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Application of LC/MS and related techniques to high-throughput drug discovery

Alfonso Espada[1], Manuel Molina-Martin[1], Jeffrey Dage[2] and Ming-Shang Kuo[2]

[1] Analytical Technologies Department, Centro de Investigación Lilly S.A., Avda. de la Industria 30, 28108 Alcobendas, Madrid, Spain
[2] Analytical Technologies, Lilly Research Laboratories, A Division of Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285, USA

The broad coordinated implementation of common platform technologies, such as LC/MS, can be a key factor in attaining increased throughput, sensitivity and data quality for pharmaceutical discovery. These platform technologies are the key tools that Medicinal Analytical Chemists rely upon to address the ever-changing needs of a drug discovery team. Despite the recent advances in key areas of sensitivity and speed for LC/MS, additional progress to address these never-ending analytical problems can be anticipated. This review will highlight current status and future advances for the applications of LC/MS and related techniques to high-throughput drug discovery.

Introduction

Despite significant advances in biomedical research to discover new molecular entities (NMEs) for the prevention, treatment and cure of human diseases, there is still serious concern about the existing strategies used to produce truly innovative drugs [1]. Currently, the pharmaceutical sector is one of the most research-intensive organizations. The investment in research and development (R&D) is necessary, owing to the limited product market timeframe and the need for public companies to maintain profitability for their investors. Although the sector has made tremendous improvements to human health, the fact remains that investment in R&D is up 147% from 1993 to 2003, and the number of FDA-approved NMEs has increased by only 7% over the same timeframe (http://www.fda.gov/oc/initiatives/criticalpath/whitepaper.html). It is this investment versus productivity dilemma that has made pharmaceutical discovery organizations to rethink their approaches and investments.

Drug discovery is a complex, multistage scientific endeavour (Figure 1) that spans diverse disciplines and often attempts to use new technologies and approaches to test or evaluate the many hypotheses generated during the course of the 12–15 year project life. Typically, the roles of medicinal chemists, biologists, pharmacologists and ADME/Tox scientists are highlighted, or routinely considered, as key to the earliest stages of the process. Throughout the long history of drug discovery, the analytical chemist has been a partner to each of these areas, developing new technologies and applications to address the key hypotheses or overcome the barriers to acquisition of key data. Such partnerships create the medicinal analytical chemist role. The medicinal analytical chemist understands many of the basic analytical technologies (liquid chromatography, mass spectrometry, magnetic resonance spectroscopy) , their strengths, limitations and key experimental design requirements. Experience in solving problems rapidly and establishing technology platforms makes this individual key to the hypothesis-driven drug discovery drill. The movement away from the simple filter-based process of drug discovery to the hypothesis-driven approach of answering key questions and solving problems quickly through rapid experimental design and application of quality science is stimulated by the desire for personalized medicines. Each stage of the drug discovery endeavour is full of examples using many new technologies and approaches, but arguably the most important and widely implemented analytical technology is LC/MS. Since the advances of the mid 1980s with atmospheric pressure ionization, LC/MS has enabled a revolution in experimental design and hypothesis testing through rapid method development and high-throughput analysis. The recent advances in sensitivity, throughput and automation have not only made LC/MS technology available but also enabled it to become the primary analytical platform throughout pharmaceutical research and development [4,5]. When coupled with the advances

Corresponding author: Espada, A. (espada_alfonso@lilly.com)

