19 High Throughput Quantitative Mass Spectrometry

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19.1 INTRODUCTION

Studies from the late 1980s showed that 40% of the attrition of all drug candidates in clinical trials could be attributed to poor pharmacokinetic (PK) properties [1]. Currently, the primary mechanisms for clinical failure are lack of efficacy and toxicological issues while PK-related failures have been reduced to <10% [2]. This dramatic change

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can be attributed to many pharmaceutical companies doing more *in vivo* PK studies and also establishing *in vitro* metabolism groups that were charged to develop and carry out a collection of *in vitro* assays to find compounds with appropriate ADME (absorption, distribution, metabolism, and excretion) properties. To respond to this daunting challenge, high throughput analytical techniques were desired to collect the data. As it turned out, quantitative liquid chromatography–mass spectrometry (LC-MS) fulfilled this need. Moreover, innovations in high throughput quantitative LC-MS have been driven by the ever-increasing need for industrialization of the bioanalytical process within the pharmaceutical industry.

The power of hyphenated chromatography/mass spectrometry techniques, that is, gas chromatography–mass spectrometry (GC-MS), was well established for the analysis of drug compounds and metabolites in the 1980s and into the early 1990s [3]. However, the GC-MS application range was limited to ~15% of the known chemical space since the compounds had to be volatile or made volatile with chemical derivatization [4]. Generally, a GC/MS method that required derivatization requires more extensive method development and sample preparation time than a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method [5]. It had long been known that mass spectrometry (MS) had the potential to supplant other liquid chromatography (LC) detection techniques. The coupling of liquid chromatography with ultraviolet detection (LC-UV) has limited detection sensitivity and was further confounded by specificity constraints, requiring longer run times to obtain sufficient chromatographic resolution. Fluorescence and electrochemical detection offer improvements in specificity and sensitivity of compound detection; these techniques were also coupled with drawbacks, such as the need for derivatization, intrinsic molecular properties, but most often, increased analytical cycle times to ensure analytical reproducibility, particularly in the case of electrochemical detection [6].

Before the advent of API (atmospheric pressure ionization), many other interfaces using various ionization sources were developed to couple LC with MS, including direct liquid introduction [7], moving belt [8,9], particle beam [10,11], thermospray [12,13], and continuous-flow fast atom bombardment (FAB) [14,15]. Intrinsic liabilities and robustness limitations precluded these interfaces from being used for high throughput quantitative analyses. For example, excessive flow splitting from an eluent flow rate of >1 mL/min to the low as 1-10 μL/min inlet flow rate was required for continuous-flow FAB. Particle beam was more suited for environmental analysis of medium polarity compounds but, in general, exhibited limited sensitivity. Thermospray, a precursor and closely related LC-MS interface to the newer electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) technologies, was the most established and utilized technique for small molecule analysis due to its ability to accommodate higher LC flow rates [12]; however, thermospray was not a truly robust technique. Extended operational use resulted in excessive source fouling, blockages in the thermospray probe, and inconvenient manual intervention, which was not a trivial exercise due to the need for operation within a high vacuum. Thermospray interfaces required extensive tuning; exhibited degradation of thermally labile functional moieties, such as acyl glucuronides; confounding mass assignment; and when compared retrospectively to API, exhibited limited structural coverage.

Publications that outlined the utilization and application of LC-MS dramatically increased through the mid-1990s, primarily because of the invention of second-generation commercial APCI [16,17] and ESI sources [18–20], which could

accommodate higher LC flow rates [21]. Their rapid uptake and acceptance is clearly demonstrated through the explosion of abstracts in the MS community at the 1994 American Society Mass Spectrometry Conference. A total of 413 LC-MS presentations (35% of all presentations) were shown, ~81% featured ESI and ~9% featured APCI. These sources provided a robust, straightforward way to couple LC to a mass spectrometer [21].

In contrast to previous LC-MS interfaces, the API interface enabled facile coupling of LC-MS systems, demonstrated improvements in ionization efficiency, and demonstrated rapid interchange between ESI and APCI probes, as the technique operated outside of the high vacuum region of the mass spectrometer. Contamination from the analysis of minimally purified samples, historically responsible for interface fouling and leading to variance and degradation in mass spectrometer ion transmission performance, could be readily rectified, as the interfaces and ion optics were accessible to the operator for cleaning between runs.

Improved robustness resulted from a number of significant evolutions in interface and inlet (orifice) designs. The original interface technologies employed an on-axis ESI, such as the Branford Analytica and Finnigan MAT first-generation interfaces. The inclusion of a heated capillary ion transfer tube resulted in a degree of gas phase ion declustering and, together with differential pumping systems and multipole ion transmission technologies, provided an analytical footprint for acceptance of LC eluent flow rates of 1 mL/min for APCI and split flow of 200 μL/min for ESI. The development and commercial launch of ESI interfaces incorporating off-axis [22] and, eventually, orthogonal [23] electrospray emitters relative to the mass spectrometer inlet (orifice), continued to dramatically increase the robustness of API interface. Also, instrument evolution of high purity curtain gas ion optics facilitated higher interface gas loads and eluent flow rates to be used [24]. Incremental improvements in differential pumping, partial pressure ion extraction technologies, interface pumping capacity, and orifice cross section resulted in improved instrument sensitivity. Moreover, these improved API interfaces enabled direct coupling of the LC eluent to the ionization source without flow splitting, reducing postcolumn resolution loss (band-broadening) inherent within early split flow API interfaces [25]. Interfaces could accommodate LC eluent flow rates up to 2 mL/min directly via APCI and up to 1.0 mL/min with pneumatically assisted ESI sources using heated N_2 gas (sometimes referred to as *ionspray*) [22]. It should be noted that the ability of API technologies to predominantly form a single, predictable precursor ion for small molecules, based on ionization mode (positive/negative), has enabled MS operation earlier in the drug discovery process. For example, open-access workflows have been developed to provide additional confidence assignment in chemical synthesis [26]. These features of API, leading to the moniker of "soft" ionization properties, have also enabled automated tuning capabilities for additional efficiency gains.

A striking example of the potential improved throughput using API was demonstrated for renin inhibitor quantitation from human serum using an isocratic LC-MS assay with an APCI source [27]. This assay described analyte peaks 3.4 min after injection, conservative numbers by today's standards, but may be indicating a degree of empirical experimental design that was ahead of its time in terms of resolution of coextracted matrix constituents, together with the chromatographic resolution needed in selected ion monitoring experiments. More importantly, the method was able to detect and quantify down to levels of 50 pg/mL of the drug, demonstrating the capabilities

necessary to compete with the existing electrochemical and fluorescence detection techniques.

Before the advent of API ion sources, it was known that compound selectivity and thus sensitivity (signal-to-noise ratio, S/N) could be enhanced by performing tandem mass spectrometry (MS/MS) [28,29]. The most commonly used MS/MS experiment in quantitative efforts is the selected reaction monitoring (SRM) scan. The experiment is typically conducted on a triple quadrupole mass spectrometer using the following conditions. The first quadrupole (Q1) transmits a precursor ion (based on mass-to-charge ratio, *m/z*) of the compound of interest, usually a protonated or deprotonated ion, representative of the original analyte. The second quadrupole (Q2, often called the *collision cell*) is filled with neutral collision gas; a collision energy (accelerating voltage) is applied to induce energy transfer through collision and create a series of product ions through collisional activation of the precursor ion. Lastly, the third quadrupole (Q3) is set to transmit a specific, prominent product ion, before signal amplification. The SRM mode of analysis requires the analyte to transit through two distinct stages of mass-to-charge filtering, thereby increasing analyte specificity, while greatly diminishing the background noise. Performing a SRM scan event, together with an orthogonal separation modality, such as an LC separation, provides three distinct filtering mechanisms to increase specificity in measurement, namely, chromatographic retention time, protonated/deprotonated molecular ion *m/z* ratio, and product ion *m/z* ratio. The enhancement in selectivity, sensitivity, and operational utility of the modern triple quadrupole mass spectrometer represents one of the most powerful analytical techniques devised to date.

LC-MS/MS soon became the indisputable gold standard technique for quantitative analysis for *in vitr*o and *in vivo* biological samples as is evident by the most commonly performed *in vitro* assays (Table 19.1). The increasing capabilities and utility of LC-MS/MS provided an enabling solution to performing *in vitro* assays on all lead compounds produced in early drug discovery. With this in mind, many researchers in the past two decades began to seek ways to improve quantitative LC-MS/MS throughput. This chapter presents the various methodologies used to obtain high throughput quantitative MS. A series of topics describes specific methodologies that have been demonstrated to directly improve the throughput of the quantitative LC-MS/MS experiment. These topics include automation, MS acquisition method setup, optimization of single and multiplexed LC systems and interfaces into the mass spectrometer, and data reduction. An additional topic covers some new and emerging non-LC-based quantitative MS techniques that show the promise of improving throughput. Although this chapter focuses on *in vitro* assays, many of the techniques described can and have been applied to *in vivo* sample analysis.

