In Vitro ADME Medium/High-Throughput Screening in Drug Preclinical Development[§]

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Abstract: The study of the ADME features of the huge number of new chemical entities (NCEs) produced mainly by combinatorial chemistry has become a bottleneck in the drug development process. In response the pharmaceutical industry is involved in the development of new medium/high-throughput screening capabilities. The aim of this paper is to review some of the available *in vitro* ADME systems adapted to screening requirements together with the technological approaches which can be linked to medium/high-throughput molecular screening.

[§]*To Prof. Senén Vilaró, Scientist, colleague, and friend. You will be always in our memories.*

Key Words: ADME, metabolism, screening, HTS, mass spectrometry.

INTRODUCTION

Since the 90s, combinatorial chemistry, genomics and proteomics have enormously increased the number of compound candidates capable of conversion into drugs. Among the major reasons for termination of development of new chemical entities (NCE) other than efficacy, the dominating factor is toxicity and unfavorable pharmacokinetic properties including poor absorption, distribution, metabolism and excretion (ADME) features. Absorption and metabolism determine to a great extent the pharmacokinetic properties of most drugs, unusual ADME parameters are behind bioavailability problems, inter-individual variations, metabolic interactions, idiosyncrasies and so on. Drug regulatory agencies such as the Food and Drug Administration (FDA) [1] and the European Agency for the Evaluation of Medicinal Products (EMEA) [2] have released different guidelines, enhancing the importance of ADME studies. This has forced the pharmaceutical industry to integrate ADME studies into the early discovery process to identify compounds that are likely to present unsatisfactory pharmacokinetic properties in later stages [3]. Nowadays, several *in vitro* preclinical screening methods are being used dealing with important issues like: absorption, inhibition/induction of drug metabolizing enzymes, compound metabolic stability, identification of the specific enzyme/s responsible for such metabolism, and inter specie metabolic profile, chemical structure of the metabolite/s, toxicity, and possible drug-drug interactions (Fig. **1**). In the last two decades, industry has invested hugely in screening technologies development to address important issues concerning drug absorption and metabolism, in order that these technologies should keep pace with the increased rate of compounds submissions [4]. As a result, laboratories have optimized their *in vitro* ADME assays to high throughput screening (HTS) protocols. The biological aspects of these assays are amenable to parallel processing using dense plate formats, thus sample capacities can be adjusted to handle large number of compounds [5]. Advances in technology, particularly in the analytical field together with assay miniaturization and automated devices allow the screening of a large number of compounds per day at the different stages of drug development, thus increasing the number of ADME assays linked to HTS [6, 7]. Here we intend to review some of the available absorption and metabolism assays adapted to screening requirements together with the technological approaches that can be combined with high-throughput molecular screening.

ABSORPTION

Oral administration is the most common desired pathway for the delivery of therapeutic compounds, but, it frequently occurs that NCEs have low absorption across biological membranes. To circumvent this problem, pharmaceutical companies have placed screening systems at the early stages of preclinical development for evaluating two key parameters, drug solubility and drug permeability [8, 9].

Traditionally, *in vivo* evaluation of drug absorption has been performed by comparison of AUCs after oral/ intraduodenal administration with those after intravenous administration [10-12]. This method although highly predictive is low throughput. As an alternative, to increase throughput of *in vivo* absorption studies, a procedure has been built up consisting of simultaneous administration of several compounds to a single animal (cassette dosing). Although this increases the assessment of the pharmacokinetics of a series of drug candidates at the same time it could also result in drug-drug interaction [13, 14]. *In situ* rat intestinal perfusion is also a reliable *ex-vivo* technique that allows drug absorption to be investigated in combination with intestinal metabolism. Despite its usefulness it is time

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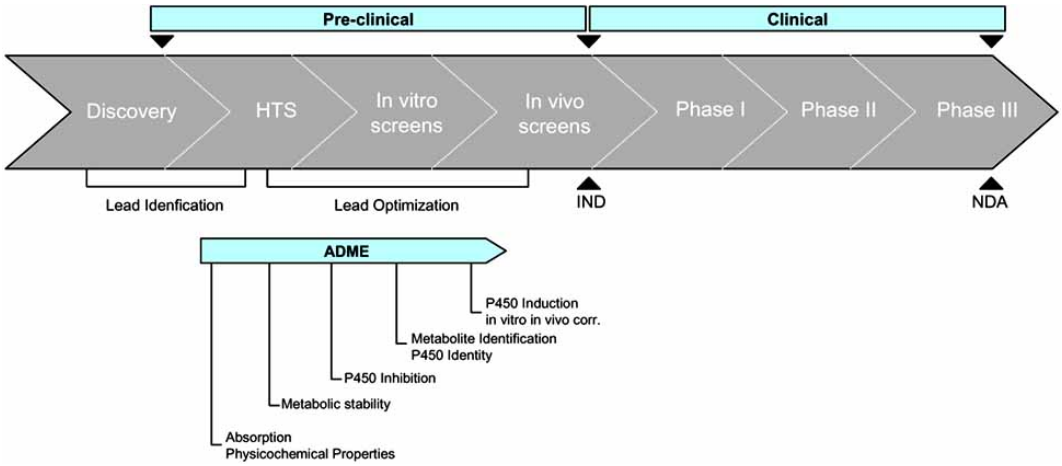

