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Review

A review of LC–MS techniques and high-throughput approaches used to investigate drug metabolism by cytochrome P450s☆

Kuresh A. Youdim*, Kenneth C. Saunders

Pfizer Global Research and Development, Pharmacokinetics Dynamics and Metabolism, Sandwich, Kent CT13 9NJ, UK

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ABSTRACT

One of the major challenges facing the pharmaceutical industry today is finding new ways to increase productivity, decrease costs whilst still ultimately developing new therapies that enhance human health. To help address these challenges the utilisation of analytical technologies and high-throughput automated platforms has been employed; in order to perform more experiments in a shorter time frame with increased data quality. One of the main *in vitro* techniques to assess new chemical entities in a discovery setting has been the use of recombinant liver enzymes, microsomes and hepatocytes. These techniques can help predict *in vivo* metabolism, clearance and potential drug–drug interactions of these new compounds by cytochrome P450s (the major drug metabolising enzymes). This *in vitro* methodology has been totally transformed in recent times by the use of automated liquid handling and HPLC tandem mass spectrometry detection techniques (LC–MS/MS). This review aims looking at recent advances in the methodology used to investigate drug metabolism by cytochrome P450s; including an up to date summary of high-throughput platforms including the use of automation and LC–MS/MS to facilitate greater throughput, chromatographic resolution and data quality.

Contents

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* Corresponding author. Tel.: +44 01304 649196; fax: +44 01304 651987.
E-mail address: kuresh.youdim@pfizer.com (K.A. Youdim).

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1. Introduction

The "drug metabolizing enzymes" (DMEs) are a diverse group of proteins that are responsible for metabolizing a vast array of xenobiotic compounds including drugs, environmental pollutants, and endogenous steroids and prostaglandins (http://www.invitrogen.com/etc/medialib/en/filelibrary/pdf.Par.58399.File.dat/O-12758DMEGuide_Intro.pdf). These enzymes can be separated into two groups, namely oxidative and conjugative. Oxidative enzymes, largely contribute to so-called Phase I metabolism, which include cytochrome P450s (CYPs) and flavin monooxygenases (FMOs), which catalyse the introduction of a reactive oxygen atom into a lipophilic compound. If the metabolites of Phase I reactions are sufficiently polar, they may be readily excreted at this point. Phase II reactions involve the addition of an endogenous substrate with either the newly incorporated functional group derived following Phase I metabolism or to that of a pre-existing functional group. This serves to increase polarity further facilitating excretion from the body. The cytochrome P450 family of enzymes have to date received the greatest attention owing to their role in the metabolism of the majority of drugs in humans. In this regard, CYPs 1A2, 2C9, 2C19, 2D6 and 3A4 together have been reported to be the major route of metabolism for almost 75% of the 200 most commonly prescribed drugs [1].

A requisite of any drug entering the marketplace is an understanding of its potential to affect one or more of these CYPs and elicit a drug–drug interaction (DDI); where the maximum plasma concentration of any one or two drugs co-administered together increase by more than 2-fold. For certain drugs this results in toxic concentration in the circulation which ultimately results in hospital admissions [2] and if reactions are significantly adverse, potentially drug withdrawal from the marketplace [3]. Given that the simultaneous co-administration of multiple drugs is common place increases the likelihood of clinically significant interaction, in particular in patients receiving multiple therapies for one disease (e.g. HIV infection) or treatment for several diseases concurrently [4,5]. The pharmaceutical industry has had to invest significant resources to understand these risks, since the progression of compounds with potential DDI liabilities will lead to labelling restrictions as well as having a potential competitive disadvantage.

Screening for unfavourable drug-like properties, such as interactions with cytochrome P450s early on in the drug discovery process can potentially avoid the excess cost of developing unfavourable drug candidates. A screening strategy is designed and implemented to provide the greatest amount of quality data to inform project decisions early in discovery, but at the same time minimise overall costs [6]. In order to meet this challenge, a significant amount of effort is concentrated towards implementation of targeted *in vitro* assays at the different stages of the drug discovery process. These *in vitro* assays are executed with sample preparation in miniaturized micro-plate formats using advanced automated liquid handling technologies; producing high numbers of experiments and samples [7]. These samples are then analysed using a number of high-throughput analytical platforms based around rapid high-performance liquid chromatography (HPLC) and tandem mass spectrometric detection (Table 1). These analytical platforms provide increased efficiency in running these screens with robust, multi-analyte quantitation capability.

This article will summarise the mass spectrometric technology, HPLC configurations, high-throughput platforms and automation utilised in the field of drug metabolism by cytochrome P450s. A summary of most recent applications of these technologies will be described in the various *in vitro* studies carried out in the pharmaceutical discovery arena.

2. Mass analysers for liquid chromatography

Due to the high specificity, speed and selectivity offered by HPLC–MS/MS, this approach has long been adopted in the pharmaceutical industry to assess certain properties of drug molecules, such as metabolic stability. Given the large number of mass spectrometer types available and that their application differs between laboratories, means the approaches used for metabolism studies will inherently differ from laboratory to laboratory. As such the data quality and reliability of the results strongly depend upon which instrumentation is optimal for the task [8]. A brief overview of the mass spectrometers used in the assessment of new chemical entities (NCEs) and their associated metabolites will be covered.

2.1. Quadrupole mass analysers

Single-Stage quadrupole mass spectrometers (SSQMS) as well as triple-stage quadrupole mass spectrometers (TSQMS) are commonly used by the pharmaceutical industry for both qualitative and quantitative studies [9]. Whilst quadrupole mass analysers have the ability to operate in both negative and positive ion modes, specific advantages of SSQMS instruments include low cost and their relatively small size, whilst TSQMS instruments have greater discrimination against chemical background resulting in real gains in selectivity and sensitivity. In TSQMS, the Q1 mass analyser filters the desired ions such that they are fragmented by Ar or N_2 within Q2, and their fragment ions are subsequently scanned by Q3 before reaching the mass detector. Consequently, given that TSQMS acquires much richer, higher value datasets than SSQMS and in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) modes would suggest it to be the instrument-of-choice in routine and high-throughput quantitative bioanalysis. However detection sensitivity decreases dramatically when wide mass range is analysed in a scanning mode; which can be a limitation in its application for screening of '*unknown*' drug metabolites.