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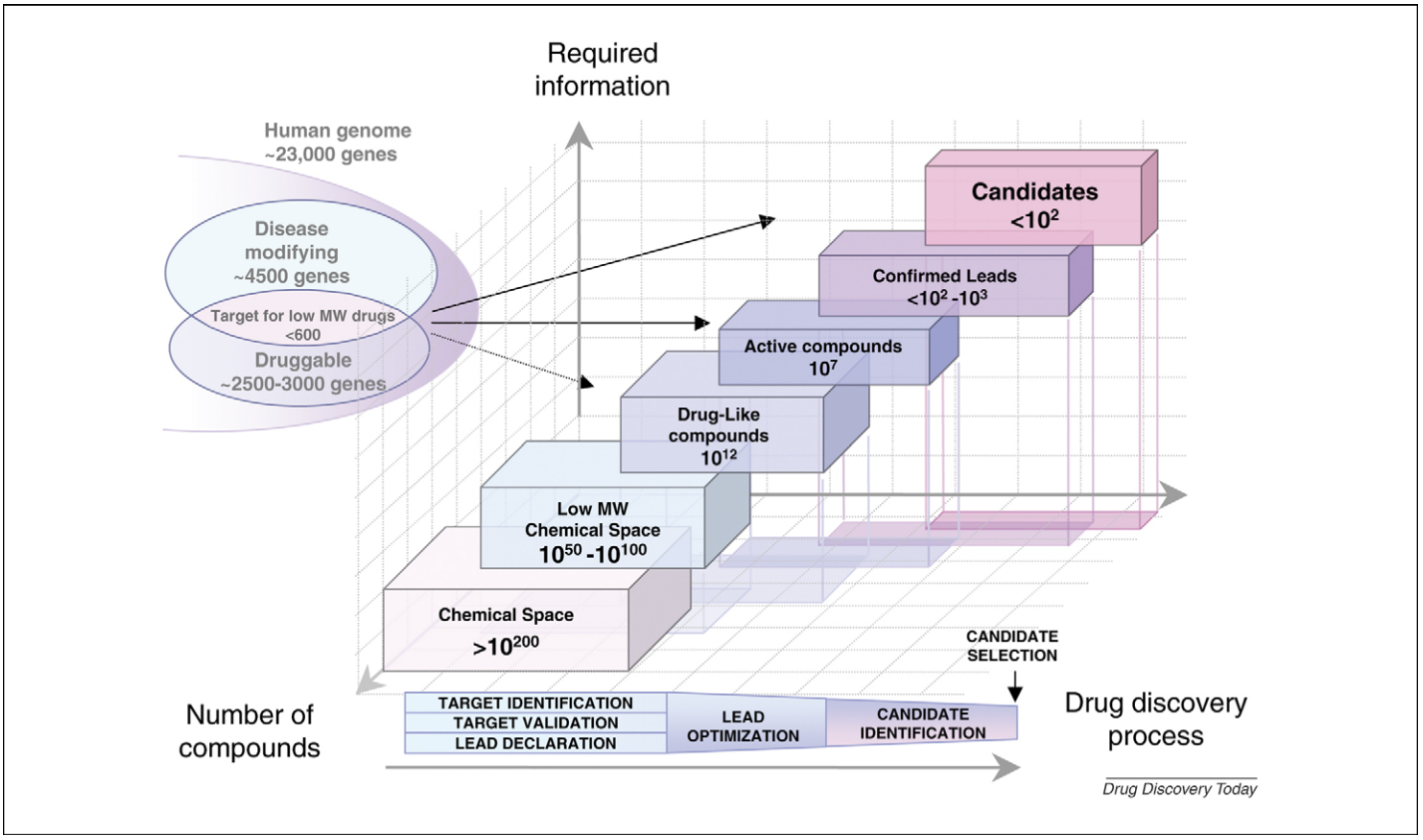

FIGURE 1

Integration of the drug discovery process in the chemical and biological space accessible by low molecular weight drug-like compounds. The most adequate regions of a huge chemical space (not coverable through collections) need to be exploited to access a limited region of the human genome [2,3]. Higher information content is needed as the molecules progress through the process.

made in the related fields of separation and automation, as well as sample introduction (ionization) technologies, the field has clearly exploded in its ability to provide meaningful impact [6].