Fig. (1). Schematic representation of drug development process, depicting the different types of ADME studies that could be perform at the various stages. Investigational New Drug (IND). New Drug Application (NDA).

consuming and therefore not suited for screening purposes [10].

Since the norm of the industry is the implementation of rapid screening systems, a tremendous number of *in vitro* tools have been developed for high throughput selection of better candidates [15, 16]. Tissue-based and cell-based model systems are useful *in vitro* techniques for HTS. Regarding the former, the Ussing Chamber technique utilizes small intestine sheets mounted between two compartments and the everted sac approach is performed by dividing rat intestine into sacs. Both systems allow the study of the so called "absorption window" (specific regions of the small intestine where drug absorption is significantly higher) in combination with intestinal metabolism [17, 18]. The major drawbacks of both models are their short life spans and the total or partial presence of muscle layers that can reduce drug absorption [19].

Cell culture models strike the right balance between predictability and throughput and thus are the method of choice for permeability assessment across the pharmaceutical industry [20]. Among them, Caco-2 cells have become one of the most popular cell culture models. The human intestinal Caco-2 cell line has been extensively used as a model of the intestinal barrier. This cell line, originally obtained from a human colon adenocarcinoma, undergoes in culture a process of spontaneous differentiation expressing several morphological and functional characteristics of the mature enterocyte [21]. Despite limitations for hydrophilic compounds that use the paracellular route; these cells are considered a good model for compounds that are transported *via* the transcellular pathway [10]. In addition, cassette dosing studies (several compounds in a mixture) in Caco-2 cell monolayers have also been found to be suitable for permeability studies [22].

To facilitate HTS, several biotech companies market Caco-2 cells pre-plated in 24-wells on collagen coated inserts (Caco-Ready^{TM}, Advancell; Caco-2 assay system, Invitrogen Corp; Caco-2 assay Kit, *In Vitro* Tech Inc.) but because this plate format limits the number of drugs that can be evaluated at a time, further miniaturization has been achieved. Thus, Caco-2 cells are now commercialized in 96-wells/plate for permeability and interaction studies of carrier-mediated transport processes (Caco-Ready^{TM}, Advancell; MultiScreen® Caco-2, Millipore). The major disadvantage of Caco-2 cells is the need for a 3 week cell culture to obtain a fully functional cell monolayer. As an alternative, an accelerated 3-7 day Caco-2 cell permeability model has been developed. This system, which is suited for rank ordering of compounds in drug discovery, is not however useful for studying cellular transport mechanisms [23].

An ideal cell-based intestinal permeability tool would be one that stimulates the human gastrointestinal enterocytes not only in lipid bilayer characteristics but also in metabolic enzyme activity. The TC7 clone of the parental Caco-2 cells [24] could be used for this purpose despite their low CYP3A4 expression. Another alternative are the cytochrome P450 (CYP) genetically manipulated Caco-2 cells [25]. However, a shortcoming of such transfected models is the lack of stringent control of expression levels of the enzymes [20].

Because Caco-2 cells lack mucus, co-cultures of Caco-2 cells and mucus-secreting HT-29/MT6 cells have been used to resemble the small intestinal mucosa *in vivo* (CacoGlobet, Advancell). Although this seems a better system for predicting paracellular transport of hydrophilic compounds, absorption rates of actively transported drugs could be underestimated [10]. The dog kidney cell line, MDCK, is another model system used for drug screening. Although they grow more rapidly (≈ 3 days) than Caco-2 cells a further characterization of various transporters and relevant enzymes has to be performed. In addition, potential inter-species differences should also be considered [8]. Since no unique model cell system is able to successfully predict intestinal permeability, a combination of the most used cell lines in drug absorption studies (Caco-2, TC7 and MDCK) have been commercialized (Transport-Plus, Advancell) in the 96 wells/plate format. Other cell lines that have also been explored as an alternative to Caco-2 cells are those derived from the porcine kidney (LLC-PK1) and rat intestinal (2/4/A1) epithelial cells [20].