2.2. Ion-trap mass analysers

Like quadrupole instruments, ion traps are relatively inexpensive and compatible with a wide range of systems. Ions generated are focused towards the centre of the trap allowing measurement of all ions retained in the trapping step. Consequently, sensitivity losses during the full-scan mode are avoidable. Whilst TSQMS retains sensitivity advantage for quantification when operated in SRM mode, ion-trap instruments provide more sensitivity for structure elucidation than TSQMS. This is due to the fact that ion traps can obtain richer mass spectra, with more efficient trapping and scanning of ions; this MS mode can be more structurally informative when compared to triple quadrupole or Quadrupole time-of-flight (Q-TOF)-mass spectrometers. The ion-trap analysers can be used for quantification in full-scan mode, with little difference between in sensitivity in SIM or MRM modes. When operated in a full-scan mode, the sensitivity gains, ability to measure a wide mass range and acquisition of full-scan data can make these instruments ideal for screening-type applications in which qualitative information is paramount (such as metabolite identification studies) [10]. With the advent of increased computer power and data storage, increased capture of full-scan data can enable mining of this qualitative information at a later date, when more is known about a compounds metabolism. These instruments have traditionally shown increased variability at the limits of detection due to the slow ion accumulation time. This coupled with the relatively slow data acquisition rate, limits their use in high-speed quantitative LC–MS applications, such as fast UPLC analyses. In recent years, linear ion traps have been developed. Their configuration is similar to quadrupoles, with a barrier at the end to prevent ions exiting. Ions

Table 1
Overview of recent automated high-throughput approaches used to assess metabolic instability.

Automation platform/liquid handlers	Mass spectrometer	Data processing tool	Data collection tool	MS/MS optimization required	Sample preparation	Analytical platform	Assay capacity	Run time* (min)	REF
Tecan Genesis RSP 150	LTQ linear ion trap	SmartReport; Microsoft Visual v6.0 & Thermo Fisher Xcalibur® Developers Toolkit v1.3	SmartReport (customized) and Xcalibur™	Yes	Precipitation	RP; Phenomenex Luna C18-2 (2 mm × 30 mm; particle size 5 μm)	96	2	[93]
Tecan Genesis RSP 150	DECA XP+ ion trap	SmartReport; Microsoft Visual v6.0 & Thermo Fisher Xcalibur® Developers Toolkit v1.3	SmartReport (customized) and Xcalibur™	Yes	Precipitation	RP; Phenomenex Luna C18-2 (2 mm × 30 mm; particle size 5 μm)	45	1.5	[24]
Hamilton Workstation STARPLUS	Micromass 4 Ultima Platinum TQ	Microsoft® Excel	QuanLynx	Yes	Precipitation	RP; ACE C18 (4.6 mm × 50 mm; particle size 3.5 μm) Column switching to waste	96 Increased further via sample pooling	2.5	[33]
Tecan EVO	Perkin Elmer Sciex API3000	Microsoft® Excel	Analyst v1.1	Yes	Precipitation	RP; Phenomenex Luna C18-2 (2.1 mm × 30 mm; particle size 5 μm) Column switching to waste	96	3	[29]
Biomek® FX	Quantum Ultra TQ	Excel & STARscreen	QuickQuan™ 2.0 and Xcalibur™	Yes	Precipitation	RP; Pursuit XR C18 (2 mm × 20 mm; particle size 3 μm) Dual injection column switching	48	1	[34]
Not reported	Perkin Elmer Sciex API3000	Not reported	Not reported	Yes	Precipitation then Cohesive Turbo Flow on-line extraction	RP; YMC C18 basic (2 mm × 50 mm; particle size 5 μm) Column switching to waste	Not reported	4.6	[19]
Packard Multiprobe™ II EX HT	Micromass Quatro Micro™ TQ	SAS V8.2	Mass Lynx v4.0	Yes	Precipitation	RP; Dual trapping cartridges (Keystone Aquasil C18 (2.1 mm × 10 mm; particle size 5 μm) Keystone Aquasil C18 (2.1 mm × 50 mm; particle size 5 μm)	Not reported	2.5	[94]
Biomek® FX	Perkin Elmer Sciex API3000	Customized script using Microsoft Visual Basic 6	Analyst v1.1	Yes	Precipitated, dried then reconstituted	SP; Atlantis dC18 (2 mm × 100 mm; particle size 5 μm)	96	5	[95]
Multimek ™ 96-channel and Biomek® 2000 multichannel	Applied Biosystems/MDS-Sciex API165 single quadrupole	Microsoft® Excel Scripts	Not reported	Yes	Precipitation & filtration using Unifilter® PKP 0.2 μm plate	RP; Eight samples injected simultaneously onto 8 separate HQ-C18 microbore columns (1 mm × 10 mm; particle size 3 μm) Column switching to waste	96	1.25	[17]
Biomek® 2000 multichannel	Micromass QToF2	Not reported	Not reported	No	Precipitation	UPLC; Acquity BEH C18 (2.1 mm × 50 mm; particle size 1.7 μm)	16	2.5	[41]

*Time between injection; TQ, triple quad; RP, reverse phase; SP, stationary phase.

are held between the quadrupole rods and experiments are carried out in a similar fashion to cylindrical ion traps [11]. The ions are then either ejected sideways through the quadrupole rods or via the end of the rods for subsequent detection. As the construction of these linear traps is very similar to quadrupoles, hybrid instruments are available, where both tandem and trap MS functionality can be used (for both quantitative and qualitative applications).

2.3. LTQ-Orbitrap mass analysers

LTQ-Orbitrap is a hybrid, high-resolution mass spectrometer composed of a 2-D linear ion trap (LTQ) front-end that is coupled to an electrostatic ion trap on the back-end (Orbitrap). Ions are formed traditionally using a wide variety of ionization techniques: APCI, ESI, and APPI (see Section 3). The orbitrap provides very high mass resolution for ions delivered by LTQ used as a pre-selection of measured ions. Given this, the LTQ-Orbitrap is an effective alternative to the TOF instruments used for metabolite profiling [12]. Also, this instrument is capable of high sensitivity screening over a wide mass range and tandem mass spectrometry with accurate mass data for both parent and fragment ions. The LTQ-Orbitrap, like other ion-trap systems suffers from a slow data acquisition rate compared to TOF instruments and hence, is not suitable for very fast chromatography applications.

