of metabolites	Test compounds in rat plasma	LLE and reconstitution	C18, 2 mm × 50 mm, 3 μm partice, isocratic	ESI	TOF, triple quadrupole, MS/MS	Accurate mass measuremnt

[76]	Four column parallel LC/MS	Test compounds in human plasma	SPE and reconstitution	C18, 2.1 mm × 50 mm, 4 μm particle	ESI	Triple quadrupole	Isocratic or gradient
[75]	Cytochrome P450 inhibition assay	Test compounds in human liver microsomes	Acetonitrile protein precipitation	Monolithic C18, 4.6 mm × 50 mm, 0.4 min gradient	ESI	Triple quadrupole, MRM	Ultrafast P450 inhibition screening
[91]	Determination of drugs of abuse	Amphetamine, methamphetamine, and derivatives in meconium	Extraction and SPE	C18, 2.1 mm × 150 mm, 5 μm particle, 30 min run time	ESI	Single quadrupole, SIM	Validation
[92]	Determination of illicit drugs	Methadone and metabolites in oral fluid	Acetonitrile protein precipitation and reconstitution	RP, 2.0 mm × 150 mm, 4 μm particle, 24 min run time	APCI	Ion trap	Validation
[93]	Quantification of drug	Capecitabine and metabolite in human plasma	Protein precipitation and reconstitution	Hypercarb, 2.1 mm × 150 mm, 5 μm particle; C18, 2.1 mm × 50 mm, 3.5 μm particle; 8 min rune time	ESI	Triple quadrupole, MRM	Column switching, validation
[94]	Quantification of glucuronide metabolites	Dexmedetomidine and glucuronide metabolites in human plasma	SPE and reconstitution	C18, 2.0 × mm × 150 mm, 5 μm particle, isocratic, 6 min run time	ESI	Triple quadrupole MRM	Validation

of interest is determined and measured under this condition by the HPLC retention time, the molecular ion, and the transition from the parent ion to the product ion, making this method extremely specific. High throughput capability[75–78] is achieved by generic (universal) LC and MS methods and simple or no sample preparation, which again is possible by specificity of the LC/MS/MS detection. Turbulent flow chromatography,[79,80] monolithic columns,[75] and column switching/parallel column approach[76] have been employed to further increase sample throughput. Quantitative LC/MS and LC/MS/MS methodology is routinely validated to support GLP and GMP projects regarding linearity, dynamic range, accuracy, intra- and inter-batch precision, recovery, and stability. As reported by many researchers in their published works, LC/MS and LC/MS/MS has become the primary analytical technique in the field of ADME and DMPK for analysis of drug compounds in biological matrices.

PROTEOMICS AND BIOLOGICAL SCREENING

The genomics revolution has increased the number of targets available to the drug industry dramatically and will continue to do so in the future. Every potential protein expressed can be deciphered from the genome sequence databases. Most pharmaceutical and biotechnology companies have been working on a narrow set of "drug-friendly" targets including proteases, kinases, transmembrane proteins, and nuclear hormone receptors.[95] Enormous effort is being made to identify novel targets via the genomics-based approach in order to expand their drug discovery pipelines. It is advantageous to acquire as much information as possible on the biological targets for the benefit of the target discovery, target validation, lead identification, and lead optimization processes. One area where mass spectrometry has developed into an indispensable essential tool is proteomics,[1,7,11,96–99] in the era of genomics-based drug discovery. Proteomics is a multifaceted and collective research field for the study of identification, characterization, and quantitation of proteins in a cell or tissue. The characteristics that make mass spectrometry such an attractive tool for proteomics include ease of use, sensitivity, speed, and rich information contents.