Computational *in silico* methods can also be used to optimize the selection of candidate drugs from a large pool of available compounds. This methodology employs drug chemical structure to model an ADME property such as absorption. Currently, the predictive capability is limited to passive, diffusional uptake and predominantly relies on a few molecular descriptors related to lipophilicity, hydrogen bonding capacity, molecular weight and charge [26, 27]. Several companies have combined these parameters in commercially available software packages. QMPRPlus™, Simulations Plus Inc is based on structural data only while GastroPlus™, Simulations Plus Inc and iDEA™, Lion Bioscience Inc allow some experimental data to be include [10]. Other attempts to increase the HTS of drug permeability are the physicochemical approaches. These models have been shown to correlate quite well with intestinal absorption, but are not useful for studying transport processes and modelling of paracellular pathways. In contrast, they are beneficial for HTS and for mechanistic studies (i.e, pH). These models include: Immobilized artificial membranes (IAM), Parallel Artificial Membrane Permeation Assay (PAMPA), Immobilized Liposome Chromatography (ILC), Micellar Liquid Chromatography (MLC), Micellar Electrokinetic Chromatography (MEKC) and Bipartitioning Micellar Chromatography (BMC). In most cases, further optimization of these methods is being currently performed [28, 29]

METABOLISM

In addition to the important connection with bioavailability, the study of metabolic parameters are very important in pharmaceutical development as they may explain inter subject variability, drug-drug interactions, non-linear pharmacokinetics and toxic effects. Once a drug is absorbed, it can be converted into metabolites (mainly in the liver) that typically are more aqueous-soluble and readily excretable than the parent compound. Inappropriate drug pharmacokinetics results in an inadequate or variable therapeutic behavior which frequently compromises its medicinal usage. Consequently the detection of possible future adverse effects is preferable during preclinical development. To speed up the selection of new drug candidates industry makes use of different biological matrices including recombinant CYPs, liver microsomes, pooled S9 fractions, hepatocytes or liver slides. Different *in vitro* assays, extensively used by the pharmaceutical industry are easily adapted for the evaluation of drug metabolic stability, metabolic profiling, and enzyme inhibition/induction potential.

Unfortunately at the present the majority of *in vitro* systems employed during preclinical screening are medium to low throughput systems. The most amenable parameters to screening techniques are metabolic stability and drug enzyme inhibition.

Metabolic Stability

Microsomes

Liver microsomes from different species (human and animal) are the simplest and best adapted tool for early drug screening strategies. Microsomes, are subcellular fractions obtained upon centrifugation of liver homogenates [30]. Microsomes prepared from a frozen pool of livers provide a general view of drug metabolism without taking into consideration any individual factors like sex, age, race, diet, etc. Alternatively, microsomes obtained from an individual liver can provide other information such as the influence of certain factors like genetic polymorphism. Microsomes hold most of the oxidative drug enzymes involved in phase I metabolism (mainly CYPs), but lack cytosolic enzymes (i.e. some conjugation enzymes). This means that the information obtained through this system could be incomplete. However, because of their easy handling, stability and availability, microsomes have become the most used *in vitro* system for studying drug metabolism in the very early stage of drug development. From a throughput point of view human liver microsomes (HLMs) are used mainly to conduct drug stability and inhibition of CYP enzymes studies [31-32]. These assays focus on providing critical information with relatively simple experimental designs to achieve the maximum throughput and speed. Microsomal stability assays provide important information on the metabolic liabilities of drug candidates. Low metabolic stability is indicative of high clearance, short half-life, and poor *in vivo* exposure. Data from these assays are used to predict *in vivo* drug metabolism.

The HTS processes generally involve highly automated systems with liquid handling and detection instruments that can screen a large number of molecules in a short period of time with little compound. Several experimental approaches using microsomes have been described to screen drug stability. In general metabolic stability assays make use of two different parameters to rank compound stability: the percentage of parent remaining after a determined period of time, and compound half-life. Di *et al.* [33] described an experimental HTS design to study microsomal stability based on using single time point, instead of using traditional multiple time points studies (more time and cost consuming). In this approach, a single time point assay is enough for ranking the compounds with subtle differences in metabolism. The limitation of this method is that it is only valid when metabolism follows first order reaction kinetics. Taking into consideration that most of compounds follow a first order kinetics when [S] <<<< Km, the model could be a useful tool for screening compounds at the early stage of drug development. However, at later stages of drug discovery multiple time point assays (several points between 10 – 60 min) are recommended to accurately determine metabolic clearance of lead candidates.