2.4. Time-of-flight mass analysers; TOF, Q-TOF

TOF MS involves measuring the time taken for an analyte ion to travel from the ion source to the detector. As ions have different masses, but similar energy, they are separated according to velocity as they pass down the flight tube. Ions of low mass reach the detector before those of higher masses. TOF instruments are generally used with electrospray ion sources in which ions are pulsed orthogonally into the flight tube. This together with the use of electrostatic mirrors can enable operation at very nigh mass resolution. This increased mass resolution can help with the accurate determination of undefined metabolites, hence TOF instruments are primarily used for metabolic identification studies [13]. Quadrupole time-of-flight (Q-TOF) mass spectrometers are relatively simple, and capable of recording all the ions produced in the source on a microsecond time scale offering increased sensitivity. The time taken for an ion to reach the detector is proportional to its m/z ratio and this is used to differentiate the ions mass down the path of the instrument flight tube. Q-TOF instruments have the ability to operate with relatively high mass resolution and to make accurate mass measurements, providing a degree of selectivity since it is able to discriminate between interference and among mass peaks having similar nominal masses but different exact masses. This instrument can operate at relatively high scanning rates which makes them ideal for use with high resolution liquid chromatography (LC) methodology such as ultra performance liquid chromatography (UPLC).

3. Ionization methods

The application of atmospheric pressure ionization (API) methods in particular electrospray and pneumatically assisted electrospray, have provided a breakthrough for the combination of liquid separation techniques with mass spectrometry. The two most significant API techniques used to date have been atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) [8]. Ionization of non-polar compounds at atmospheric pressure was made possible following the introduction of atmospheric pressure photoionization (APPI) [8,14].

3.1. Atmospheric pressure chemical ionization (APCI)

In APCI, the HPLC eluent components and analytes are vaporized before the initialization of ionization process by a corona discharge needle [15]. APCI is well suited for the analysis of neutral compounds and has proven to be more adaptable than ESI requiring less sample clean-up and is easily interfaced with mobile phase flow rates of conventional chromatography columns (1 mL/min). This has become less of a concern following the introduction of the pneumatically assisted electrospray interface. In general, APCI is a mass-flux sensitive detector where sensitivity is dependent on the volatility of the compounds.

3.2. Electrospray ionization (ESI)

ESI is a technique ideally suited to polar, thermally labile compounds requiring ionization of the analyte within solution prior to introduction into the ion source and thus works best for fairly basic or acidic compounds. This technique is commonly employed in the drug discovery field since the physicochemical properties of drugs are ideally suited to it. The ions in solution are emitted into the gas phase without the application of heat. $[M+H]^+$ or $[M-H]^-$ ions are generated from very labile compounds with no thermal degradation [21]. Depending on the chemical structure of an analyte, multiply charged molecular ions can also be formed, a significant advantage in the analysis of biological macromolecules such as peptides and low molecular weight proteins.

3.3. Atmospheric pressure photoionization (APPI)

A new form of APCI for LC/MS application, atmospheric pressure photoionization [16], has facilitated the analysis of unionizable or poorly ionizable compounds. The HPLC eluent is evaporated in the same way as with APCI (heated nebuliser), but significantly, a UV lamp replaces the corona discharge present on an APCI source. Photons are used to ionise gas phase analytes at atmospheric pressure. Direct ionization can occur when photons the analyte to give radical ions, M^+. Alternatively a dopant can be used, where the dopant APPI process involves the addition of a dopant compound, such as toluene, into the nebulisation process. The photons first ionise the dopant molecules which in turn act as reagent for the formation of analyte ions (MH^+) in an APCI-type process. APPI can give the capability to analyse non-polar neutral compounds that are not as amenable to APCI or ESI techniques for example neutral steroids.

4. Approaches used in a high-throughput screening process

Screening hundreds of compounds ideally requires a process flow that incorporates functions such as sample preparation, qualitative compound purity and integrity verifications, has adaptable and sophisticated quantitative MS method selection, bioanalytical sample analysis, data processing, and one where result reporting is coordinated by a sample and data management software. Given that high-throughput screening is characterized by screening hundreds of different compounds that have no regulatory submission concerns, the screening process can be dissected in five distinct steps [6]:

- Plate management of compounds in need of *in vitro* ADME (absorption, distribution, metabolism and excretion) data.
- Optimization of the MS/MS method for the compounds.
- Automated *in vitro* ADME experiments and sample clean-up.
- Collection and reduction of the raw LC–MS/MS data.
- Archival of the processed ADME data.

4.1. Automation approaches

Automation for use in drug metabolism *in vitro* ADME studies can be utilised to varying degrees depending on the data required to advance discovery projects. Fully automated systems are available that are adapted to high capacity and simple assay protocols [7], these tend to be implemented at early stages in the drug discovery continuum where sparse sampling (e.g. single time point) screens are often operated. These systems are optimised for the relatively high compound numbers encountered. A fully automated approach can be adopted, using a centralised robotic arm integrated with other robotic liquid handlers, which carry out the assays [17]. These systems are designed for fully automated sample preparation, data analysis and management of results generated from *in vitro* ADME assays. Although these systems take longer to develop and require more capital investment, they can be very successful in situations where maximum efficiency, short data feedback times, high-throughput and unattended (overnight) operation is required. Introduction of semi-automated approaches for *in vitro* ADME assays have the advantage of offering sufficient flexibility to operate different assay configurations, enabling manual intervention at crucial stages of the assay. It also provides a robotic platform that is easily reconfigured when assays evolve.

4.2. Sample preparation

Sample preparation is a key step in quantitative bioanalysis and can potentially be a bottleneck in the process in developing robust and efficient screening methodologies. This is attributed to the complex nature of macromolecular compounds, such as proteins and non-volatile endogenous substances, that have to be removed from the *in vitro* sample and separated from the analytes to eliminate matrix interferences prior to the LC–MS/MS analyses. Due to the high specificity of LC–MS/MS detection, sample preparation is typically achieved by protein precipitation with an organic solvent such as acetonitrile and subsequent on-line HPLC separation [18]. The advantage of using a protein precipitation technique is that this sample preparation stage is usually incorporated into the *in vitro* assay protocol, by utilising it as the actual termination step.