Today, LC/MS-based methodologies are an essential tool in all the key steps in the development of small-molecule therapeutics, including target identification, synthesis, assessment of compound identity/purity, lead identification, pharmacokinetics and drug metabolism as part of ADME/Tox, clinical assessment of activity or efficacy and quality control of bulk drug substance (scale-up process, formulations). In this article, we present an overview of the diverse applications of LC/MS to meet the fundamental analytical requirement involved in high-throughput drug discovery and a key technology platform for the medicinal analytical chemist.

LC/MS applications and advances for discovery chemistry

Accessing compound identity and purity

The medicinal chemist's objective is to develop drug-like molecules with high affinity/activity to test in the clinic. This is met by building a broad understanding of the structure–activity and structure–property relationships (SAR, SPR) through an iterative process of molecular design, synthesis and hypothesis testing (activity or molecular properties). The timeframe for this multiple compound iteration and the ability to test multiple hypotheses drives the demand for rapid turnaround times within each step in the process. In this fast-moving environment, the goal of the analytical scientist is to implement LC/MS systems that yield maximum efficiency for laboratories challenged by throughput requirements. LC/MS open access service (analysis carried out by chemists with minimum training on sample submission) has become the standard technique to monitor the progress of synthetic reactions in real time and/or verify the identity and purity of compounds from structure–activity relationship studies [7,8]. Fast generic LC methods, with run times between 4 and 10 min with acceptable peak capacity (PC) values, are routinely used to provide excellent selectivity without compromising either chromatographic resolution or speed of analysis. PC is the measurement of the separation resolving power under gradient conditions (number of peaks that can be fit into a chromatogram), and it is also used to monitor the LC/MS systems' performance. Depending on the fundamental analytical requirements (Figure 2), PC requirements can vary between that needed for hit finding (i.e. 50) and that necessary to support clinical candidate selection (i.e. >120) [9].

Over the past several years, fundamental improvements in LC technology have changed the toolbox of the medicinal analytical chemist. Recent instrumental improvements, as well as the advent of new column technologies operated at higher flow rates, elevated temperature and/or ultra-high pressure allow both analytical scientists and medicinal chemists to obtain results more rapidly with the highest information content from a sample [10,11]. In our laboratory, ultra-fast LC separations have been implemented on conventional LC/MS systems for the analysis of large