Different approaches have been proposed for reducing the number of samples generated during multiple time point *in vitro* ADME assays. Several reports have described the development of sampling pooled methods (PMs) [34, 35] which can be classified into three categories: i) Cassette dosing, compounds are combined before biological assay, assayed and analyzed as mixtures, ii) Cassette analysis, compounds are individually biologically assayed, and mixed before analysis, and iii) Pooled analysis, compounds are assayed separately with samples from an individual compound being combined for analysis. These methods present serious drawbacks such as: drug-drug interactions in cassette dosing, limits of drug detection in cassette analysis, and assay volumes in pooled samples. Recently, Sabrina *et*

al. [36] successfully addressed this issue by a novel strategy which involves a method for pooling samples from a multiple time point assay; the method avoids the potential drug-drug interaction and sensitivity drawbacks observed with cassette dosing and cassette analysis methods. This strategy can be used in order to decrease the number of samples generated during multiple time point assays (the method reduces the number of samples per compound from 11 to 5, representing a 54% reduction in sample load).

In order to support throughput in drug stability screening, systems are becoming more robotized [37]. In this context, Jenkins *et al.* [38] have implemented in their high-throughput capabilities a robotic core system (SAGIAN™) for automated sample preparation for human liver microsomes incubation. The system employs a Multinek™ 96 channel pippetor for liquid handling and robotic sample incubation. The sample plates are transferred off-line for a final semi-quantitative analysis using a high throughput parallel liquid chromatography/mass spectrometer to determine the percentage of parent compound remaining. Such an automated solution led to increased in capacity, throughput and reliability for *in vitro* assays. Di *et al*. [33, 38] also make use of robotic components to integrate them in a high-throughput microsomal stability method using a 96 well plate format. The system consists of two automated components; a robotic sample device for incubation and clean up, and a rapid liquid chromatography/mass spectrometry analysis to determine percent of the parent compound remaining. The system performance is comparable to a validated standard metabolic stability method used in lead optimization, in which multiple times are used.

Most of the described assays involved HLMs and consequently provide partial information on drug metabolism (membrane bound enzymes). The use of S9 liver fractions has become increasingly popular in metabolic stability assays, as this subcellular liver fraction contains both microsomal and cytosolic enzymes and conserves the whole Phase I and Phase II enzymes. Metabolic stability assays using S9 fraction are conducted similarly to those carried out with microsomes [39, 40] and, although not as well characterized as microsomes, have the potential to provide more information about *in vivo* metabolism.

Hepatocytes

Cell cultures or cell suspensions may be used to study multiple aspects of drug metabolism: drug transport across cell membranes, cytotoxicity, and enzyme inhibition/ induction. Using such *in vitro* methods cellular integrity is maintained and enzymes and cofactors are present in normal physiological concentrations. The availability of human liver for cell harvesting has increased over the years, which has indirectly led to an increased use of human hepatocytes for research and screening purposes. Nowadays, primary cultured hepatocytes are recognised by the scientific community and authorities as the most suitable tool to investigate *in vitro* drug metabolism [1]. Hepatocytes can be isolated from different types of liver tissue samples (surgical biopsy, non implanted liver grafts). The functionality of the isolated hepatocytes is assessed by measuring their drug oxidative capability. Commonly 7-ethoxycoumarin O-dethylation (ECOD) is measured as representative of the total CYP activity. Other probe substrates for each of the major CYP enzymes have been used and reported in literature [41-43] as for example testosterone 6-β-Hydroxilation (6-β-OH) as CYP3A4 activity indicative. Traditionally hepatocytes are used in the late stages of drug metabolism studies when the assays are running using multiple time points and a more reliable half-life determination is needed. The hepatocytes model, when compared with subcellular models such as microsomes or S9 fractions, present several intrinsic advantages. They express all the metabolic liver enzymes (phase I and phase II) and therefore provide a better approximation of liver metabolism. Hepatocytes conserve intact cell membranes and express membrane transporters, resulting in a more realistic barrier that the compound must through. This helps to alleviate the inherent problems in cell free systems of metabolism overestimation or false positives. Furthermore, hepatocytes offer the possibility of screening potential CYP inducers, which cannot be done in microsomes as a cellular system fully capable of expressing CYP genes is required (Table **1**). For this reason, primary hepatocytes are the unique *in vitro* model for global metabolism studies. The speed of testing compounds in cell culture and the obvious advantages of using intact cells have made cell based testing a key component of drug discovery programs.