Mass spectrometry with ionization techniques such as ESI and MALDI has made a remarkable contribution to the determination of primary structures of proteins and peptides. There are two major MS-based approaches to identify proteins, namely peptide mass mapping and sequencing via tandem mass spectrometry. In peptide mass mapping, the protein is subjected to sequence-specific proteolysis followed by mass spectral determination of resultant peptides. For example, an enzyme trypsin cleaves proteins at arginine or lysine residues on its carboxyl terminus side producing tryptic peptides. A protein with a particular amino acid sequence has a unique

specific peptide mass fingerprint after trypsin-proteolysis. By comparing the mass spectral data of the trypsin-digested sample and known proteins in a sequence database with in-silico digestion, it is possible to identify the protein. DNA sequence databases could be used for this exercise as well, upon translation of the DNA sequences into protein sequences. This approach of proteomics using protein digestion is termed as "bottom-up." On the other hand, the second approach utilizes tandem mass spectrometry to characterize the sequence of peptides. Peptide fragmentation through collision induced dissociation (CID) has been extensively studied and well characterized along the peptide backbone for the 20 naturally occurring amino acids, and standard nomenclature was established to denote the MS fragmentation. After proteolysis by a specific protease, the peptide mixture is typically separated by capillary electrophoresis (CE) or HPLC then characterized by MS. Sequential MS/MS analysis of observed peptide molecular ions is performed and fragment ion spectra are generated. Full or partial sequencing of the peptide can be obtained from the fragment ion spectra, and the sequence is searched against a database to identify the protein. Apparently, if the protein of interest is unknown and not present in the database, de-novo sequencing becomes necessary. The strategy of sequencing intact whole protein ions by tandem mass spectrometry is termed as "top-down" approach.

Mass spectrometry in conjunction with two-dimensional polyacrylamide gel electrophoresis (2D PAGE) was widely employed to profile protein expression in disease states.[100] In this approach, the solubilized protein contents of entire cells or tissues were separated by 2D gels and visualized by silver staining, followed by mass spectral analysis. By comparing the protein expression of the normal and disease states (such as tumour tissues), disease markers could be identified. The expression proteomics could also provide the basis of understanding functional significance of the proteome levels and post-translational modification of the proteins, so termed "functional proteomics." The traditional proteomic analysis of gel electrophoresis followed by mass spectrometry is tedious, time-consuming, and not easily amenable to automation. An alternative to 2D SDS-PAGE with the mass spectrometry method is a shotgun approach. Instead of fractionating the proteins on gels prior to mass spectral analysis, in a shotgun approach[101] proteins are first digested to produce complex peptide mixtures, and the mixtures are analyzed by LC/MS. Wu and coworkers[101] reported the use of the shotgun methodology combined with multidimensional protein identification technology (MudPIT), where a desalting column and triphasic column packed with materials of C_{18}, strong cation exchanger, and hydrophilic interaction were employed. The peptides eluted from the column were directly analyzed by an on-line ion trap mass spectrometer equipped with electrospray ionization interface for identification. Ion trap mass spectrometers used in proteomic analysis were typically operated in a data-dependent MS/MS

mode. This signifies that tandem mass spectral analysis is performed on the fly during the data acquisition for the ions that are observed in the previous scan based on user-determined criteria, for example, the three most intensive ions, or preset list of ions, effectively increasing information content of the data.

For quantitative analysis of peptides to study protein expression levels in specific cell organelles or at a different physiological or pathological state, stable isotope dilution technique has been used together with LC/MS/MS.[11,102] Two chemically identical species with different stable isotopes can be resolved by mass spectrometry due to the mass difference. Provided that the compounds with stable isotopes are treated equally during biological and chemical reactions, the ratio of the isotope peaks in MS data represents the relative abundances of the two analytes. This principle has been utilized to differentiate b- and y-series ions for peptide sequencing, using ordinary and heavy water with ^{18}O in the tryptic digest step.[102] The C-terminus of the tryptic peptides would incorporate either ^{16}O or ^{18}O depending on the medium used during the digestion step. Therefore, by utilizing a 50 : 50 mixture of $H_2^{16}O$ and $H_2^{18}O$, y-series ions that bears C-terminus would show up as doublets in the mass spectrometric data, on the other hand b-series ions that bear N-terminus would appear as a single peak, facilitating easy identification of the fragment directionality. John et al.[103] reviewed quantification of peptides in pharmaceutical research by LC/MS including discussions on sample preparation, chromatographic separation, and mass spectrometric detection.