19.2 BASIC CONSIDERATIONS FOR HIGH THROUGHPUT LC-MS/MS

To obtain the full advantage of high throughput MS, the entire experimental process requires optimization and understanding of the timelines for each component in the overall process. A question of key consideration is “what is high throughput?” Frankly, the definition of high throughput is somewhat arbitrary; however, the time frames for independent components in the process are germane to defining an approximation to the answer. Perhaps the more telling parameter for high throughput is the cycle time

TABLE 19.1 ***In Vitro*** **Metabolism Assays**

Test	Technique(s)	Analyte Monitored	Sample Matrix	Throughput Bottleneck/Comments
CYP inhibition (drug–drug interactions)	LC/MS/MS	Specific probe molecules	Liver microsomes	Number of compounds tested; number of enzymes to be tested; LC run time
	Fluorescence	Fluorogenic probes	Recombinant proteins/buffers	None: relevancy of recombinant protein used, interference from fluorescent compounds
	Luminescence	Luminescent compounds	Recombinant proteins/buffers	Limited types of CYP isoforms
CYP 3A4 induction	Luminescence	PXRE-luciferase	Cells	Highly tuned specific assay
CYP isozyme profiling	LC/MS/MS	Drug and individual metabolites	Recombinant proteins or liver microsomes	Tuning of the mass spectrometer for individual compounds
Caco-2/efflux transport	LC/MS/MS	Drug compound	Buffers	Tuning of the mass spectrometer for individual compounds
PAMPA	96-well plate with UV detection	Drug compound	Buffer	Multiplexed detection, cost-effective—useful in early discovery phase
Metabolic stability	LC/MS/MS	Drug compound	Extract from hepatocytes	Tuning of the mass spectrometer for individual compounds

Abbreviations: PXRE, progesterone X receptor element; PAMPA, parallel artificial membrane permeability assay

for each event. Sample preparation includes off-line or on-line extraction/filtration techniques to reduce sample complexity and novel methodologies to pool samples. Sample preparation has evolved to become generic with/without zwitterionic analog or stable labeled internal standards. Incorporating 96-well plating automation has realized 10 s per sample [30] for preparation schemes, additional parallelization into 384-well plates and above has further reduced these steps to the 2- to 5-s time frame. This at least gives the process a throughput goal for optimization. Sample introduction into the LC-MS system, discounting the influence of carryover, has been realized in the 5- to 10-s time frame, although typically occurs in the 45- to 90-s cycle time time frame in which carryover control is desirable, utilizing generic autosampler wash protocols [31–34]. Chromatographic peak widths in the subsecond regime are now readily achievable, utilizing gradient/recycle single-plex LC-MS systems with cycle times of 60 s down to <10 s [35]. Introduction of multiplexing LC systems facilitates eight samples in <60 s, together with incorporation of multianalyte per sample analysis, effectively realizes 1–2 s per analyte in targeted SRM mode and 1–2 s for full scan data [36]. Interfaces can now provide stable response in the 10- to 100-ms time regime; as such, these are now no longer not truly additive to the cycle time. As alluded to above, MS acquisition time frames are in the 1- to 2-s to subsecond regime as a function of the scan events required. It should be noted that the time frames to open and close files has been evaluated as a time-critical variable, and the 1–3 s required for this event have been eradicated from some processes in which a single file is acquired and deconvoluted postanalytically [37]. Lastly, data reduction and posting of data has been somewhat lagging. Functionally, 10 s per sample in review is an appropriate time frame for current workflows. This leaves us with a somewhat realistic argument to the proposed question; it appears that high throughput is 1–2 s per sample, irrespective of the number of independent analytes being assayed.

To achieve these throughput requirements, generic process systems are required. Method development is a key component in targeted assay measurement; however, in high throughput quantitative analysis of multiple chemically diverse compounds, process development is paramount. For quantitative LC-MS/MS, the goal is to produce a separation that resolves the analyte from background components. Unlike LC with a UV detector, the compound can coelute with many compounds because the tandem mass spectrometer will filter out the analyte precursor and product ions away from interfering species via mass discrimination. Generic LC separation, wherein desalting is functionally embedded in the separation, is the common and often only requirement for the LC separation. MS method development includes finding the appropriate precursor and product ion pair transitions for the analyte and maximizing the signal response by adjusting the collision cell pressure and voltage. Data reduction method development includes integration of the chromatographic peaks, assigning calibrators, quality controls, expected concentrations, and acceptability criteria, before posting data to a corporate database.

The key bottleneck for an assay depends on the type of analysis that is being performed (Table 19.1). For example, metabolic stability assays in which 96-well plates filled with unique compounds are analyzed, the critical bottleneck is optimizing the response of the mass spectrometer, that is, selecting the appropriate precursor and product ions and collision conditions for the SRM experiment, with saturation of response mitigated. In contrast, for a P450 inhibition assay in which the same analyte(s) are detected for each sample, the most important throughput constraint is sample analysis

time, predominantly linked to the time of the LC method, requiring development of a short, robust, and generically applicable LC method.

19.3 AUTOMATION OF SAMPLE PREPARATION AND BATCH PROCESSING

Automation of processes is an integral component of the high throughput quantitative ADME workflow, intrinsically linked to the type of *in vitro* assays and projected sample volumes. Detailed process review is necessary to assist infrastructure planning, such as sample tracking, software customization, and amount and types of automated sample preparation equipment, personnel (skill sets and number), and LC-MS/MS systems. Automation of processes requires significant investment capital and long developmental lead times. Ideally, the throughput of sample incubation/preparation should exceed the LC/MS/MS analytical throughput. As the analysis time is reduced, preanalytical systems will be required to provide increased numbers of samples to optimize the usage of the analytical equipment.

In a recent review of automation for *in vitro* analytical laboratories by Saunders [38], key examples of equipment used to automate sample incubation and preparation using sample-plate-based robotic systems were described. The review detailed the extent of automation required for sample volume and types of analyses performed. The type and extent of automation can range from very little (i.e., an autosampler in the LC-MS/MS system), which is well suited for a small start-up company or a university laboratory, to a fully automated system for a larger pharmaceutical or contract service laboratory. Fully automated systems control sample incubations, sample preparation, tuning of the mass spectrometer, automatic transfer of samples to the mass spectrometer, data acquisition, and data processing. These systems are custom built by the company and require extensive internal resources to establish communication and control all the diverse components within the analytical workflow. Examples of fully automated systems have been reported by Janiszewski *et al*. [39] and, more recently, by Laycock [40]. The majority of laboratories fall short of these modular and fully implemented automation solutions. In a typical laboratory, a multiuse multitip liquid handling platform is tasked with dispensing samples, performing off-line extractions, reaction quenching, and dispensing into new plates for subsequent LC-MS/MS analysis. As volume and sample needs increase, these "islands" of automation are readily scaled to match throughput requirements.

19.4 SAMPLE PREPARATION AND POOLING

Numerous and varied ways to perform sample preparation have been described in this volume and are detailed elsewhere (see chapter titled ***Direct Biofluid Analysis Systems***) [41]. It cannot be overstated that sample preparation is crucial for the reliability and robustness of the assay using the current LC-MS/MS workflows. The clean-up process not only removes interfering analytes but also enables the filtration of particulates (i.e., microsomes) and reduction of proteins that eventually diminish the performance of the LC separation. Just as the instrumental parts of the analysis need to be optimized for high throughput, so does sample preparation. Off-line procedures such as solid-phase

extraction, sample dilution, and protein precipitation can be multiplexed and allow for plates to be transferred directly for LC-MS/MS analysis [42]. On-line sample clean-up methods have been demonstrated using restricted access media columns and turbulent flow columns and are summarized by Xu *et al*. [41].

Sample pooling is an effective strategy that is employed to improve throughput by reducing the number of analyses conducted. Multiple analytes can be combined [43] and run simultaneously through the biological system (biological cassetting). Smalley *et al*. [44] successfully demonstrated Caco-2 permeability analysis using a biological cassette of 10 samples. The method used on-line solid phase extraction (SPE) LC-MS/MS and achieved a standard curve range of 10–2500 nM with a run time of 3.5 min. Sample pooling involving post dosing sample combination (analytical cassetting) may also be performed after the biological assay is completed [45]. This strategy is generally preferred to eliminate any questions that arise from competition effects (i.e., inhibition) of compounds when presented simultaneously to the enzyme in question. Compounds that can be pooled together typically have different retention times, to minimize the chance of ion suppression effects resulting from coelution with other compounds within the pool.

Another potential approach to reduce the number of analyses, not widely deployed for ADME analyses, is the use of subsetting compounds based on chemical properties. Using *in silico* methods, specified compounds with diverse chemical motifs are selected for potency screening from a limited number of well plates. Statistical models are used to predict compounds that have similar chemical properties to the training set hits, to "cherry pick" from the compound collection for additional screening [46]. The authors noted that the results from the subsetting approach compared favorably with data obtained from a traditional screening approach and led to a reduced sample analysis burden of ~75%.

Another pooling approach is to determine the effect the compound has on multiple enzymes in a single analysis using a cocktail of probe substrates. This workflow was demonstrated by Kim *et al*. [47] for the analysis of inhibitory potential of nine CYPs by measuring the metabolized products formed from microsome incubations containing six- and three-probe compounds, respectively. The incubations were pooled together before analysis. The LC/MS/MS run time was 6.5 min for each pooled sample. The methodology provided only a modest improvement in analytical throughput because of the slow LC analysis conditions when compared to what can be achieved using the RapidFire™ (see below). However, this assay did effectively increase the coverage of CYP enzymes studied and somewhat minimized material usage.

19.5 MASS SPECTROMETRY METHOD DEVELOPMENT—TUNING AND CHOICE OF MASS ANALYZER

For experiments that perform a single or a limited number of samples for each analyte (i.e., metabolic stability, Caco-2), mass spectrometer method development becomes a critical factor in throughput enhancement. Practically, sample is infused or loop injected into the mass spectrometer for selection of precursor and product ions to be monitored on a triple quadrupole instrument. Additionally, interface gas flows, polarity, and ion optics setting are tuned to optimize transmission of the precursor ion. Subsequently, the collision cell accelerating voltage is adjusted to identify the most prominent product ion, before fine-tuning of collision cell conditions to optimize

the response of the SRM transition. Computer-driven programs have been developed to automatically perform this task [37,48–52]. These programs perform a series of multiple loop injection experiments to optimize these previously described variables and can significantly reduce the development time requirements. Success with these technologies does come at a price. When loop injection is performed, the signal profile changes quickly (i.e., peaks can become as narrow as or narrower than a LC peak), thus the analyte signal is changing very quickly as a function of time. This can result in limited time for algorithms to find optimal settings for each parameter. Reduction in the flow rate to allow increased residence time of the flow injected analyte bolus is often used to extend the amount of time a steady signal is delivered to the instrument. This process overcomes constraints within the selection of SRM transitions; however, tuning of the ion source parameters may be compromised since optimization of some of ion source voltages can be dependent on eluent flow rate. Ideally, obtaining a steady flow of sample at the same flow rate used for the chromatography method is required. Conversely, performing these procedures manually consumes a significant amount of time (4–10 min per sample); thus, a 96-well plate with eight discrete analytes would require 32–80 min to complete tuning of the instrument. Moreover, choosing the appropriate analyte(s) serving as tuning compound is critical since carryover issues can occur, which for sticky compounds could require extensive time (hours) to flush the compound out of the instrumentation setup. In many cases, instrument tuning time is greater than the overall analytical time frame for quantitative LC-MS/MS analysis of the samples themselves.