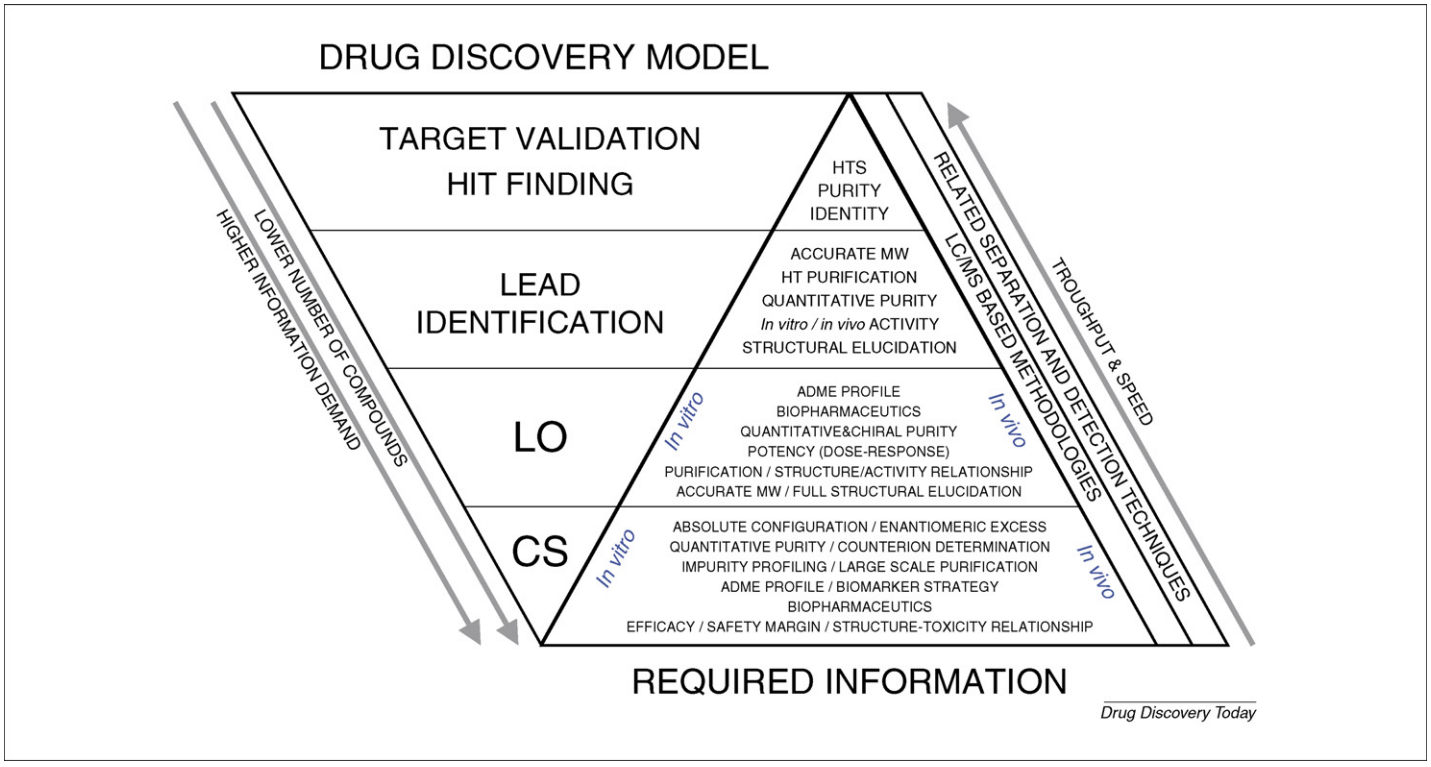

FIGURE 2

Available analytical tools (mainly LC/MS and related techniques) support drug discovery across the whole process. Throughout, attrition decreases the number of compounds, while the amount and quality of information required is continuously growing. Diverse simple or hyphenated instrumental configurations can be used to provide complementary data.

compound libraries derived from combinatorial chemistry and to identify and quantify reaction products from reaction screening (A. Espada *et al.*, unpublished). The application of these advances on a small column architecture allows routine analysis of >400 samples/day per LC or per LC/MS open access system with typical run times of 1.25 min (4× 'normal') and still delivers acceptable PC values up to 50 (Figure 3a). Ultra-high-pressure liquid chromatography (UHPLC) in conjunction with TOF–MS detection is another technical advancement in the field that is providing a drastic increase in capacity for medicinal chemistry labs. UHPLC technology exploits the theoretical advantages of sub-2 μm particles to yield faster, more sensitive and high-resolution separations. Rapid UHPLC/TOF–MS methods tolerate sample throughput, around 1.5 min/sample for the analysis of compound libraries (http://www.waters.com/WatersDivision/SiteSearch/AppLibDetails.asp?LibNum=720002099EN). Often, when faced with complex samples or samples close to clinical testing (requires limiting false negatives when looking for impurities), an analytical chemist would have used high-quality long columns and long run times [12]. More recently, strategies for alternating orthogonal (i.e. low, medium and/or high pH mobile phase) LC/MS and LC/MS/MS-based methods on a single system with short columns have been reported for a wide range of applications [13,14]. A similar multicolumn orthogonal approach has been applied for enantiopurity determination that can be crucial to understanding the SAR. There are also emerging technologies in the field of microfluidics gaining widespread attention for fast, efficient and high throughput chiral separation. Sajonz *et al.* have recently reported the application of this technology for catalyst screening and performed complete method development in 1 h for up to 8 substrates [15].