Lai and Khojasteh-Bakht [19] reported the use of an automated on-line method that gave a good approximation of the prodrug conversion, as compared to the conventional protein precipitation method. The authors suggested that their approach offered multiple advantages including immediate sampling and analysis of the incubation sample, eliminating pipetting, deproteination, drydown, and reconstitution steps (as in the conventional method), and saving time, space and cost of equipment and solvents. Moreover the method was argued to be able to provide more accurate internal timing and minimise errors from multi-step transfers in sample preparation that sometimes lead to compound degradation. The method also generated real-time chromatographic results during incubation such that the operator can adjust time points as they see fit.

An alternative approach is solid phase extraction (SPE). In this regard, Kerns et al. [20] have reported an on-line alternating parallel SPE column with MS/MS detection. However, this system required a cycle time of 1.1 min, and has the risk of potential carry over from the use of the same SPE cartridge. Qi and Danielson [21] described customized mini-SPE method with nano-electrospray mass spectrometry with improved sensitivity. Davies et al. [22] also described an automated SPE-LC–MS/MS system with a 2.5 min cycle time and the use of an analytical column. More recently Inman et al. [23] demonstrated how eluting metabolic stability samples directly from a SPE card into the mass spectrometer for MS/MS analysis circumvents the lengthy HPLC run used in routine ADME analysis. When combined with sample pooling, their SPE-MS system acquired 480 data points in 1hour on a single MS instrument.

4.3. LC–MS; a pre-requisite for high-throughput metabolism studies

Due to the structural diversity and large number of the NCE compound sets in the discovery phase, there is a significant challenge to develop compound specific bioanalytical methods in a relatively short time frame. The most time-consuming step in establishing a high-throughput approach to study a drug property can be the method development to quantify low concentration levels of compounds in a biological matrix (for example microsomal incubations, hepatocyte culture, etc.). This challenge together with subsequent analysis of large numbers of *in vitro* samples requires both robust, specific and sensitive bioanalyis for ADME assays. In this regard, the speed and sensitivity associated with liquid chromatography/tandem mass spectrometry (LC–MS/MS) lends itself to supporting high-throughput analyses from drug-metabolism studies. However, recent advances in ion-trap mass analysers make these systems a suitable alternative [24].

Drexler et al. [24] recently described an iterative two-step process addressing some of these processes involved in a screening strategy. Compounds were initially analysed by LC-ultraviolet (UV)/MS for purity (UV detection) and identity confirmation (MS detection) in the structural integrity (SI) assay, ensuring that the correct and verified compound with sufficient purity is used in an assay. This should not necessarily be assumed to be the case, especially in a high-throughput screening (HTS) environment where compounds are processed by an automated central compound management system [25]. Analytical information was gathered in up to four different ionization and polarity modes under "universal" full-scan MS/MS conditions that provide the necessary details required for the automated development of specific and sensitive MS methods for the subsequent quantitative LC–MS analyses. The need to quickly obtain optimized selected reaction monitoring MS/MS conditions for hundreds or even thousands of discrete compounds per week, and to easily review, archive and retrieve the optimized conditions for subsequent sample poses a significant challenge. Although there are differences in the infrastructure of HTS groups across the pharmaceutical industry, there is a commonality in the systems being used. Owing to the need for sensitivity and efficiency most laboratories use tandem MS (MS/MS) scan functions (i.e. selected reaction monitoring [SRM] or full-scan MS/MS), which monitor the transition of collision-induced dissociation (CID) of the precursor ion, preferably the deprotonated $[M-H]^-$ or protonated molecular ion $[M+H]^+$, to product ion(s). The MS/MS data acquired by selecting the $[M+H]^+$ or $[M-H]^-$ ions and the declustering potential and collision energy are optimized using step functions and the methods generally saved in a suitable database [6]. This approach has utilised software tools either developed in-house [26–28] or from vendors [29,30].

There have been multiple attempts however to reduce/eliminate this MS/MS method optimization step through use of alternative mass analysers. These have included obtaining SRM MS/MS parameters under a 'universal' collision-induced dissociation condition on ion-trap mass spectrometers for MS/MS [24] and single quadrupole mass spectrometers analyzing samples without MS method development [31]. Support of *in vitro* screens by LC–MS quantitation without the need for MS/MS method development has also been demonstrated using TOF analysers with accurate mass measurement [32]. Following the optimization of these properties they are generally archived within a suitable accessible application for assessing the ADME properties of NCEs.

4.4. HPLC approaches used to facilitate high-throughput LC–MS analysis

High-throughput analytical systems have been adapted to accommodate the large sample numbers and the challenge of measuring multiple analytes simultaneously in a complex biological matrix. Examples of approaches/techniques that have been developed, include but are not limited to on-line sample preparation [19], cassette dosing or analysis of compounds (i.e. where multiple compounds are administered simultaneously to animal or samples pooled and simultaneously analysed) [33], and staggered parallel high-performance liquid chromatography/tandem mass spectrometry [34]. Further efficiency gains can be made through the use of different LC–MS/MS approaches. These include the use of parallel cartridge based systems, narrow-bore and short HPLC columns, monolithic HPLC columns and high pressure systems and are briefly discussed below.

4.4.1. Conventional and narrow-bore HPLC

These analytical platforms are set up to use a conventional HPLC system capable of operating a fast binary gradient with narrow-bore (approximately 3.2 mm i.d. or less) short (50 mm or less) analytical column. The analytical columns are packed with small particles (1.8–3 μm) and operated at higher than optimal column flow rates, this to enable faster analysis cycles with a more efficient gradient per unit time. A fast reversed-phase gradient is employed to enable shortest possible analytical run times with acceptable chromatographic resolution of the analyte from any spiked probe substrates; as these components can be present at relatively high concentrations in the sample. Additionally this chromatographic set up, should give the resolution required to separate the interfering components from the sample matrix (in the shortest possible time), so as to minimise any ion suppression phenomena [35]. This phenomenon can also be reduced by injecting less sample, or diluting with an appropriate aqueous solvent, whilst ensuring acceptable detection limits are maintained.