Alternate approaches to relieve this bottleneck have been described by Kieltyka *et al*. [53] utilizing an autosampler to infuse samples into the MS system. Besides delivering sample to the source, the system using QuickQuan 2.0 was able to tune the mass spectrometer at a rate of 2.5 min per analyte. For a plate consisting of 48 discrete compounds, tuning was completed within just <2 h. These investigators reported that using this tuning procedure and a dual chromatography system for analysis, they were able to obtain a throughput of 1300 compound assays with 500 discrete compounds per instrument per month with a 95% success rate. Figure 19.1 summarizes the informatics workflow for this automated tuning process.

In larger laboratories with common vendor-supplied triple quadrupole mass spectrometers, databases have been developed to store and recall analyte tuning parameter files, reducing the time requirements for subsequent method development for the same analyte [40]. The realization of this protocol owes much to the reproducibility of MS systems manufacturing. For successful implementation, instruments using shared tuning parameters must meet a specific performance requirement, using a standard compound analyzed using fixed ionization source and collision cell conditions, to ensure that each instrument is performing optimally.

A strategy to avoid optimizing the SRM transition is to use another type of mass analyzer. For example, a single quadrupole in full scan [54] or single ion monitoring mode [55] has been utilized for quantitative ADME experiments. As discussed previously, this leads to compromised selectivity, decreased sensitivity, and concordantly, increased sample consumption. Ion trap mass spectrometers have also been utilized, whereby the ion trap is operated in product ion MS/MS mode. Quantitation is established by extracting the analyte-specific product ion(s) produced from a given precursor ion and is performed during postacquisition analysis from the total ion chromatogram. Typically, the mass window for these mass chromatograms is approximately ± 0.5 Da

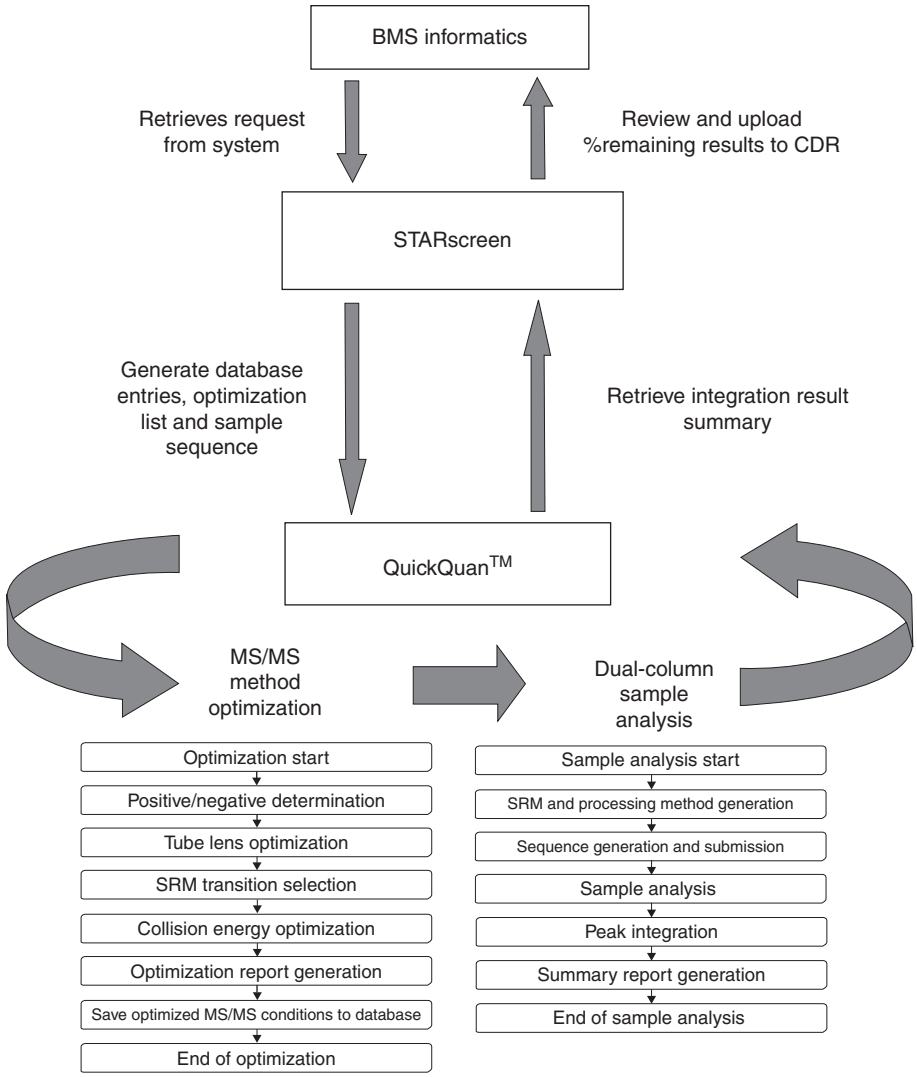


Figure 19.1 The integrated informatics workflow employed by Kieltyka *et al*. [53]. A customized software tool, STARscreen, provides corporate gate-keeping function and tasking lists for QuickQuan 2.0, including all relevant import list details and final data release/upload. The automated analyte MS optimization process is executed via QuickQuan 2.0 using infusion syringe driven optimization, allowing appropriate time to generate high quality MS acquisition methods. Batch import and process method assignment is controlled automatically through QuickQuan 2.0, including user-specified generic methods. Staggered dual-column analysis is executed for accelerated throughput, of particular note in the process; the import list generated by STARscreen incorporates the analytical staggering process automatically, using alternating samples from two discreet plates. Manual data review and data release processes follow initial autointegration.

wide, a function of the unit mass operation of the ion traps used. A key efficiency advantage to this approach is the use of "universal" collision conditions, circumventing tuning for specific SRM transitions [56]. Typically, this platform is 10 times less sensitive than the current triple quadrupole MS-based platform [53].

Another application involves the use of an orthogonal acceleration time-of-flight (TOF) mass spectrometer [57,58]. The TOF detects all ions within a given mass range (i.e., it cannot be fixed to monitor for a specific mass) and can be more selective than the quadrupole based on its higher mass resolution capability. Moreover, current TOF mass analyzers have a very fast duty cycle—20 Hz or faster, which improves its detection capabilities for sampling subsecond wide chromatographic peaks that elute in an ultrahigh performance liquid chromatography (UHPLC) separation. Initial experiments performed with a TOF with a resolving power of 5000 showed ~5–25 times less sensitivity when compared to the triple quadrupole together with limited dynamic range [57]. Using narrow mass width extracted ion chromatograms of 0.1 Da for quantitation, linearity and sensitivity (signal-to-noise ratio) approached that produced by a triple quadrupole system. However, at this resolving power, chemical noise is not always fully resolved from the analyte, so higher resolving power would be of further utility. There is growing interest in revisiting the TOF experiment now that TOF/Q-TOF (quadrupole–time-of-flight) instruments with resolving powers exceeding 20,000 and better sensitivity are commercially available. These technologies permit the use of even narrower mass width ion-extraction chromatograms (0.01–0.005 Da), to produce an extracted ion mass chromatogram for the molecular ion with minimal contributions from chemical noise. Since data are acquired over a wide mass range without *a priori* mass spectrometer tuning, acquired data can be processed to find masses of expected analytes and metabolites, especially when accurate mass measurements are performed [59]. This approach has been termed as *quantitative/qualitative* (*Quan/Qual*) *workflow* [60] and has been applied routinely for metabolic stability assays. Moreover, when a Q-TOF instrument is utilized, data-dependent MS/MS experiments can be triggered to provide additional structural information [58]. Even higher resolving powers 100,000 full width at half maximum (FWHM) in a 1-s scan cycle can be achieved using the Exactive™, a benchtop orbitrap mass spectrometer [61]. Reports have shown this system supporting metabolic Quan/Qual stability experiments [60].

19.6 LC METHOD CONSIDERATIONS

The orthogonal nature of LC-MS/MS and relative differences in cycle times between these techniques has realized the greatest diversity and efforts in enhancement of throughput. The mass spectrometer, when set in SRM mode, is capable of detecting analyte response every 10–100 ms, whereas the LC system typically requires minutes to deliver the sample to the mass spectrometer. Thus, sample analysis throughput improvements are primarily dependent on optimized sample delivery to the mass spectrometer. This can be accomplished by reducing LC separation time or multiplexing (multiple LC separations to a single MS system) to align the throughput scales of the systems. The main function of the LC separation in a SRM experiment is to separate analyte from species that cause ion suppression, such as nonvolatile salts, proteins, or phospholipids.

In early experiments, isocratic high performance liquid chromatography (HPLC) methods were common and achieved run times of <3 min per injection [6,27]. However, isocratic methods require redevelopment of the LC method conditions each time a different analyte is selected for analysis. Moreover, isocratic methods suffer from column contamination from retained lipids and proteins.