Maximizing purification throughput

Another area where advances in LC and application of MS have made tremendous impact is the purification of single compounds and libraries. Purification of compounds typically takes a medicinal chemist from 25 to 50% of their laboratory time. Many pharmaceutical companies have developed purification laboratories to build a technology platform that integrates processes, technologies, methodologies and data management. Centralized purification laboratories with experimental knowledge in analytical/medicinal chemistry and a high level of automation are becoming a powerful line of attack to sustain small-scale and large-scale achiral/chiral purification at different levels of throughput. The leading technology involved in this scenario is LC with UV and/or MS-guided collection, which can deliver thousands of purified samples/week [16,17]. The 'purification factory' is another approach that is entirely aimed at small-scale purification of samples from parallel chemistry campaigns. It employs a four-channel LC/MUX–MS purification system that is capable of sustaining a mean throughput of 528 samples/day [18]. Monolithic column technology, as well as supercritical fluid chromatography (SFC), represents newer technological advances in the field of analytical science with a significant impact in high-throughput analysis and purification of drug discovery compounds [19,20].

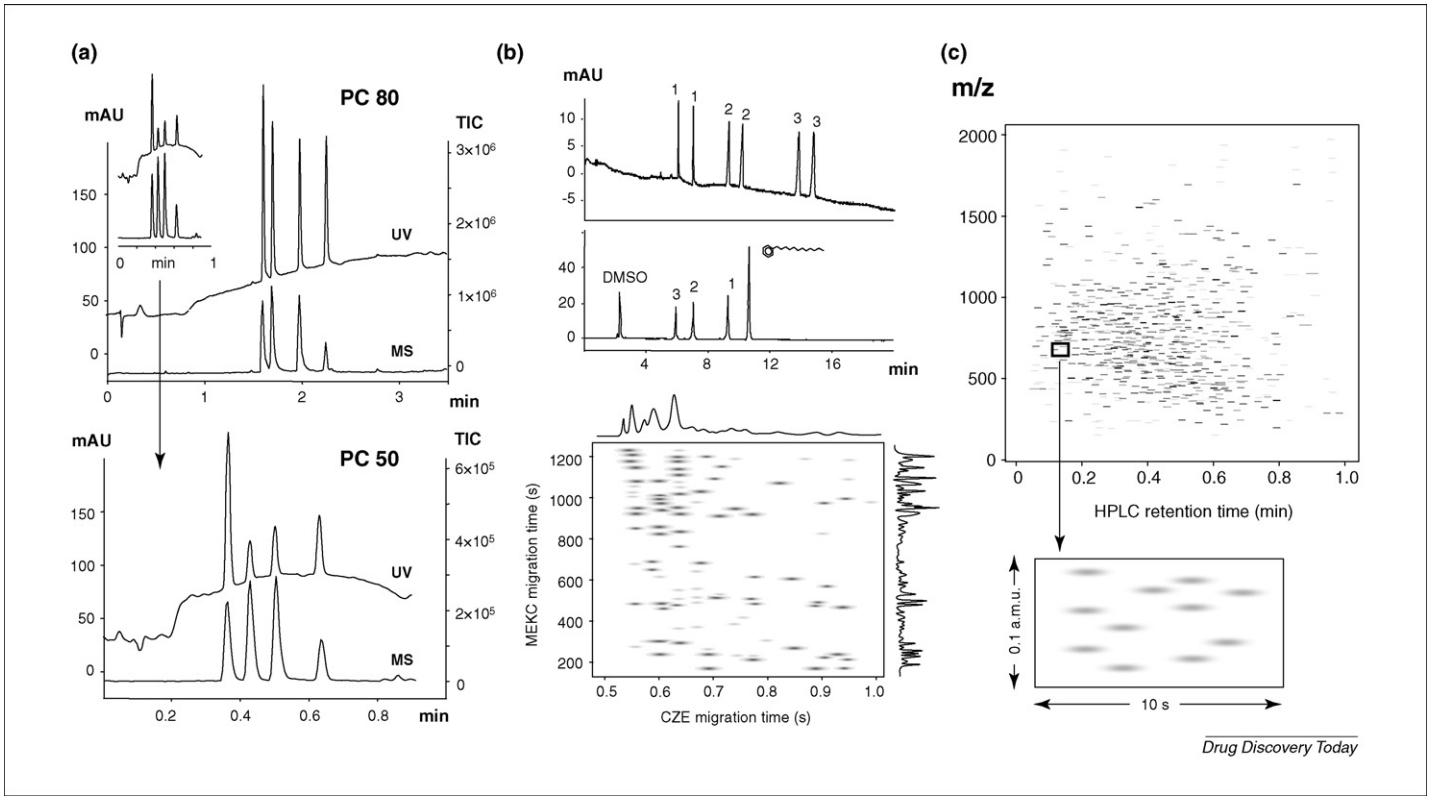

FIGURE 3

Trends in LC/MS and related techniques for a new paradigm in HT drug discovery. (a) Speed and capacity in fast HPLC: instrumental improvements and new particle technologies are making possible a 4-fold increase in the capacity of HPLC-based analysis. The cycle time to get every single datum is reduced by the same factor. PC: peak capacity. (b) Orthogonal information and bidimensional separations: the same analytical technique (CE) in different modalities is used to get complementary information from the compounds (top: chiral analyses and log *P* determination of a pool of three enantiomerically pure drugs) or increase the peak capacity in the separation for complex samples (bottom: schematic diagram of a two-dimensional separation of a complex biological sample using MEKC and CZE). (c) Idealistic representation of a fully integrated HT high content HPLC/MS separation: the advent of ultra-fast HPLC and ultra-fast MS detectors provides an enormous amount of information to support biomarker strategy. The bottleneck is being displaced from analytical capacity and data acquisition to obtaining the required information and validating the methodologies.

Bridging analytical and medicinal chemistry: testing hypothesis