Based on stable isotope dilution quantification and biotin affinity tag, Gygi and coworkers[104] devised isotope-coded affinity tags (ICAT) for quantitative proteomic analysis. The novel class of reagents, ICAT, consists of three functional elements: a thiol specific reactive group, an isotopically coded linker, and a biotin affinity tag. The light linker contains only ^1H (no deuterium, d0), whereas the heavy isotopically-coded linker has eight deuterium (d8). Via the reaction of the thiol specific group with cysteinyl residues in a reduced protein sample in one cell state, the sample is coded with d0 reagent. On the other hand, a protein sample representing a second cell state (e.g. disease state) is derivatized with the heavy d8 linker. These two derivatized samples are combined and digested enzymatically to generate peptide fragments, some of these which contain cysteine are tagged with ICAT reagents. The tagged peptide fragments are isolated by avidin affinity chromatography and analyzed by LC/MS using data-dependent MS/MS. A pair of d0/d8 ICAT-labeled peptides are chemically identical, thus the chemical reaction and biological digestion would happen in the same manner, making them ideal internal standards for each other to achieve accurate relative quantitation. Quantitative information of the peptides is obtained by measuring the relative signal intensities of d0/d8 derivatized forms in the full scan MS mode, while the sequence information is obtained from the data in the MS/MS mode. The ratio of d0/d8 reagents exhibited expected quantitative ratios for standard protein mixtures, and this

strategy was applied to quantitatively analyze protein expression levels in different cell states. Simultaneously, the peptide MS/MS data acquired was searched against a protein database automatically, in order to identify proteins. Thus, the ICAT approach demonstrated that it could accomplish protein identification and quantification in a single LC/MS experiment. The proteomic analysis furthers understanding of protein functions and interactions, possibly providing new biological targets for drug discovery.

Another biological area where LC/MS has been applied is high throughput screening. Biological high throughput screenings for discovery of pharmacologically active compounds have been performed[105,106] by using LC/MS. Yan et al.[105] reported the use of LC/MS/MS for monoamine oxidase (MAO) inhibition assay. A non-selective substrate, kynuramine, was used to assess MAO activity and the appearance of the substrate conversion product, 4-hydroxyquinoline (4-HQ), was monitored by LC/MS/MS in an MRM mode. When MAO inhibitors are incubated with MAO, the conversion of kynuramine to 4-HQ is hindered and, therefore, the signal intensity of the MRM peak would decrease. This method was validated using known inhibitors by comparing IC_{50} values. Experiment run time per sample was 2 minutes employing isocratic HPLC conditions, owing to the fact that less efficient separation is tolerated because of the MS specificity. The method was sensitive, high throughput, and did not require handling of radioactive material compared to other alternatives. In addition, the determination of reversible and irreversible inhibition was demonstrated using this method, which is important in drug discovery since irreversible inhibitors are less favorable as drug candidates due to safety concerns.

Boer et al.[106] reported screening of complex natural product mixtures using on-line LC/MS to identify bioactive compounds. In this scheme, the sample mixture was introduced to an HPLC column for separation, and the partially resolved analytes were subjected to incubation with an enzyme of interest in an on-line reaction coil. A known substrate of the enzyme was then mixed with the incubation mixture in a second reaction coil. If no bioactive compounds were eluting from the column, then the enzyme converted the substrate into products that were continuously monitored by MS. However, bioactive compounds bound to the enzyme caused a decrease in the product turnover. By monitoring the turnover product signal by MS, the retention time and peak shape of the bioactive compounds were characterized. Further, based on the retention time match and peak shape comparison, the molecular ions of the bioactive compounds were identified from a complicated natural product mixture. Because of the MS selectivity it was not necessary to have base-line resolution in the HPLC separation of the mixture. In addition, MS-based detection did not require synthetic sample preparation in comparison with fluorescence or colorimetric-based detection.