A more practical approach involves the inclusion of a binary gradient HPLC method, the goal being to provide appropriate LC separation quality across the majority of chemical space, without individual analyte reoptimization, minimizing ion suppression, and column contamination. An important consideration to achieve high throughput LC separations is reduction of system volume for gradient mixing experiments. System volume is composed of a delay volume in pumping systems, solvent mixing, extra-column tubing, LC column volume (at least 60% of the LC column volume unpacked), and postcolumn interface volume. Pump dead volume results in a gradient delivery delay to the column and also lengthens column reequilibration time. Manufacturers have reduced extra-column volume significantly with the development of UHPLC systems. Improvements in column packing materials have resulted in reduced variability from residual silanol activity. Better reproducibility of silica particle cross section has resulted in a reduction in tortuosity, the A term in the van Deemter equation. Manufacturing processes have also improved column packing reproducibility. Finally, gradient separations result in chromatographic peaks with reduced peak tailing, leading to improved detection limits.

A generic gradient LC separation will include a 3- to 5-μm, C18-functionalized silica particle packed into a column of dimension 2.1 mm in diameter by 30–50 mm in length. Flow rates are typically delivered in the 0.5–1.0 mL/min range. Generic reverse-phase linear gradient separations start at a low organic concentration, typically, acetonitrile at 0–5%, 95–100% aqueous 1 mM ammonium formate, and 0.1% formic acid, and elute at a sufficient organic concentration to remove proteins and some lipids (95–100% acetonitrile). The solvent flow rate and slope (gradient time), along with the stationary phase of the column, are principle drivers in determining the speed of the LC-MS/MS analysis and the chromatographic resolution achieved. Generic LC conditions become more tailored and refined if insufficient compound detection occurs or if the compound will be continuously analyzed for many studies. During process refinement, different variations of C18 columns or stationary phases (i.e., C8, cyano, phenyhexyl, etc.) may be investigated to obtain increased throughput and improved selectivity.

Recent advances in LC column technologies have resulted in further choices for high throughput LC-MS. Good results have been demonstrated with the use of monolithic columns [62,63], where the intrinsic porosity (∼15% > standard columns) provides for separations at significantly higher flow rates (up to 5 mL/min). With preinterface solvent flow splitting, chromatographic separation of buproprion and its metabolites was achieved in 23 s [64]. Similarly, fused-core columns have become available that provide higher resolution separations than conventional spherical semiporous 3-μm particles, with reduced separation timescales. Fused-core LC columns enable higher flow rates (>1 mL/min) without producing a pressure drop that exceeds the 400-b pressure limits of standard HPLC systems. These benefits do come at a price. Gradient reconditioning before the next assay is required, which is essentially dead time in an assay. Alterations in the workflow, such as multiplexed LC analysis (see below), are necessary for realization of the true chromatographic efficiency gains.

The evolution of UHPLC has provided an alternate methodology to accelerate LC cycle time [65]. With UHPLC, better chromatographic separations can be achieved in a shorter timescale than that with conventional HPLC columns, since columns with smaller (sub-2 μm) particle size particles realize improved theoretical plate heights at the same linear velocity, when compared to columns with 3- to 5-μm particle sized particles. Moreover, UHPLC pumping systems have been designed with reduced system volume (~60 μL) and higher operating pressure regimens, up to 1800 b. Cycle time reductions of two- to threefold have been demonstrated by multiple laboratories [66–68], with a concomitant increase in sensitivity through chromatographic peak compression.

Sample throughput is impaired by autosampler cycle time constraints. For a highly optimized LC method (<60 s), a significant portion of the injection-to-injection cycle time is invested in a number of autosampler tasks. Typically, the autosampler receives a signal from either the mass spectrometer or the data system that the previous analysis is complete and starts a cycle. The autosampler aspirates the next sample in the queue, transports the sample or an injection needle to enable injection, and injects the sample. Following injection, the autosampler is tasked with preparing the system for the subsequent injection (i.e., washing the sampling valve). As LC throughput increases, the cycle time of the autosampler becomes the dominant factor in efficiency enhancement as preinjection delays of up to 20 s can occur. Technologies such as "inject ahead" have been developed to reduce this problem [69]. Inject ahead enables the autosampler to wash the injection device and inject the next sample during the analysis of the previous sample.

19.7 HIGHER THROUGHPUT SERIAL LC-MS/MS

Solid-phase extraction provides less chromatographic resolution than that of LC; however, sufficient resolution can be obtained to desalt the analyte and reduce the levels of proteins, salts, and detergents that can suppress the analyte response in a rapid timescale MS analysis. Inman *et al*. [70] described an off-line SPE method that enabled 24 s per sample MS analysis times for metabolic stability samples. The method used SPE cards in a 96-well plate format. Samples were processed using a SPExpress system, composed of a Harvex liquid handler with an array of 96 needles, and transferred into the SPE elution zones on the card-containing Empore C18 media. After that, the analytes were washed with aqueous mobile phase to remove buffer salts. The sample plates were transferred into the Elutrix unit and eluted from the SPE resin into the mass spectrometer with a C18 guard column coupled in between to enhance peak shape. The Elutrix unit had the capacity to hold 35 × 96-well plates for unattended analyses. Good correlation between traditional LC-MS/MS and SPE-MS (solid-phase extraction–mass spectrometry) were observed ($R^2 > 0.92$) for *in vitro* clearance samples from human and dog microsomes. This chapter described the impact of limited chromatographic resolution and reduced recoveries for highly polar analytes, which affected the results obtained by the SPE method.

An innovative on-line SPE instrument, RapidFire, has been introduced by Biocius (formerly BioTrove). Originally designed as system for high throughput biochemical screening [71,72], the system has recently been applied to *in vitro* screening of CYP inhibition [73], Caco-2 (Özbal 2009, (Fig. 19.2) Personal communication), and

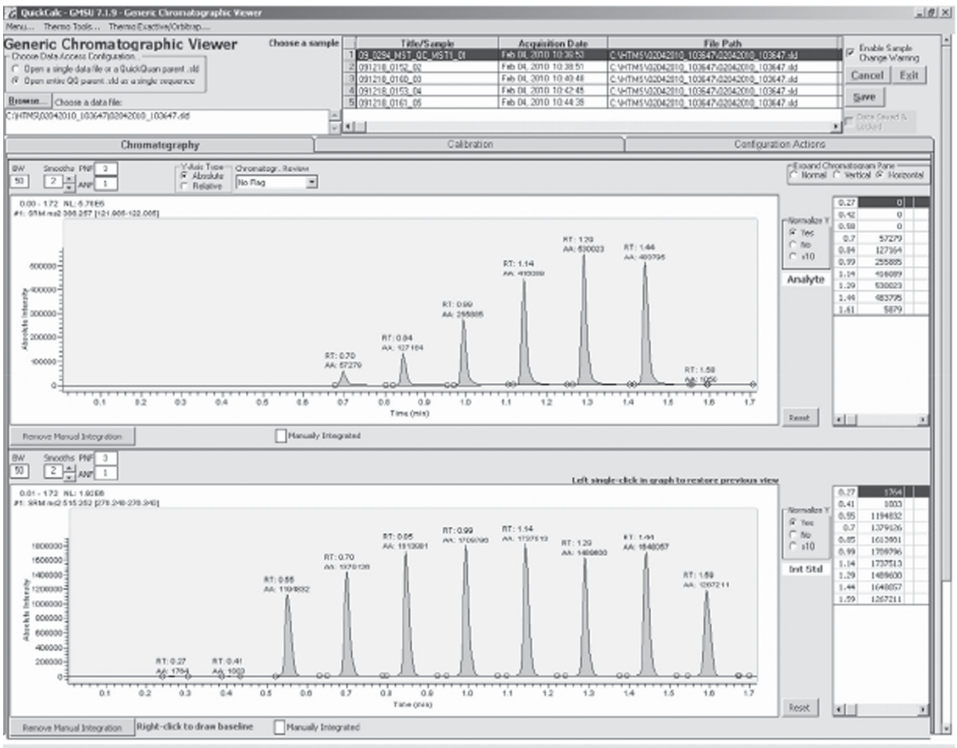
metabolic stability [74] assays. The RapidFire system utilizes three isocratic pumps to deliver a low organic strength mobile phase and two eluents with high organic strength mobile phases delivered at flow rates of 1–1.5 mL/min. Columns similar to those described by Janiszewski *et al*. [51] but with smaller bed volumes are utilized. The system consists of four valves (three high speed–low dead volume switching valves and a four-port valve), an injection needle that utilizes vacuum aspiration of the sample to an injection loop, a set of wash stations, a three-dimensional translatable well plate stage, and a well plate stacker. An injection cycle consists of three distinct steps: load, desalt, and analysis. The valve timings are set so that a single sample can be analyzed every 6–10 s. Recent CYP450 inhibition assay studies screened for inhibition of 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4 isoforms [73]. Good correlation was obtained for data acquired on pooled samples analyzed by RapidFire, MS/MS and a 2-min LC-MS/MS method. Lim *et al*. [73] reported that a 96-well plate was analyzed in 15 min by the RapidFire method, while the LC-MS/MS method required 4 h. A drawback of the reported data was significant carryover, between 1% and 4.1%.

Multiple column functionalities are available for highly polar compound extraction (hydrophobic interaction liquid chromatography (HILIC) extraction media), overcoming a number of the analytical constraints observed by Inman. When coupled with quadrupole TOF system, it is plausible to obtain both quantitative and qualitative information on consecutive injections of the same sample through postacquisition data mining.