4.4.2. Parallel HPLC systems

This analytical approach has been developed to support higher throughput screening assays, using more simplified experimental protocols, where the HPLC–MS/MS systems are set up to analyse the larger sample numbers [36]. These systems are necessary as the HPLC run time can become the rate limiting step in the assay work flow. In settings where high throughput is paramount, cartridge columns can be employed in a parallel system, which allow the analyte components to elute off simultaneously, thus fully utilising the specificity of the MS detection system. Multi arm, multi injections port auto samplers linked to two or more columns can be set up, in order that HPLC cycle times of less than 30 s can be realised [37]. The samples eluting from the different cartridges are switched into, staggered or "pipelined" into the mass spectrometer for analysis. Due to the vast number of samples analysed and subsequent chromatograms generated, various automated methods for data handling are used [6,27].

In a similar manner more conventional separations using gradient or isocratic column switched HPLC systems can be configured for higher throughput. A HPLC column switching system is configured with 2 or more HPLC columns into a single mass spectrometer. Samples are injected alternately onto each column in a staggered manner and the mass spectrometer is utilised to continually monitor the elution of the analyte peaks.

4.4.3. Monolithic HPLC

Reversed-phase monolithic LC–MS/MS has been successfully implemented to give improvements in efficiency and resolution for *in vitro* assays [38]. Monolithic silica columns can be operated at higher flow rates and lower back pressures compared to other HPLC columns, Additionally their higher permeability makes them ideal for some bioanalytical applications where high throughput and robustness to biomatrices is required [39,40]. Monolithic phase HPLC can also be used for fast reversed-phase gradient separations. This fast binary gradient HPLC system allows for direct analysis of *in vitro* samples that have had minimal sample pre-treatment (e.g. protein precipitation using acetonitrile). A divert valve is often used to direct the HPLC flow to waste at the start of the analysis and therefore reduce the introduction into the mass spectrometer of polar components, non volatile salts, soluble proteins and other endogenous material. Employment of a fast gradient achieves separation of the analytes from the remaining endogenous material. The fast gradient systems are simple to set up, requiring only a binary HPLC pump and a single switching valve. These switching valves are often integrated on many modern mass spectrometers, therefore easily controlled and configured using the mass spectrometer software.

4.4.4. Ultra performance liquid chromatography and fused core technology

Ultra performance liquid chromatography (UPLC) employs particles smaller than 2 μm in diameter to achieve superior resolution, speed, and sensitivity compared with HPLC [32,41]. The benefits of UPLC vs. HPLC were originally demonstrated for small molecules (<500 Da) with reversed-phase columns. Significant improvements in resolving power, sensitivity and separation speed were demonstrated for many different applications [42], using MS as the primary detection end point. These benefits derive through the use of the separation material of very fine particle size (sub 2 μm) and core chemistry used in UPLC columns. This stationary phase creates a higher working back pressure, due to the increased number of particles packed per unit volume packed in the UPLC column. To enable fast separations on this material, the column hardware and instrument have significant design modifications from typical HPLC. UPLC systems can operate at higher column back pressures (up to 15,000 psi). The systems inject samples into a smaller system dwell volume (to preserve the high-efficiency separations), capture detector signals at fast scan rates for fast eluting peaks, thus lowering of limits of quantitation (LOQ). This improved resolution has helped separation of complex mixtures such as metabolite profiling in biomatrices, where chromatography separation of structurally similar metabolites can be very important (due to the analytes being unable to be resolved by the mass spectrometer). Very fast UPLC separations have been used to resolve the multi-analyte mixtures in *in vitro* samples [43]. Further sensitivity enhancement can be gained by optimization of the detection method and ion source (e.g. ESI-MS, APCI-MS), as mentioned previously, these factors are dependent on matching the detection endpoint to the analyte properties (e.g. polarity).

Fused core or core shell HPLC technology has been utilised for similar high-throughput bioanalytical applications as an alternative to sub 2 μm UPLC technology, without the use of such high column back pressures [44]. These phases are designed primarily for speed with very fast separations arising from both the small particle size (2.7 μm) but also from the particle technology that creates a thin porous shell (0.5 μm) of stationary phase fused to a solid core particle. Fused core columns have the potential to deliver more separating power per unit time than columns of the same length packed with conventional phases. This means that shorter columns operated at higher flow rates can be used to achieve remarkably fast high resolution separations, without the back pressure observed with sub 2 μm UPLC set ups. This has the advantage of utilising conventional HPLC instrumentation and not requiring a dedicated UPLC instrument with high pressure capability.

Consequently, the use of short HPLC columns packed with either small particle sizes in UPLC or fused core (1.5–2.5 μm) configurations has therefore been utilised to good effect for high-throughput separations [45]. When compared to established HPLC techniques, these approaches can yield an increase in resolution of analytes and/or metabolites, throughput and therefore increase data quality. To achieve this goal, there has been a recent trend in bioanalysis to shift HPLC ADME assays towards UPLC systems. Due to the crude nature of the samples that are injected onto these type of systems (acetonitrile precipitate supernatant), smaller sample injection volumes must be used to avoid column instability or blockages. This can be balanced with the advantage of using less sample (and hence expensive reagents); improved MS sensitivity gained from the sharp chromatographic peaks and more importantly enhanced chromatographic resolution per unit time.

5. Approaches used in the assessment of cytochrome P450 mediated metabolism

Drug-metabolism studies have a key role in medicinal chemistry, extending to lead optimization, detection of potentially toxic metabolites, and in the identification of the rate(s) and route(s) of drug clearance from the body. A particular example of early-stage cytochrome P450 liability profiling of drug candidates is the assessment of metabolic stability. Data from these studies, utilising liver microsomes from different species (e.g. human, rat, mouse, dog) can be employed to predict *in vivo* clearance rates of compounds [46]. Given that chemistry departments are now able to produce larger numbers of compounds, there has been a move to employ higher throughput approaches, to provide the data in a timely fashion such that it can have impact on the decision to advance a drug and on drug design efforts [7].