A summary of experimental procedures in terms of analytes, sample preparation protocols, LC methods, and MS conditions used is provided in Table 2,

Table 2. Summary of experimental procedures of LC/MS applications on proteomics and biological screening

Ref. no.	Activity	Analyte of interest	Sample prep	LC	MS ionization	MS instruments	Comment
[39]	Compare ESI and MALDI	DNA-binding proteins of E. coli	Trypsin digest	C18, 5 μm particle, 75 μm × 150 mm, 300 nL/min, 80 min gradient	ESE, MALDI	ESI Q-TOF; MALDI TOF-TOF	ESI for hydrophobic peptides; MALDI for basic and aromatic peptides; MASCOT search
[2]	Protein characterization	SARS virus	SDS-PAGE, proteolytic digestion, HPLC fractionation	C18, 5 μm particle, 150 μm × 150 mm, 4 μL/min flow rate, 60 min gradient	MALDI	QqTOF	De-novo sequencing
[96]	LC evaluation	Mouse cortical neuron proteome	Lysis, trypsin digest, desalting using Oasis MCX extraction cartridge	C18, 5 μm particle, 75 μm × 300 mm, 75 μm × 100 mm, 50 μm × 100 mm, 500 nL/min flow rate, 180 min gradient	ESI	Ion trap, data-dependent MS/MS	100 unique peptides identified from 5 ng of protein digest, SEQUEST search
[102]	Stop and Go extraction (Stage)	BSA	Tryptic digest	C18, 3 μm particle, 0.4 mm × 0.5 mm (L)	ESI, MALDI	TOF	New procedure for self-packed micro-columns in pipet tips
[107]	Peptide identification algorithm modification	Human embryonic kidney, microtubule-associated protein (MAP), rat hippocampus	Trypsin, EndoK-C, proteinase K digests	C18, 100 μm × 100 mm, SCX	ESI	Ion trap, data-dependent MS/MS	SEQUEST search

[108]	preparation of integral plasma membrane proteins	Integral plasma membrane proteins	Biotin-directed affinity purification (BDAP); SDS-gel; trypsin digest	C18, 75 μm × 50 mm, 5 μm particle, 30 min gradient	ESI	Ion trap, data-dependent MS/MS	MASCOT search
[101]	Proteomic analysis of membrane proteins	Brain and liver homogenate	High pH; proteinase K (hpPK)	Triphasic column; C18, 100 μm × 70 mm, 5 μm particle; SCX 100 μm × 30 mm, 5 μm particle; hydrophilic interaction 100 μm × 30 mm, 5 μm particle; 350 min gradient	ESI	Ion trap, data-dependent MS/MS	Shotgun approach; MudPIT analysis; SEQUEST search
[104]	Quantitative analysis of protein mixtures	Yeast metabolic protein under glucose-repressed conditions	Isotope-coded affinity tag (ICAT)	Spherical silica, 100 m × 120 mm	ESI	Ion trap, data-dependent MS/MS	ICAT reagent; SEQUEST search
[109]	Ultrasensitive nanoscale proteomic analysis	BSA tryptic digest	On-line microSPE precolumn	C18, 15 μm × 850 mm, 3 μm particle, ca. 20 nL/min, 180 min gradient	Nano ESI	FT-ICR, ion trap MS/MS	Protein identification from 0.5 pg of whole proteome extracts
[110]	Identification of an a priori unknown modified protein	Whole cell lysate fraction of S. cerevisiae	Centrifugation	Fractionation by reversed phase LC, 2.1 mm × 100 mm, 10 μm particle, 12 min gradient	Nano ESI	Ion trap	Top down approach; identification and characterization of an unknown protein as large as 11.6 kDa

(*continued*)