Once the medicinal chemist has successfully synthesized and purified their designed compound, it is time for the medicinal analytical chemist to come up with ways to test their design hypothesis. Since it is well understood that SPR are important in understanding, or sometimes in predicting the likelihood that a compound will have good absorption, distribution or metabolism properties, every compound is evaluated for physical properties, such as lipophilicity, pK_a, stability, or solubility through computational tools or analytical testing. Since lipophilicity and pK_a are traditionally low-throughput shake-flask or titration methods, these were the areas first evaluated by analytical chemists to come up with higher efficiency techniques. Owing to its ease of automation, ease of use and intrinsic separation mechanism, capillary electrophoresis (CE) is being adopted as the technique of choice for the estimation of pK_a values [21]. Pressure-modified CE (PACE) affords a simple and automated way to run up to 1 sample/h in conventional instruments running serial injections [22]. A recent paper reports an analogous multiplexed system suitable for HT purposes. Vacuum-assisted multiplexed CE (VAMCE) in a 96-capillary array allowed pK_a determination of 8 samples (12 different running buffers/sample) in a single shot [23]. This platform has been optimized to yield a maximum throughput of 128–168 compounds/8 h. Lipophilicity is a complex physicochemical property; therefore the use of a suitable surrogate, such as the octanol–water partition coefficient (log *P*), is often adopted as an aid in testing the effect of lipophilicity against desired characteristics. There have been several reversed-phase LC and electrokinetic chromatography techniques used to develop correlations to traditional, lower throughput methods for log *P* determinations. Chromatographic hydrophobic index (CHI) is used as a direct measurement to characterize lipophilicity, and the employment of parallel microchip cartridges has dramatically increased the capacity (up to 24 columns can be run simultaneously with a very low solvent consumption) without negatively affecting precision (predicted CHI values differing less than 5% with respect to conventional HPLC methodologies (http://www.nanostream.com/support/downloads/AD1005_CHI.pdf). Log *P* values can also be determined in this microchip system, with an estimated throughput of 7 samples/h including analyses of the standards and the unknown samples as replicates (http://www.nanostream.com/support/downloads/AD1002_Log_P.pdf). A better correlation between retention and log *P* is typical of micellar and microemulsion electrokinetic chromatography systems, owing to the absence of hydrogen bonding interactions. Availability of 96-capillary arrays in commercially available instruments allows a maximum throughput of 80 samples/h in a highly automated mode. Multiplexed Microemulsion Electrokinetic Chromatography

(MMEEKC) provides a rapid, accurate and reproducible approach for obtaining log *P* values of basic and neutral compounds [24,25].