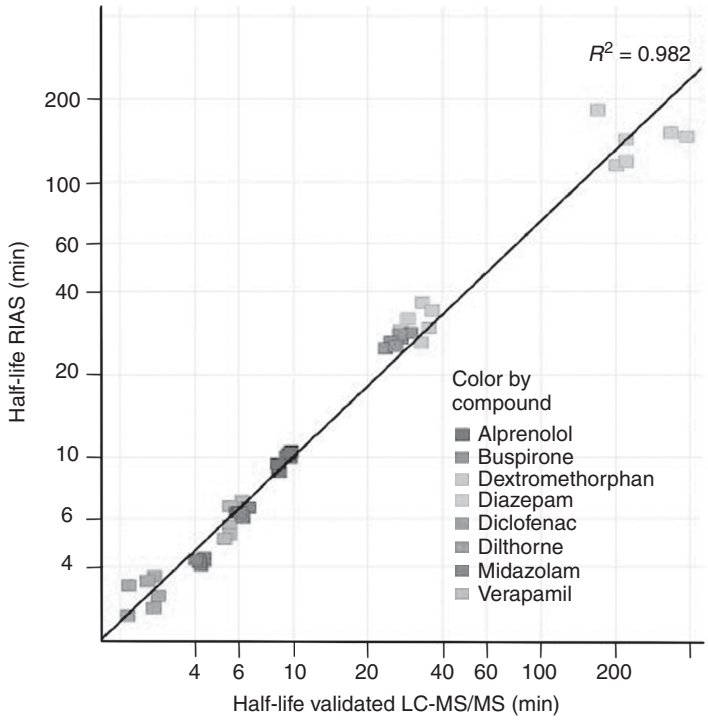
19.8 MULTIPLEXED SYSTEMS

Currently, the most efficient LC-MS systems have maximized MS data collection rate and minimized the time spent detecting noise. These designs have moved away from the serially delivered single LC-MS/MS platform, requiring sacrifices in LC resolving power, while retaining the inherent lag time of LC reconditioning and autosampler processes. When a single analyte is being analyzed in a very fast UHPLC run, the peak width is approximately one-thirtieth (2 s wide/60 s gradient) the time of the separation. Many techniques have been devised to increase the number of analyte signals detected during a single MS acquisition. Multiplexing the number of chromatography columns attached to a single mass spectrometer is a commonly used option. There are three major families of multiplexed experiments: staggered parallel dual-column analysis with off-line column regeneration, true parallel chromatographic separations (MUX), and staggered parallel.

Figure 19.2 (a) An example of data generated using the RapidFire system for metabolic liability analysis, as describe by Luippold *et al*. [74]. Single data files are acquired, following similar preanalytical principles as described in Fig. 19.1, with 8 s per sample acquisition rates. Analyte (top) and internal standard (bottom) are automatically integrated using QuicCalc, area responses are displayed in a tabular format on for rapid data review and release. Of particular note, the process assays the latest time points first (likely the lowest concentration) to minimize errors associated with carryover effects in quantitation. (b) Studies comparing the RIAS (rapid and integrated analysis system, utilizing the RapidFire) and a validated LC-MS/MS assay (10-fold lower throughput). Reference compounds were assayed in duplicate across three separate days using both processes; calculated half-lives ($t_{1/2}$) between platforms indicate excellent correlation. (See color insert.)


QuickCalc - GMSU 7.1.9 - Generic Chromatographic Viewer
Generic Chromatographic Viewer
Choose a sample
Title/Sample
Acquisition Date
File Path
Cancel
Exit
Save
Chromatography
Calibration
Configuration Actions
Analyte
Int Std
Remove Manual Integration
Manually Integrated
Right-click to draw baseline
Time (min)
Absolute Intensity

(a)


R^2 = 0.982
Half-life RIAS (min)
Half-life validated LC-MS/MS (min)
Color by compound
Alprenolol
Buspirone
Dextromethorphan
Diazepam
Diclofenac
Dilthorne
Midazolam
Verapamil

(b)

19.9 STAGGERED PARALLEL DUAL COLUMN WITH COLUMN RECONDITIONING

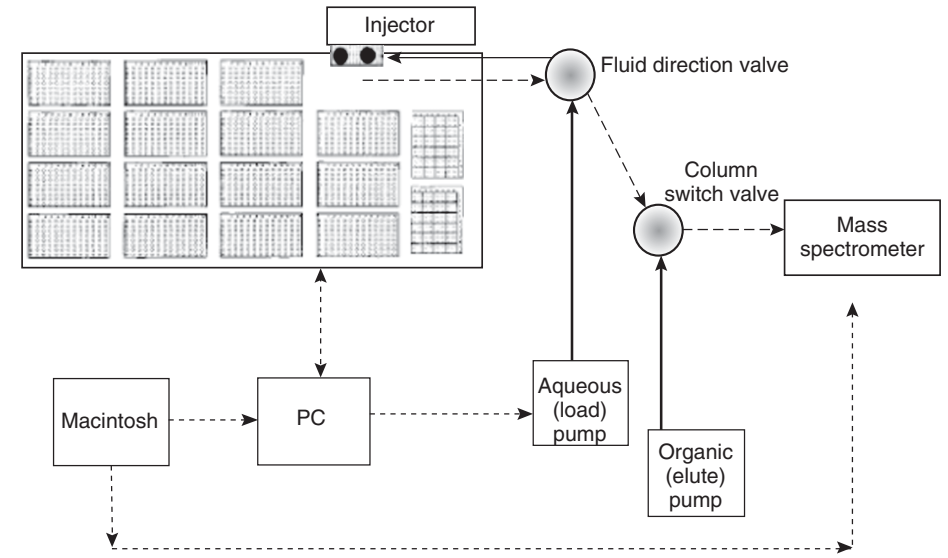
A simple method to overcome the gradient reconditioning time involves a dual-column analytical separation with a secondary pump for off-line column reconditioning. The analytical setup is composed of two 6-port valves, a gradient mixing LC pump, and an isocratic or gradient conditioning pump. This setup enables parallel processing of LC columns to be effected. When an analytical separation is being delivered to the mass spectrometer from the gradient LC pump, the second column undergoes reconditioning with the starting conditions for the gradient separation, isocratically delivered by a secondary LC pump. Subsequently, valve switching occurs to index the reconditioned column in-line to the autosampler and detector for the next assay while reconditioning the previously used LC column. Enhancements such as inject ahead autosampler operation may be employed to further improve throughput. The dual column configuration has been successfully deployed to support a variety of *in vitro* assays.

An example of this analytical setup was described by Janiszewsk *et al*. [51], using an OptiLynx 1 mm $\times$15 mm, 40-μm pellicular pore C18 columns. The system consisted of a dual-arm autosampler with a dual chromatography system to provide column cycling and peak stacking. Two isocratic pumps delivered low and high organic strength mobile phase to the columns, which were set on opposing arms of a 10-port switching valve. The system timing delivered high organic strength mobile phase 18 s after injection onto column 1 to elute the analytes to the mass spectrometer. At 24 s after injection, aqueous flow was diverted through the autosampler and the second sample was injected onto the second column. During this time, the first injection needle was washed before subsequent injections and the process was repeated. The system was utilized for a variety of ADME studies including metabolic stability and Caco-2 assays and was reported to have a capacity to run over 2000 samples per instrument per day (Fig. 19.3).

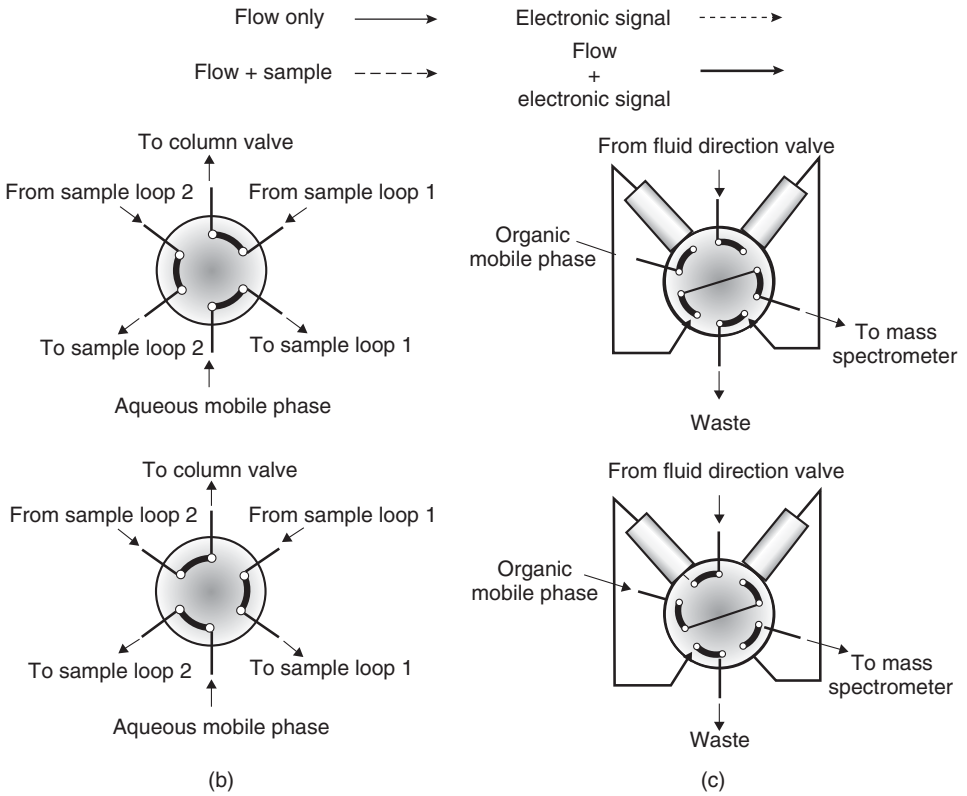
19.10 MULTICOLUMN PARALLEL CHROMATOGRAPHY—SINGLE IONIZATION SOURCE

Xu *et al*. [55] demonstrated the utility of an eight-way "true" parallel LC-MS system for the analysis of metabolic stability samples. The system consisted of an LC pump, Gilson 215 eight-tip autosampler, an AB/MDS-Sciex API 165 mass spectrometer, two Valco valve manifolds, eight 10 mm $\times$ 1 mm i.d., 3-μm particle sized HQC18 PEEK microbore columns, and a Valco switching valve to divert the solvent front to waste.