Table 2. Continued

Ref. no.	Activity	Analyte of interest	Sample prep	LC	MS ionization	MS instruments	Comment
[111]	Strong cation exchange (SCX) coupled with RPLC for high dynamic range	Human plasma proteome	Dilution and trypsin digest	Micro SPE C18, 75 μm × 40 mm, 5 μm particle; nano LC C18, 30 μm × 850 mm, 3 μm particle; 300 min gradient; SCX polysulfoethyl aspartamide-bonded silica, 320 μm × 800 mm, 3 μm particle, 200 min gradient	ESI	Ion trap, data-dependent MS/MS	2D LC separation of SCX adn RP, dynamic range of 8 orders of magnitude in protein relative abundance
[112]	Post-translational modification (PTM) detection and localization	S. cerevisiae cell lysate	PAGE fractionation	C4, 4.6 mm × 50 mm; C18 4.6 × 14 mm, 20 min gradient	Nano ESI infrared multi-photon dissociation (IRMPD)	Q-FTMS, Linear ion trap FT-ICR	Top down proteomics (fragmentation of intact protein ions)
[113]	Absolute quantification of membrane protein	G Protein-Coupled Receptor (GPCR) rhodopsin	Trypsin digest	C18, 1.0 × mm × 50 mm, 5 μm particle; 17 min gradient	ESI	Triple quadrupole MS; SRM	Use of synthetic isotopically labeled peptide as true quantitative standard
[114]	Peptide sequencing and quantification	Trypsin digest of proteins	Synthesis of stable isotope label and guanidination of lysine residue	C18, 0.32 mm × 10 mm, 50 min gradient	MALDI	TOF, ion trap	Stable-isotope based MS quantification; automated MALDI spotting system

[115]	On-line capillary isoelectric focusing (CIEF) with RP LC/MS to compare with 2D-PAGE analysis	Protein mixtures		Capillary isoelectric focusing; trap column C18, 300 μm × 5 mm; analytical column C4, 300 μm × 50 mm	ESI	Q-TOF	Remove ampholyte prior to MS analysis to prevent ion suppression
[106]	Continuous-flow enzyme assay	Red clover extract, fungi extract	Extraction	C18, 2.1 mm × 100 mm, 5 μm particle, continuous flow isocractic and gradient	ESI	Single quadrupole MS; full scan and SIM	Binding of enzyme and substrate in reaction coils
[105]	Monoamine oxidase (MAO) inhibition assay	Known MAO inhibitors to validate method	Dilution	CN, 2.1 mm × 50 mm, 5 μm particle, 2 min isocratic	ESI	Triple quadrupole MS; MRM	No sample preparation, high sensitivity and specificity, high throughput, low consumable cost
[116]	Analysis of oligosaccharides by normal-phase LC/MS	N-glycans	Gel filtration and trypsin digest	Amide-80, 75 μm × 100 mm, 5 μm particle; 200 min gradient	Nano ESI	Ion trap	Use of normal phase column for polar interactions with glycans
[117]	Characterization of proteins in a complex protein mixtures	Human growth homone	Intact protein mixture or trypsin digest	Intact protein mixture analysis, C8, 150 μm × 100 mm, 100 min gradient; peptide analysis, C18, 75 μm × 100 mm, 60 min gradient	Nano ESI	Linear ion trap FT-ICR	Top down analysis; direct analysis of proteins by LC/MS; identification of post-translational modification; accurate mass measurement

for selected recent publications focused on proteomic analysis and high throughput biological screening using LC/MS techniques. The scope of LC/MS applications in diverse areas of proteomic and biological research is expanding, with the creativity of researchers pursuing more, better, faster, and deeper.

CONCLUSION

Widespread applications of liquid chromatography coupled with mass spectrometry in various stages of drug discovery and development were reviewed with examples of published work. Employed in the areas encompassing the biological target discovery, high throughput biological screening, drug candidate characterization, pre-clinical and clinical studies, LC/MS is undoubtedly one of the most essential analytical methods employed in the pharmaceutical industry. The diversity of methodology can be showcased from the experiment run time per sample, ranging from cycle time of 23 seconds under isocratic conditions for bioanalysis, to over 350-minute gradient LC for proteomic analysis, depending on the goals the LC/MS method serves. The ease of use, sensitivity, specificity, and speed of analysis are the factors that make LC/MS such a powerful and attractive technique for both quantitative and qualitative purposes. Advances made in the fields of separation technologies, understanding of ionization process, faster and more accurate instrumentation, and flexible and user-friendly software will certainly expand the capability and increase the throughput of the technique in the coming years.

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