Applications of high-throughput microsomal stability screening in drug discovery are based on the premise that *in vitro* microsomal stability data correlates with *in vivo* PK parameters such as plasma clearance. To this end there have been a number of reports of the past few years having described the most recent application of automated high-throughput approaches used in assessing the metabolic liability of NCEs (Table 1). Whilst reverse phase chromatography is widely used for these types of studies, the recent introduction into the field of bioanalysis of UPLC has allowed significant improvements in analytical speed and chromatographic resolution [47]. Several studies have illustrated the capabilities of UPLC/TOFMS for the determination of metabolic routes [42,48,49]. O'Connor et al. [32] reported a 96-well plate based metabolic stability assay where using TOF detection, full-scan data was acquired, with run times of 2.5–3.5 min, from which narrow window extracted ion chromatograms were generated, producing quantitative data for the test compound equivalent to that obtained by HPLC with tandem mass spectrometric detection on a triple quadrupole instrument. The use of TOF detection offered two advantages over MRM detection for bioanalytical applications; i) specific MS methods for each analyte are not required, since specificity for the analyte is derived from the extraction of narrow window extracted ion chromatograms from full-scan data and ii) preliminary evaluation of metabolic routes are collected at no cost in terms of sensitivity to the test compound or method development time.

As has already been alluded to, a common goal in ADME screening is development of a generic LCMS/MS method to simultaneously determine a wide range of NCEs and their metabolites in *in vitro* or *in vivo* samples. RP chromatography is as stated above the most widely employed technique in pharmaceutical analysis. However, for the determination of NCEs or existing drugs with lower octanol–water partition coefficient, log *P*, it is very challenging to establish a reliable RP-LC–MS/MS method. This can be due to the fact that many polar compounds/metabolites show little to no retention on traditional RP columns, under HPLC conditions where the mobile phase pH has not been evaluated with respect to the analytes charge and thus potential interaction/retention with the stationary phase. Hydrophilic interaction chromatography (HILIC) with low-aqueous/high-organic mobile phase is emerging as a valuable supplement to the reverse-phase-HPLC–MS/MS for the retention of polar analytes such as amino acids and pharmaceuticals [50–54]. In contrast to normal phase HPLC, where elution is promoted by the use of polar organic mobile phase, in HILIC an appropriate amount of water (usually 5–15%) in the mobile phase is suggested for maintaining a stagnant enriched water layer on the surface of the polar stationary phase where the analytes partition. Using this technique compounds by eluting with a strong organic mobile phase against a hydrophilic stationary phase where elution is driven by increasing the water content in the mobile phase. The highly volatile organic mobile phases such as methanol and acetonitrile used in HILIC provide low column back pressure and also an increased API ionization efficiency for MS/MS detection. A review of literature found that no high-throughput studies have been published using HILIC. Readers are however, referred to a recent review by Hsieh [51] which eloquently presented the potential of HILIC-MS/MS in quantitative bioanalysis of individual drugs and metabolites.

6. Mass spectrometry in studies of drug biotransformation

Understanding the metabolic fate of a drug is highly important, since some metabolites could potentially be (a) pharmacologically active [55], (b) toxic [56], (c) involved in drug–drug interactions *via* inhibition or induction of drug metabolizing enzymes [57,58]. Active metabolites may have superior pharmacology, pharmacokinetics and safety profiles compared to their respective parent molecules. Although metabolite characterization has only recently become important to drug discovery, it has been a valuable part of the drug development and approval process for several decades [59,60].

The main requirements for metabolite identification are good chromatographic separations, full-scan sensitivity and exact mass in full-scan mode and MS/MS [8,61]. Detection of common metabolites can be carried out using full-scan MS followed by extracted ion chromatographic analysis or using list-dependent tandem mass spectrometric (MS/MS) acquisition methods [62]. Table 2 shows the utility of different mass spectrometers in these endeavours. In this mode, full-scan MS acquisition is employed as a survey scan to search for predicted metabolite ions listed in an acquisition method. Once a listed metabolite ion is found, the MS/MS acquisition of its product ion spectrum is triggered. The list-dependent approach allows the detection of common metabolites and the acquisition of their product ion spectra in a single LC/MS run [63]. Recently, a novel mass defect filter (MDF) technique has been reported [64,65], which enables high-resolution mass spectrometers to be utilised for detecting both predicted and unexpected drug metabolites based on narrow, well-defined mass defect ranges for these metabolites. This approach is completely different from, but complementary to, traditional molecular mass- or MS/MS fragmentation-based LC/MS approaches.

Typically, the samples to be analysed vary greatly, differing in source, i.e. *in vitro* or *in vivo* samples. *In vitro* samples tend to produce less complex results than *in vivo* samples and, therefore, are easier to analyse [66]. In most cases, when analyzing *in vitro* samples, only the major metabolites are reported. At this stage of the drug-screening process, it is important to have evidence about the major metabolic route of the drug of interest. By contrast, *in vivo*

Table 2
Common type mass spectrometers and the features applicable to *in vitro* drug metabolite profiling (adapted from [8]).

	Optimal use	Full-scan detection sensitivity	Mass accuracy (exact mass measurement)	Data acquisition rate for fast chromatography	MS/MS capability (elucidation of site of biotransformation)	Linearity of response (quantitative studies)	Precursor ion or neutral loss scanning	Detection sensitivity for unknowns
Time-of-flight (TOF)	Fast metabolite screening; identification of biotransformations	High	Good	High	No	Good	No	High
Quadrupole-TOF (Q-TOF)	Fast metabolite screening; identification of biotransformation and their sites	High	Good	High	Yes	Good	No	High
Ion trap	Fast metabolite screening; identification of biotransformation sites	Moderate	Poor	Low/moderate (3D trap/linear trap)	Yes	God	No	High
Triple quadrupole	Supplemental MS/MS data for elucidation of biotransformation sites	Low	Poor	Low/very high (scan mode dependent)	Yes	Very good	Yes	Very high
Linear ion trap-triple quadrupole	Metabolite screening; identification of biotransformation sites	Moderate	Poor	Moderate/very high (scan mode dependent)	Yes	Very good	Yes	Very high
Orbitrap	Metabolite screening; identification of biotransformation and their sites	Moderate	Good	Low/moderate	Yes	Moderate	No	High

samples are more complex because they contain many endogenous compounds, and the xenobiotics tend to be present at much lower concentrations than in the corresponding experiments *in vitro*. Typically, the metabolites are not clearly visible in the total ion current chromatogram and, therefore, their detection is difficult [65]. This is especially true for first-in-human experiments when knowing the circulating metabolites might provide valuable information to refine the strategy for clinical development [67]. The use of radio-labelled compounds makes this process easier [68], but by the time the labelled compound is available, resources might have been wasted.