Figure 19.3 (a) The dual-column high capacity LC-MS configuration, as described by Janiszewski *et al*. [51]. The system schematic indicates the positioning of custom dual injection system and the 17-plate deck of the Gilson 215 multiprobe autosampler, together with in-line selector valves for dual-column/dual-pump operation, all operated under computer and pump signal control. (b) The specific valving configurations for the aqueous solvent flow (sample collection from the dual injection loops, LC column reconditioning, and gradient starting conditions). (c) The valve orientation to provide sample loading and subsequent elution to the mass spectrometer for dual-column analysis.


Injector
Fluid direction valve
Column switch valve
Mass spectrometer
Macintosh
PC
Aqueous (load) pump
Organic (elute) pump

(a)


Flow only
Flow + sample
Electronic signal
Flow + electronic signal
To column valve
From sample loop 2
From sample loop 1
To sample loop 2
To sample loop 1
Aqueous mobile phase
From fluid direction valve
Organic mobile phase
To mass spectrometer
Waste
To column valve
From sample loop 2
From sample loop 1
To sample loop 2
To sample loop 1
Aqueous mobile phase
From fluid direction valve
Organic mobile phase
To mass spectrometer
Waste

(b) (c)

Using an accelerated (ballistic) gradient separation, of ~0.8 min per cycle, the system was capable of running 240 samples per hour. To enable analyte focusing during injection, sample preparation utilized trichloroacetic acid instead of acetonitrile. The group reported that on average, 65–70% of samples were successfully analyzed by the system. Three major reasons for failure included poor compound solubility, weak ion signal, or experimental pipetting/injection errors.

19.11 MULTIPLE COLUMN PARALLEL CHROMATOGRAPHY WITH MULTIPLE ESI SOURCES

A variety of multiple electrospray systems have been studied for many different MS applications [75–78] and were later investigated for the use in quantitative efforts [79–81]. Multiple spray sources can be termed as *nonindexed* or *indexed spray sources*. For nonindexed spray sources, spray is continually sampled by the mass spectrometer from all sources. Indexed sources enable only one channel to be sampled by the mass spectrometer at a time. For quantitative efforts, nonisobaric species must be analyzed from each channel (e.g., mass spectrometrically distinct compounds) when a nonindexed source is utilized.

A simple dual-spray system was described by Hiller *et al*. [81] using a Sciex 3000 triple quadrupole MS system. A Turbospray source with two electrospray and heater units was developed. The dual sprayers were coupled to two separate LC systems each of which had column regeneration capabilities. The autosamplers were synchronized to simultaneously inject sample so that data could be recorded in a single data file to generate a cycle time per compound of 30 s. Utilization of the dual-probe approach generated a twofold improvement in throughput with data quality that was deemed adequate by the authors for discovery-based quantitative applications.

The most widely used multiple sprayer source was the multiplexed electrospray interface, MUX, introduced by Waters in 1999 [75]. The device consists of API interface that composed of either four or eight sprayers. A rotating valve with a machined slot is located between the fixed electrospray probe tips and the skimmer cone of the instrument. The rotating valve serially selects an electrospray probe to sample for a finite period (50–200 ms), while excluding the majority of eluent sampling from the other electrospray emitters. The rotating valve indexes to the next sprayer (50 ms) and so forth to realize a sampling duty cycle of 400–1000 ms for a four-channel system and 800–2000 ms for an eight-channel system. The rotating valve was designed to reduce the gas load on the vacuum system at the interface and eliminate any ion suppression effects caused by the other sprayers. Independent LC columns were connected to each sprayer tip and solvent delivery was provided by either a single LC pump, split across all columns and sprayers, or column-/sprayer-independent LC pumps. Data for each column were encoded into individual channels and into a single data file. With such a system, the same or different analytes could be analyzed on all channels.

Morrison *et al*. [82] and others have demonstrated that the system does suffer from reduced sensitivity related to the duty cycle constraints. Lower flow rates or flow splitting was required for optimal performance to further reduce the load of liquid introduced to the API region. Cross talk between spray channels have also been a body of concern with this method, although selection of the appropriate assay type and

sampling strategy negated this bias [82], as was demonstrated by Fung *et al*. [49] in utilizing the MUX interface to multiplex their Caco-2 permeability assay.

The dual-channel MUX interface is more commonly implemented now and has been successfully employed on TOF and Q-TOF instruments to enable the introduction of reference masses to obtain accurate mass measurements [77,78]. Utilization of a lock mass enables on-the-fly correction of the mass-scale and improves mass axis stability. When coupled with higher resolution measurements, improved mass axis stability enables the use of narrower mass widths (i.e., increased specificity) when extracting the signal for an analyte from the mass chromatogram, leading to improved sensitivity and specificity.

An alternative dual- or four-spray-indexed interface was described by Schneider *et al*. [80] In this interface, an electrospray probe was paired with an ion lens located near the tip of the probe. A voltage was applied to synchronously enable or disable the spray from reaching the entrance lens to the mass spectrometer. The signal emitted from a probe could be fully turned-off or -on rapidly, within 50 ms of the change in the lens voltage.

In terms of throughput, multiple sprayers increase throughput as a function of the number of sprayers introduced into the system. After their initial introduction, these systems have lost favor because of the reduction in sensitivity and increased complexity in terms of MS hardware when compared to other multiplexed systems.

19.12 STAGGERED PARALLEL CHROMATOGRAPHY

The use of staggered parallel technologies has ameliorated a number of the issues associated with the parallel systems described above. The concept of staggered parallel chromatography is shown schematically in Fig 19.4. Sample is injected onto column 1, using an independent LC pump, and most often, acquired to an independent data file. After a fixed time (Δt_{inj}), another sample is injected onto column 2. The same steps are repeated for columns 3 and 4 in a four-channel multiplexing system. Before elution of analytes from column 1, eluent streams (analytes) are delivered into the mass spectrometer by means of a selector valve. Following a predefined acquisition window, column 1 eluent is diverted to waste and column 2 is selected for elution to the mass spectrometer. The same steps are repeated for columns 3 and 4, and the cycle repeats again. The advantage of such a system is that it enables multiple columns to be coupled to a mass spectrometer fitted with a conventional ESI/APCI source for class-specific analysis. The staggered parallel strategy maximizes the time spent by the mass spectrometer detecting analytes, which elute in 3- to 10-s peak widths and minimizes the time spent detecting background and chemical noise. With a minimal increase in run time compared to a single LC system, over a threefold improvement in sample throughput is typically achieved.

The concept was originally described by Van Pelt *et al*. [83] using a single LC with pressure regulation to control the flow to the individual columns and calibrated loops to delay to the delivery of mobile phase to the columns. The concept was refined by using three [84] or four [85] columns with multiple LC pumps and a multiport autosampler for *in vitro* Caco-2 screens. With the four-column system, it was reported that 600 samples could be analyzed during an overnight period (~16 h). These technologies have now been commercialized into a dual-CTC arm,

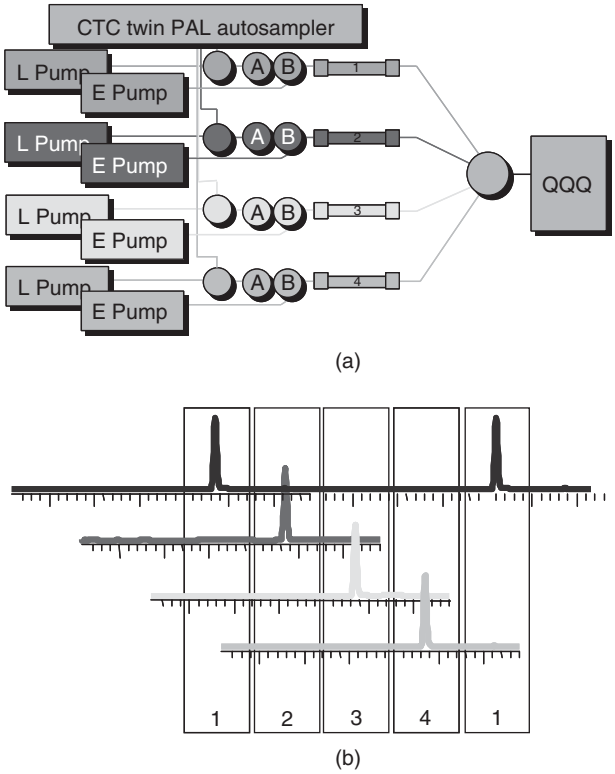


Figure 19.4 (a) The four- or eight-channel LC-MS configuration, as commercialized by Thermo Fisher. The system schematic indicates eight LC pump systems, facilitating either four-channel operation with column switching or eight-channel operation as single dimension LC separations. A dual-arm CTC autosampler with two- to eight-injection valves provides sample aspiration and injection. Dual-switching valves (A/B) enable column switching selection for analysis and a selection valve gates the appropriate LC eluent stream to the triple quadrupole mass spectrometer (QQQ). (b) Sample delivery is staggered in time such that the appropriate eluent stream windows (shaded and numbered) are serially selected for MS detection with an interacquisition delay for independent acquisition file open/closure events. (See color insert.)

two- to eight-pump, and one- to eight-channel staggered parallel system, as shown in Fig. 19.4. Where the same assay is run across multiple channels, there has been concern raised to intercolumn retention time variability. Variability can be reduced by purchasing columns with the same lot of packing material. Briem *et al*. [85] reported that 2000 injections could be made on the columns without notable increase in backpressure or change in chromatographic properties.

19.13 INFORMATICS

As the number of data points generated increases, so does the burden on data processing and reporting. This requires increased efficiency and robustness in the procedures used in method development (discussed above), sample tracking, data analysis, data processing, and posting, and reviewing [39].