Stability in solution is a fundamental property of successful drug candidates. A solution stability assay provides an early flag for potential liabilities of compound series under study. The information gained can be used to guide SAR decision making, to develop strategies to overcome stability issues and to enable stable formulation development to be successful in the clinic. A fully automated solution stability assay using the advanced capabilities of LC/MS instruments has been recently reported. For example, Di *et al.* have developed an automated process, without operator intervention, that allows 96 experiments to be run using only 0.5 h of a scientist's time, compared with 8 h for the same study when performed manually [26]. Using fast LC gradients with narrow-bore or uHPLC columns and MS in ultra-fast scan mode, more degradants are quickly resolved and identified from the parent compound [27]. This approach adds a new dimension to stability assays, enabling us to obtain better quality data, better throughput and increased chromatographic resolution.

LC/MS applications and advances for *in vitro* biology

The primary goal of high-throughput screening (HTS) campaigns is to identify active compounds, or 'hits', against a therapeutic target of interest, from which lead series may be derived (Figure 2). Typically, the hits are identified by ligand-binding assays in plate readers that analyze outputs in high density (96, 384 and 1536) microtitre plates in a parallel fashion. However, the outputs of some enzymatic reactions cannot be measured conveniently, or it is impossible to do so using these readers. In such cases, LC/MS-based assays have proven to be a viable alternative when the library size is ~200 k or less. As such, this technology falls into the medium-throughput screening (MTS) category. Fast analysis time, high sample-throughput and high capacity sample-inputting devices are key factors for success. One advantage of this technology is that it detects enzymatic products without labelling or coupling reagents, potentially improving data quality and minimizing the need for preparation of labels/reagents. Thus, this technology sometimes is dubbed a 'label free' method. The LC/MS/MS analysis time is typically in the order of ~6–8 s/data point. When the throughput is this high, one has to minimize other essential steps to reduce the overall duty cycle time. Steps like washing injection needles, sample aspiration and injection, need to be optimized to improve the throughput further. Overall, the throughput is around 1 h to analyze a 384-well plate, depending on the configuration and sample-inputting device used [28]. One can further improve the throughput by utilizing a MUX set-up where the injections are staggered. For example, recently Ruddy *et al.* have developed an assay based on such a system with a throughput of 37 min/384-well plate [29]. Another successful case is the development of an acetyl cholinesterase MS-based screen using a proprietary microfluidic system that includes an online chromatographic step before introducing samples to an MS. In this study, 4608 compounds were screened in a total of 48 plates (96-well) in less than 2 h [30]. Recently, this microfluidic device has been commercialized by BioTrove (Rapidfire^{TM}). Clearly, LC–MS/MS-based assays can be strategically and successfully implemented when conventional HTS methods cannot be properly deployed.

Alternatively, it is also possible to detect directly, the binding of target (proteins or nucleic acids) with small molecules, normally present as a group of compounds. An example of this approach is the so-called multitarget affinity/specificity screening (MASS) technology developed by Sannes-Lowery *et al.* They employed electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI–FTICR–MS) to interrogate the interaction between RNA and small molecules [31]. In a single assay, this platform can determine the chemical composition of ligands that bind to an RNA target, relative/absolute dissociation constants and the specificity of binding to one RNA target relative to other RNA targets, as a result of the extremely high resolving power of the FTMS. Another advantage of such an assay, based on the equilibrium conditions reached between target and ligand, is that it provides a 'snapshot' of the species present in solution. As a result, the solution dissociation constants can be measured from the observed ion abundances of the free and complexed RNA. Ligand–RNA complexes with affinity ranging from 10 nM to at least 1 mM can be observed in the MASS assays.