A number of recent reviews have discussed in detail the role of bioanalysis in metabolite identification [8,61,69–71]. A consequence of recent advances to address high-throughput technology for screening the metabolic liability of compounds is the demand for more rapid methods for metabolite identification [72–74]. The bioanalytical approaches which appear most amenable to support these demands utilise UPLC and HILIC [32,41,51], whose applications have already been presented earlier in the context of metabolic stability studies.

7. Approaches to investigate enzyme inhibition

The preceding sections have discussed the application of mass spectrometry tools and analytical platforms in assessment of NCEs and their associated metabolites to understand metabolic liability. This information is not only useful in understanding the rate of clearance but can also be used to estimate the contribution certain P450s make to the overall metabolism of a NCE (fraction metabolized; Fm). This value is important when it comes to predicting the likely magnitude of a clinical interaction, should this NCE reach the market and need to be co-medicated with another drug that may inhibit that specific route of metabolism, thus increased exposure of the drug potentially leads to a toxic effect. In order to understand a risk of co-medication with another drug, one would need to have an understanding of that drugs ability to inhibit the enzyme responsible for that clearance route. In this regard assessment of the compounds inhibitory potential (of pertinence to this review; cytochrome P450s) needs to be examined.

A number of *in vitro* assays to assess CYP inhibition have been developed for drug discovery. The differences between these systems are enzyme source and composition, i.e. human recombinant CYPs (rhCYPs), human liver microsomes (HLMs), probe substrates, and detection methods (radioactivity, fluorescence, luminescence and LC–MS/MS) [75]. Although DDI fluorescent inhibition assays are amenable to high-throughput screening configurations, they can be more prone to assay interference issues (i.e. resulting from natively fluorescent test compounds and/or fluorescent quenching by the test compound). In contrast, LC–MS/MS set-ups have the advantages of higher coverage of test compound chemical space, specificity and robustness when compared to other LC based detection end points such as UV and fluorescence. Cytochrome P450 inhibition assessed using human liver microsomes with LC–MS/MS platforms and specific drug probe substrates [76] has long been endorsed by the Food and Drug Administration (FDA; http://www.fda.gov/cder/drug/drugInteractions/tableSubstrates.htm#inVitro). Using this LC–MS/MS methodology was initially seen to be considerably more expensive to set up, labour intensive and required relatively long analytical run times. Until more recent times, this has meant LC–MS/MS has not been that easily amenable to high-throughput demands required in an early drug discovery setting.

Advances in these high-throughput analytical approaches have been applied and culminated in numerous laboratories reporting the utilisation of cocktail incubation assays or screens [38,77–90]. Here, the specific cytochrome P450 probes are added as a mixture (or cocktail) in a single experiment and the test compounds added to this to evaluate multiple CYP450 DDI activities simultaneously. Identifying the appropriate incubation time and amount of microsomal protein required to establish initial rates of metabo-

Table 3
Overview of recent cocktail approaches and the cytochrome P450 probe substrates used to assess inhibitory potency of new chemical entities against cytochrome P450.

Cytochrome P450s	Probes	Instrument	Set-up	Run-time (min)	Reference
CYP1A2 CYP2A6 CYP2B6 CYP2C8 CYP2C9 CYP2C19 CYP2D6 CYP2E1 CYP3A4 CYP3A4	Melatonin Coumarin Bupropion Amodiaquine Tolbutamide Omeprazole Dextromethorphan Chlorzoxazone Midazolam Testosterone	Micromass Quattro Micro™ triple quadrupole	Multiple reaction monitoring (MRM) mode using polarity switching between positive and negative ion modes	8	[77]
CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	Tacrine Diclofenac S-mephenytoin Dextromethorphan Midazolam	Perkin Elmer SCIEX API4000	ESI Positive ion mode	<1	[78]
CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	Tacrine Diclofenac S-mephenytoin Dextromethorphan Midazolam	Perkin Elmer SCIEX API4000	ESI Positive ion mode	2–4	[79]
CYP1A2 CYP2B6 CYP2C8 CYP2C19	Phenacetin Bupropion Amodiaquine Omeprazole	Waters Alliance 2690	ESI Positive ion mode	13	[80]
CYP2C9 CYP2D6 CYP3A4	Tolbutamide Dextromethorphan Midazolam	Waters Alliance 2690	ESI Positive ion mode	13	[80]
CYP2B6 CYP2C8 CYP3A5	Bupropion Amodiaquine Midazolam	Micromass Quattro Micro™ triple quadrupole	ESI Positive ion mode	5	[81]
CYP2C9 CYP2D6 CYP3A4	Diclofenac Bufuralol Midazolam	Micromass Quattro Micro™ triple quadrupole	ESI Positive ion mode	3	[82]
CYP1A2 CYP2A6 CYP2B6 CYP2C8 CYP2C9 CYP2C19 CYP2D6 CYP2E1 CYP3A4	Phenacetin Coumarin Bupropion Paclitaxel Tolbutamide S-mephenytoin Dextromethorphan Chlorzoxazone Midazolam	PE SCIEX API 3000	ESI using polarity switching between positive and negative ion modes	6.5	[83]
CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	Phenacetin Tolbutamide Omeprazole Bufuralol Midazolam	Finnigan TSQ 7000 triple-stage quadrupole	ESI Positive ion mode	5.5	[84]
CYP1A2 CYP2A6 CYP2C8 CYP2C9 CYP2C19 CYP2D6 CYP3A4	Ethoxyresorufin Coumarin Paclitaxel Tolbutamide Omeprazole Bufuralol Midazolam	Finnigan TSQ 7000 triple-stage quadrupole	APCI Positive ion mode	4	[84,85]
CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4 CYP3A4 CYP2E1	Phenacetin Tolbutamide* Omeprazole Dextromethorphan Midazolam Dextromethorphan Chlorzoxazone*	Finnigan TSQ 7000 triple-stage quadrupole	APCI Positive ion mode ESI Negative Ion Mode used for compounds marked*	<4	[86]
CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4	Ethoxyresorufin Diclofenac S-mephenytoin Bufuralol Testosterone	Micromass Quattro Micro™ triple quadrupole	ESI Positive ion mode	15	[87]
CYP1A2 CYP2C9 CYP2C19 CYP2D6 CYP3A4 CYP3A4	Phenacetin Tolbutamide S-mephenytoin Dextromethorphan Testosterone Midazolam	Micromass Quattro Micro™ triple quadrupole	ESI Positive ion mode	<1	[88]