Data processing mainly consists of peak integration, generation of calibration functions from fitting standards, and converting sample signal response into a representative concentration and has almost exclusively been developed inside each company by customizing vendor-provided software with the use of visual basic scripting. Most MS vendors have fully automated peak integration routines. However, automated procedures can fail to pick non-Gaussian peaks, especially those at low S/N ratios. These results require manual review and integration, which for large data sets is very time consuming. For single-shot data, the data can be directly posted into the corporate database for the compound tested. For dosing experiments, response curves are plotted and fitted to find the IC_{50} values, which are subsequently reported to the database. Finally, after data posting is completed, the data are reviewed to determine what compounds have favorable or unfavorable *in vitro* ADME properties. Using defined search criteria, the results can be automatically filtered to provide this information. An excellent example of the interconnectivity and rational design of automation, auto-tuning, multiplexed measurement, library searching and final results posting was recently shown by Laycock [40]. Proprietary data processing systems have been developed and described, accommodating many of the principles described so far, namely, single data file acquisition and rapid manual data review processes, to achieve manual data review and release of 96-well plate data in 5 min [86].Ascent™ is a commercial solution to instrument-independent peak processing and automatic data release, which has been developed by Indigo Biosystems. Software algorithms provide a numerous array of chromatographic, analytical troubleshooting, and quality scoring solutions to remove the majority of manual peak review. While these technologies have been predominantly employed within the high throughput clinical diagnostic laboratory, the analytical efficiency needs are similar, and it is anticipated that the quantitative *in vitro* setting will embrace this approach [87,88].

19.14 NON-LC-BASED HIGH THROUGHPUT MASS SPECTROMETRY

A continual theme in the previous sections involved efficiency gains through the sampling capabilities of LC-MS/MS. This has been predominantly achieved by sample pooling, reducing the autosampler and LC cycle times, or multiplexing the number of LC systems connected to the mass spectrometer. An alternate approach is to eradicate the LC dimension entirely. The gains would be simplification of the equipment burden and removal of the cycle time limitation imposed by the autosampler/LC hardware. The obvious concerns are reliability, reproducibility, and robustness of these approaches, particularly in light of the inherent reduction in matrix effects observed when an appropriate LC separation is utilized. *In vitro* ADME samples are less complex than plasma-based samples and may be more amenable to such approaches. A newer suite of technologies have been proposed, including MALDI or the recently introduced ambient ionization techniques including desorption electrospray ionization (DESI), direct analysis in real-time (DART), or laser diode thermal desorption–atmospheric pressure chemical ionization (LDTD-APCI). Each of these techniques proposes the potential for no or minimal up-front sample preparation before analysis and depends on product ion MS/MS or single ion reaction monitoring to provide an effective method filtering out the response of contaminant species.

19.15 LASER-BASED IONIZATION METHODS

One alternative that was commercialized under the product name FlashQuant consisted of a MALDI ionization source coupled to a triple quadrupole instrument (MALDI-QQQ) [89–92]. Samples were spotted onto MALDI target plates with matrix and were analyzed by rastering the MALDI plate through the laser beam. The fastest sample analysis rates ever reported were achieved by this equipment at 1.2 s per sample (Fig. 19.5) to enable analysis from 384 wells in <7.7 min [93].

Since a triple quadrupole was utilized, SRM transitions were used to provide compound specificity and removed the difficulties of detecting small molecules in the presence of interfering ions. This technique did not resolve ion suppression effects

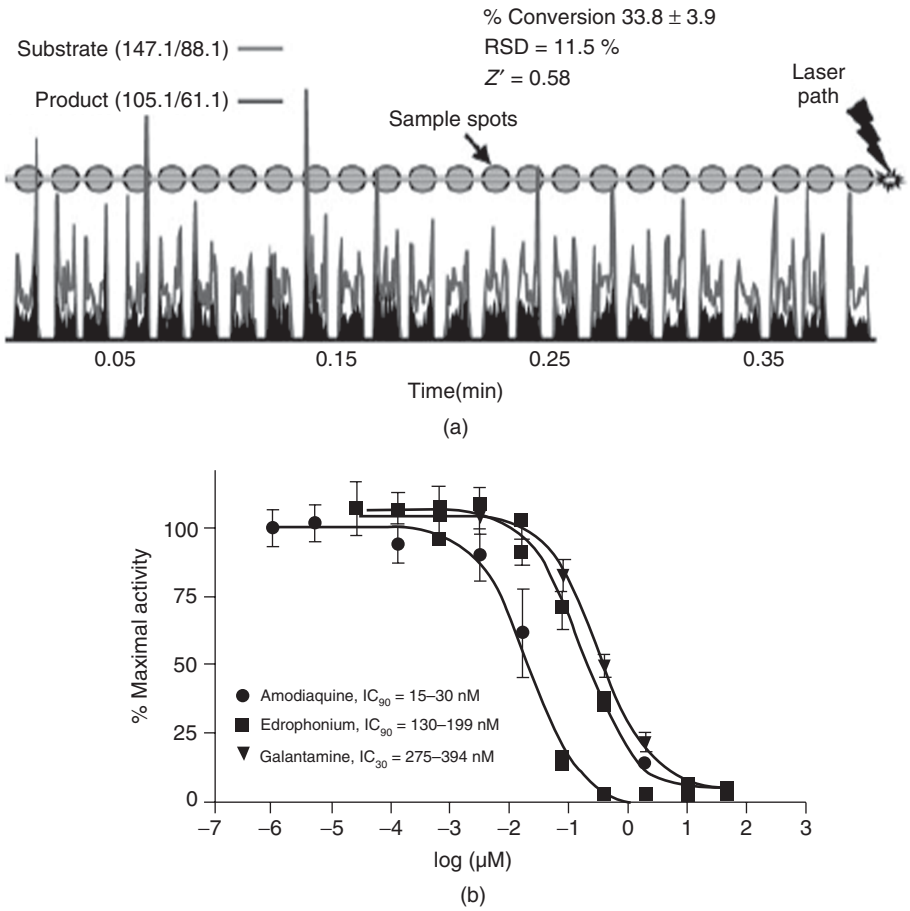


Figure 19.5 (a)MALDI-QQQ readout of the substrate acetylcholinesterase (light gray) and enzyme product, choline (black) used to probe AChE inhibitors as described by Rathore *et al*. [93]. Rastering rate of the laser beam was set at 9 mm/s, identical to the spot width of a 384-well plate, realizing a sampling rate of 1.2 s per sample, accounting for plate stage movement. Reproducibility (%RSD) of measurement of substrate and product was <12%. (b) Ten-point concentration-dependant inhibition assays for AChE against known inhibitors, results are plotted from triplicate analysis with mean and ±standard deviations shown.

from the presence of salts, proteins, and other molecules, which will generally reduce sensitivity. For less complex biochemical systems, no sample clean-up was typically required [93]. However, for more complex sample mixtures such as microsomes or plasma, an off-line liquid–liquid extraction or SPE clean-up step was typically required, limiting the overall process sample throughput. The effect of ionization suppression effects was illustrated by Kovarik *et al*. [92]. Using protein precipitation of plasma and 4 times washing *in situ* on the MALDI plate, acceptable accuracy and sensitivity of 1 ng/mL was obtained for propranolol. Addition of an off-line LC separation using a 1-mm LC column with fraction collection before spotting improved analytical sensitivity to 50 pg/mL.

The throughput of MALDI-QQQ clearly demonstrates the capability to acquire data representative of high throughput, even at the single analyte per spot rate; however, new bottlenecks such as spotting compound and matrix to MALDI plates would be added to the workflow, which require additional automation and degrade the overall process efficiency. Further, the original FlashQuant system lacked a plate-stacking capability, thus removing the capacity for sustained, unattended automated analysis.

Another laser-based methodology that has recently been introduced for high throughput quantitative MS is LDTD-APCI [94]. In this ionization source, 2–10 μL of sample are spotted into a 96-well plate made of a polypropylene with stainless steel well walls. The plate sits on an $x-y$ translatable stage, and the sample is moved to align with the source assembly. Once in position, a plunger from the source is placed into the well to seal off the sample well. An infrared (IR) laser thermally desorbs analyte into the vapor phase; vaporized sample is swept through a transfer tube by a carrier gas (air), to a corona discharge needle for ionization, before sampling by a mass spectrometer.

Wu *et al*. monitored competitive inhibition for the CYP isozymes 1A2, 2C9, 2D6, and 3A4 using human liver microsomes. For the metabolic probe molecules monitored in this study, acetaminophen, 4′-hydroxydiclofenac, dextrorphan, and 6β-hydroxytestosterone, the signal obtained for 5 μM of each analyte in 0.25 mg/mL human liver microsome data were similar and, in some cases, better than data generated by flow-injection with APCI or ESI. When operated in fully automated mode, a 96-well plate was analyzed in 18 min for a sample throughput of 11.3 s per sample.

19.16 THE FUTURE PROMISE OF AMBIENT IONIZATION METHODS

During the past five years, many new ionization techniques have been introduced in the field of MS, which enable direct analysis of samples with minimal or no sample clean-up. These ionization techniques have been classified as ambient ionization techniques. The fundamental principles and applications for ambient ionization are described in more detail in this volume in the chapter titled ***A Perspective on the Evolving Field of Ambient Ionization Mass Spectrometry***. An inherent advantage of these techniques is the generation of ions in an "open-source" before transmission into the mass spectrometer; thus, samples can be introduced from alternate technologies to the mass spectrometer (i.e., from glass slides or capillaries, thin-layer chromatography (TLC) plates, or paper). The major constraint for analyzing a batch of samples is physically introducing the samples (on a sampling device) to the mass spectrometer.