LC/MS applications and advances for *in vivo* pharmacology and biomarkers

Analytical chemistry has always played a significant role in pharmacology and with the recent interest in leveraging biomarkers to increase, potentially, the speed with which a drug can be developed and potentially tailored to a specific patient or patient population [32]; it is no wonder that LC/MS is seeing such widespread use. The technologies of proteomics and metabonomics have been around for several years and are being implemented in areas of lipid metabolism, oncology and neuroscience [33–35]. There have also been new approaches where tracer compounds are used to assess either receptor localization or occupancy by a different ligand [36]. Although all of these areas are too large to go into significant depth in this review, the most interesting aspect of these developments for the medicinal analytical chemist is the widespread ability to use LC/MS in many different disease states and approaches to understand pharmacology and pharmacodynamics during drug discovery.

Future directions of LC/MS

It is clear that the properly trained medicinal analytical chemist has the ability to increase productivity and efficiency within drug discovery and development, through the application of a growing toolbox to the real questions facing project teams: 'Is this compound a drug?' Furthermore, the broad coordinated implementation of common platform technologies, such as LC/MS, can be a key factor in attaining increased throughput, sensitivity and data quality at reduced costs. But what's next? The key to the question is the problems that remain despite any advances. Can we develop methods faster? Do we run samples fast enough? Can we use this technology to test biological hypotheses? Are there better biomarkers still below our detection limit? The future advances and applications of LC/MS are coming from these age-old questions.

The latest developments in LC/MS have made possible a more than 4-fold increase in throughput without any corresponding increase in cost or loss of resolution. Figure 3a illustrates this assessment showing a conventional instrument using short columns (30 mm length) packed with 2.7–3.5 μm particle size. Even

faster running times and higher separation efficiencies are typically achieved with currently available sub-2 μm columns and instruments designed to operate at ultra-high pressure (http://www.chem.agilent.com/scripts/LiteraturePDF.asp?iWHID=51514). New materials adjustable at the molecular scale, such as monoliths and nanoparticles, for example, have the potential to shift chromatography limits even beyond these expectations [37]. In a fast-moving environment, the reduction in cycle time adds as much value as an increase in capacity, so reduced analysis times in multiplexed, miniaturized platforms will combine to boost productivity [38,39]. Micro-LC and nano-LC and microfluidic (lab-on-a-chip) separation methods will have an increasingly important role as these technologies spread in pharmaceutical laboratories [40–42].

The electropherograms in Figure 3b show an example of the accessibility of complementary information with orthogonal separations run in a sequential way (top). Fully integrated, comprehensive analyses (bottom) can be used for the same purpose and also to multiply peak capacity in extremely complex samples. 2-D (in general nD) separations are becoming invaluable tools to attain more rigorous results, such as are needed in the –omics worlds (proteomics, metabolomics, lipidomics, and so on). Multidimensional separations are also playing a fundamental role in the biomarker strategy, widely recognized as a key driver for personalized medicines, which are expected to deliver safer and more efficient drugs to the market with lower development costs [43–46].

Within this context, powerful (mass) spectrometric detectors (TOF, FT and ICR MS, as well as combined techniques) can generate an enormous amount of information (Figure 3c). Fast-scan acquisition and accurate mass determinations provide high content analyses, releasing a plethora of new research tools [47]. Competitive interactions and allosteric inhibitors have been investigated by this means [48,49], and pooling of samples or targets of interest has been demonstrated to be applicable to maximising the extracted information from every experiment [50].

Given the potential of current instrumentation and foreseeable advances, the bottleneck is being displaced from analytical capacity and data acquisition to chemometrics as well as to the validation of analytical methodologies. Automation of these last steps of the analytical process and delivery of computational tools to acquire the maximum value from all the available information can be a key factor in driving the right decisions at the right time.

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