lite formation for all the enzyme/substrate pairs with minimal substrate depletion are imperative in establishing a robust CYP cocktail assay. In this regard use of probe mixtures some of which exhibit rapid metabolism, i.e. midazolam and diclofenac and those with the slow metabolism, i.e. (*S*)-mephenytoin provide one of the biggest challenges not only establishing optimal experimental conditions but also the sensitivity of the analytical system being used to measure metabolite formation. When considering the biochemical construct of such experiments, the reaction velocities observed in the cocktail assay relative to the single substrate reactions under the same conditions should be comparable. Zientek et al. [90] recently showed that the lower rates seen in their cocktail approach could not be ascribed to any specific interference between CYP reactions. Importantly the impact on the derived IC_{50} did not impact the accuracy of the DDI readout. A likely explanation may be isoform competition for NADPH reductase and/or for cytochrome b_5 [91]. The velocity differences observed with the cocktail format vs. single substrate format could imply specific isoform affinity differences for the CYP reductase.

This cocktail approach for each experiment allows simultaneous determination of a test compounds DDI activity towards a number of DMEs (Table 3) without significantly compromising detection selectivity or sensitivity. This experimental configuration has a number of advantages; the mass spectrometer is utilised to quantify all of the probe metabolites simultaneously, less samples are required and this in turn increases throughput and significantly reduces reagent costs (~5–6 fold). DDI cocktail approaches have been applied successfully providing that safeguards and control samples are put in place in the assay work flow. In this regard probe substrates and their metabolites present on the cocktail mixture should exhibit minimal interference or detection cross talk with each other. In addition, where ever possible isotopically labelled internal standards should be included to help correct for any suppression effects that may occur. Table 3 highlights a number of recent publications where different approaches and analytical setups have been successfully applied to measure, as part of a cocktail mixture, P450 probes metabolites. Moreover, the analytical run times reported in these various approaches range from as long as fifteen minutes to less than one minute. Given the volume of samples potentially being generated in higher throughput inhibition screens, analytical assays that are short, robust, reliable and reproducible are favoured over longer run times [78,88]. Findings from the authors laboratory [78] have shown that low volume injections of 1–5 μL achieved using a novel "sandwich injection" technique are possible; here a small volume of sample is sandwiched between two higher volumes of aqueous solvent in the autosampler syringe and then the whole sandwiched sample injected into the sample loop. This provides reproducible introduction of low volumes of biofluid extracts and band focusing of the analytes, resulting in an eloquent separation of six P450s metabolites and their respective isotopically labelled internal standards, in less than 30 s. Importantly, the approach demonstrated sensitivity gain for *S*-mephenytoin, the most analytically challenging probe of the cocktail mixture. The small sample volume together with this separation ensured that all the analytes were separated from the solvent front and any endogenous interference from the matrix. Peng et al. [88] have also reported a method where samples containing six substrates and an internal standard are separated and detected in only 24 s. This was achieved via a generic method which used an ultrafast liquid chromatography with tandem mass spectrometry. The method utilised a monolithic silica rod column to allow fast flow rates to significantly reduce chromatographic run time. These two examples exemplify how analytical approaches can be set up to meet the demands of screening large volumes of compounds in as an efficient manner as possible, but without comprising on quality of data.

8. Future directions

Understanding DDI through clinical studies is both costly and resource intense. Mitigating the potential for DDIs during the discovery phase ensures that clinical studies can be performed further down stream within the development phase of a NCEs lifetime, during which time other clinical studies may highlight deficiencies that result in it being discontinued. Combinatorial chemistry/parallel synthesis has resulted in a large amount of substrate for assessment of DDI potential. Whilst this has led to a heavier reliance on *in vitro* screening assays in order to cope with the large numbers of compounds processed during early discovery, it has enabled companies to establish *in silico* models that can provide early risk assessment, even before compounds are synthesised. *In silico* modelling can be described as the use of computers to model how chemical structures will behave either *in vitro* or *in vivo* via computer-aided simulations. The current *in silico* DDI methodologies generally focus on the main CYP enzymes (CYP3A4, CYP2D6, CYP1A2, and CYP2C9). High-throughput data generation has yielded vast amounts of screening data which has enabled multiple approaches to computational prediction. As a result, these *in silico* models could potentially provide the first-line screen for virtual compounds and compound libraries, thereby highlighting potential DDI liabilities at a very early stage.

Improvements in the efficiency of the analytical process are constantly being sought after in the pharmaceutical industry. The next generation of mass spectrometers are becoming more and more sensitive, giving the scientist more options in terms of ADME assay configuration. Increased MS instrument capability is continually being investigated by the major LC–MS vendors. Features include faster scanning rates, high resolution full-scan data acquisition, ion mobility (for matrix noise reduction), surface ionization techniques (for isolated samples) and hybrid instruments. These features allow the scientists increased experimental flexibility with more valuable information yielded from each sample. Simultaneous quantitative and qualitative assessments could be performed with very fast scanning TOF instruments, for example analysis of ADME *in vitro* samples generated for metabolic clearance and metabolic profiling studies.

When the HPLC system is established for a particular assay, other formats and scalability could be further explored, with a view to achieving miniaturisation and high-throughput analyses. The assay formats can be scaled down to 384 and 1536 micro plate formats, enabling savings on cost of reagents and performing many experiments on a single plate.

Capillary and nano LC configurations are attractive both from a mass sensitivity and environmental perspective. This is an area of emerging interest as samples are decreasing in size using isolating small volumes (micro sampling techniques) for subsequent analysis or on surfaces (for example dried blood spots). This may give rise to assays specific LC stationary phases and microchip-based formats. These lab-on-a-chip techniques can combine nanoscale chromatographic separation and delivery of analytes into the mass spectrometer in a single small chip. These techniques are already commercially available and enable sample analysis from submicroliter sample volumes [92].

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