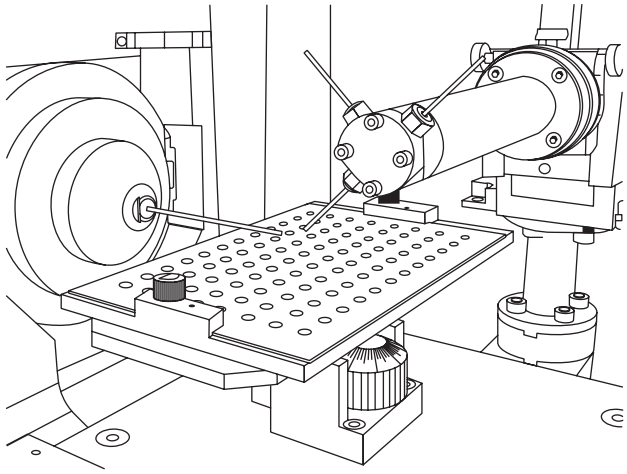
A second potential advantage these techniques offer is eliminating time spent developing a specific separation method along with concomitant reduction in SRM method development time.

DESI and DART have been utilized to provide quantitative information at increased throughputs from biological samples. Initial reports demonstrated many utilities of the DESI source for the detection of specific analytes from a variety of surfaces [95]. The high throughput capabilities were demonstrated by moving pharmaceutical tablets using a conveyer belt at a rate as fast as 0.33 s per sample [96]. For liquid samples, a DESI source with a 3D stage (automated in two dimensions) has been developed and interfaced to a linear ion trap mass spectrometer by Manicke *et al*. [97] (Fig. 19.6). They demonstrated the ability to analyze samples containing either propranolol (PRN) or carbamzepine (CBZ) at a sampling rate of 2.5 s per sample for analytes in 10% urine or porcine brain total lipid extract from a 96-sample array plate for with a total analysis time of ~3 min per plate. Moreover, good precision data were obtained for the chosen analytes: relative standard deviation (RSD), 10% from urine and <5% from brain lipid extract with deuterium-labeled internal standards. Full scan MS mode enabled a limit of detection of 10 fmol for PRN from a neat solution and increased to 400 and 200 fmol for 10% urine and brain lipid extract samples, respectively. As expected, the biological extracts increased the observed chemical noise. Notably, these detection limits could have been markedly improved with the use of MS/MS scan on the ion trap or a triple quadrupole operated in SRM mode.

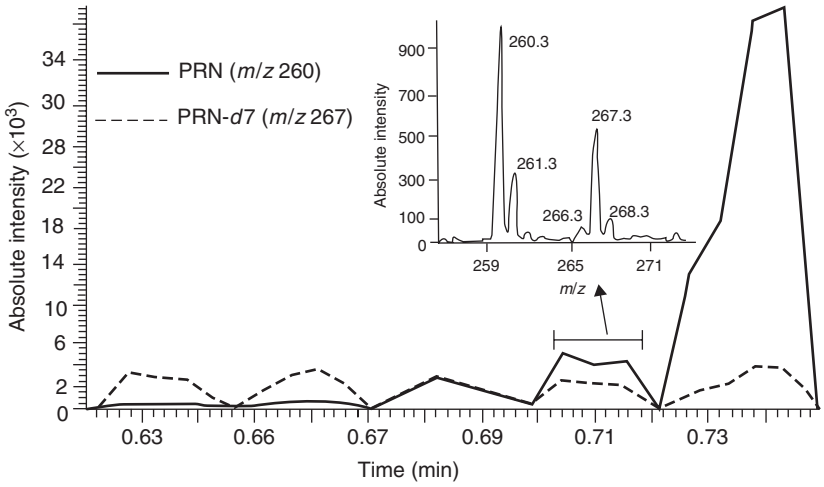
One key attribute of DESI is the low protein ionization efficiency, compared to small molecules and peptides, resulting in the potential for very little sample preparation before analysis. Additional ionization modes may be explored, such as desorption atmospheric chemical ionization (DAPCI) [98]. An automated DESI source coupled to a tandem mass spectrometer would be highly applicable for a full suite of *in vitro* ADME techniques, once automated plate handling coupled to the source and automated spotting from 96- and 384-well plates were implemented. For example, for metabolic stability, samples from various time points could be analyzed quantitatively and qualitatively using the same sample spots if Q-TOF, linear ion orbitrap, or quadrupole ion trap mass spectrometers were utilized. Using many of the same principles discussed above (sample pooling and using multiple probe molecules), CYP inhibition assays could be performed at the fastest cycle times ever recorded.

The DART ionization source developed by Cody *et al*. [99,100] has also been shown to perform well in a variety of qualitative and semiquantitative analyses. Liquid

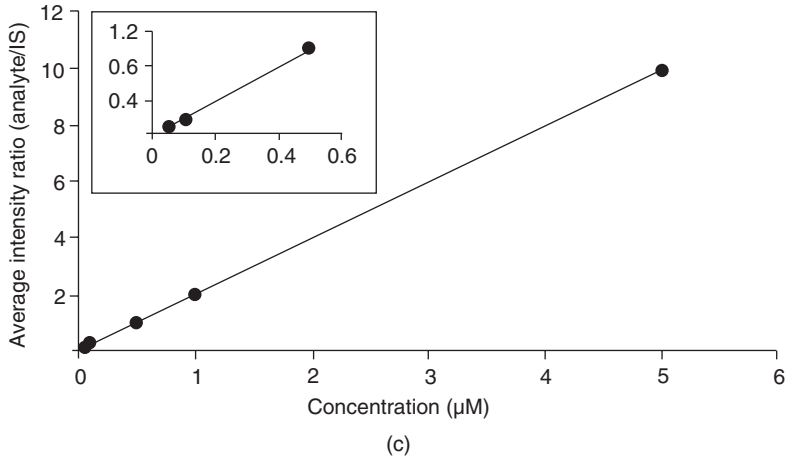
Figure 19.6 (a) A schematic diagram of the automated 2D stage DESI sampling system, as described by Manicke *et al*. [97]. The "96-well" layout is composed of a glass slide and raised printed hydrophobic ink containing polytetrafluoroethylene (PTFE) in the geometrical arrangement of a standard 96-well plate. The custom built $X-Y$ moving table aligns the appropriate spot to the DESI sampling probe for analysis, following positional and incident angle alignment of the DESI tip. (b) The sampling rate of the system of 1.5 s per sample from neat solutions, composed of 0.5-s interwell movement and 1 s of spot analysis. Propranolol (PRN) (solid line) and propranolol-*d*7 (PRN-d7) internal standard (dashed) chromatograms are shown from an expanded region of the 96-well plate analysis. (c) The representative calibration curves (intensity ratio vs concentration for propranolol) generated from neat solutions of propranolol and stable labeled internal standard ($N = 5$ replicates per concentration level). Error bars indicate reproducibility (%RSD) of 5–10% for the lowest standard and <5% at other concentrations.



(a)


PRN (m/z 260)
PRN-d7 (m/z 267)
Absolute intensity (×10³)
Time (min)
260.3
267.3
261.3
266.3
268.3
Absolute intensity
m/z

(b)


Average intensity ratio (analyte/IS)
Concentration (μM)

(c)

samples are introduced into the ionization source region using a glass TLC microcapillary tube, solvent is evaporated, and ions are generated by chemical ionization processes. Cycle times in the <5 s per sample regime have been shown, from the time of sample introduction until complete evolution of the analyte from the tube. With these principles in mind, Yu *et al*. [101] developed a DART source interfaced with an automated sampling system to enable analysis of drugs from plasma samples using a triple quadrupole mass spectrometer. A critical modification to enhance the sensitivity of the DART source was an additional pumping interface between the exit of the DART source and the entrance of the ABI 4000 mass spectrometer. This reduced the gas load of He produced by the DART source to enable sufficient pumping by the mass spectrometer and contained analyte enriched carrier gas to the API interface. The second improvement involved automating the procedure for transferring sample from a plate to the ionization region using a HTC-PAL autosampler with a glass dip tube instead of a syringe for sampling.

Matrix effects varied for each analyte. For example, praparacaine gave a response of 5.4% of the neat solution response when sampled from plasma. Linearity was achievable for the compounds studied, and the sensitivity was within the range that was usable for bioanalytical studies, that is, verapamil was detectable at a level of 0.1 ng/mL. The overall sensitivity was 2–10 times lower than that achieved by electrospray for the compounds surveyed. The cycle time for each sample was at ~0.8 min per sample. The sampling rate was dictated by the speed of the HTC-PAL for fetching and placing samples into the source. This time could be further reduced using a multiplexed HTC-PAL system in which the next sample could be dipped while the current sample is analyzed.

The authors noted that DART ionization is unable to maintain intact glucuronide conjugates during the ionization process, thereby yielding higher apparent amounts of the parent molecule. Moreover, the system is limited to analyzing molecules with a molecular weight (MW) of <1000 Da.

Recently, IonSense has announced a 3D sampling stage for the DART source, the SVP 3+D scanner at the 2010 Pittcon Conference (Musselman 2010, Personal communication). It is likely that cycle times for this implementation of DART will compare favorably with DESI and FlashQuant.

The application of laser-based and ambient ionization techniques can truly increase the throughput achieved by quantitative MS. By eliminating the LC, the complexity of the analytical system is reduced. These techniques show great capability to reduce sample analysis times from minutes down to seconds. However, for these techniques to be successfully implemented, mechanisms to automatically handle plate loading and removal from the source region must be made available along with the ability to store and access a large number of plates. Moreover, new advancements in data handling and processing will also need to be developed.

19.17 SUMMARY

The realization of high throughput quantitative MS has required a significant internal investment of resources for the development of customized workflows and human interfaces. In many instances, the techniques being employed have achieved the 1–2 s per analyte goal of "high throughput," most notably in minimizing the dead time associated

with each discreet event and removing manual processes. A number of inefficiencies still remain within the process, most notably the LC separation time domain and post-analytical data review. The nascent potential of ambient ionization methods may play a significant role in eliminating these steps to match the analytical throughput of the mass spectrometer. Recently developed instrument-independent quantitative peak processing tools may also further align these throughput efficiencies.

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