In HTS, hepatocytes are mostly used in suspension or seeded onto plates, frequently 96-well plates or 384-well plates, depending on the throughput required and resources available. The metabolic stability and metabolic profile of new chemicals can be easily investigated by incubating the drugs with the fully competent metabolic hepatocytes either in suspension or in cultured hepatocytes. The incubation period ranges from 30min to 24 h. After incubation the enzymatic reaction is quenched using different methods (adding acetonitrile, or another organic solvent like ice cold methanol). The resulting samples are stored for further analysis involving different techniques which will be discussed later in this review. Recently Gebhardt *et al.* [44] have developed and optimised a new *in vitro* system which uses hepatocytes. The system consists in a 96 well plate bioreactor, which runs 96 modules in parallel for pharmacokinetic testing under aerobic conditions. This system combines the advantages of 3D culture systems in collagen gel, controlled oxygen supply and constant culture medium conditions with the possibility of high throughput and automatization.

When available, hepatic samples can render more cells than required for immediate use, thus cryopreservation allows long term storage of isolated hepatocytes for further use for research purposes and HTS platforms [41]. Several groups have used cryopreserved hepatocytes for drug metabolism purposes. HTS assays using cryopreserved human and animal hepatocytes have been developed for hepatotoxicity, metabolic stability and inhibitory interactions [45]. Reddy *et al.* [46] have described a semi-automated high throughput system using cryopreserved hepatocytes and 96 well plates and a Tecam Geminis™ workstation. The system enables to determine human intrinsic clearance with up to 10 time points. In general, good correlations between the

Table 1. Comparison of Different *In Vitro* Systems Used in Medium/High-Throughput Screening

Systems	Advantages	Disadvantages	*In vitro* screening
Microsomes	Availability, long storage Characterized HTS	Phase I enzymes and UGTs.	Metabolic Stability Metabolite identification
S9 fraction	Availability, long storage Contain almost all hepatic enzymes HTS	Not well characterized Liver architecture lost	Metabolic Stability
Recombinant Enzymes	Availability HTS Role of individual CYP CYP Inhibition	Only one enzyme at a time can be examined	Enzyme Inhibition
Hepatocytes	Integrate cellular system Whole enzymatic component Plasmatic membrane (transporters) CYP Induction Cryopreservation allow HTS	Difficult to obtain Well establish procedures Better cryopreservation	Metabolic Stability Drug-drug interactions

manually executed experiments, the known/literature reported experimental results and the results obtained with the semi-automated system are found. The assay allows for simultaneous testing of 48 compounds with multiple time points, even a large number of compounds can be tested using 384 well plates where up to 192 compounds can be simultaneously tested. The limitations of the system are that this assay does not take into consideration the effect of protein binding and only accurately predicts *in vivo* clearance *via* hepatic metabolism.

Drug Inhibition

Interesting features of CYP enzymes are their catalytic versatility and broad substrate specificity. A relatively reduced number of CYP enzymes metabolises hundreds of drugs and other foreign compounds. The ability of an individual CYP to metabolise multiple substrates is responsible for a large number of drug to drug interactions associated with CYP inhibition. The high cost associated with drug development programmes have focused attention on predicting, identifying, and avoiding inhibitory potential early in the discovery process. Considerable progress has been made in the development of reliable *in vitro* screening methods to identify potent CYP inhibitors.

Drug-drug interactions result from catalytic inhibition of the enzymes involved and, therefore, activity endpoints are the most relevant. Classical inhibition assays involve the co-incubation of HLMs with several concentrations of the new molecule and selective substrates for individual CYP enzymes [4, 47]. Probe substrate concentrations at or below the Km value and validation of inhibition experiments by testing known specific CYP inhibitors (positive controls) are recommended. Inhibitory effects are usually expressed as percentage of the control activity value and IC_{50} are calculated by interpolation. As IC_{50} values depend on concentration of the substrate, a comparison of the Ki value among new drug candidates is more useful to rank order the compounds as a function of their inhibitory potency. The use of substrate concentrations close to Km values of the reaction allows the application of simple inhibition kinetic relations for estimating Ki from IC_{50} values, assuming competitive inhibition, according to the following relationship [43, 48]: $Ki = (Km \times IC_{50})/(Km + S)$, where Km is the affinity constant and S is the substrate concentration used, thus when $S = Km$, $Ki = IC_{50}/2$.

Inhibition assays in HLM require the use of selective probe substrates form each CYP enzyme. Precise information on selective substrates for individual human CYP is now available in the scientific literature [47, 49-51]. Most of these compounds can be obtained from commercial sources and their use has become routine in the characterisation of CYP-dependent activities. The use of specific reaction markers for individual CYPs allow the incubation of full competent *in vitro* models (liver microsomes, hepatocytes or liver slices) with a mixture ("cocktail") of selective probes [52, 53]. This strategy provides information on both inhibition selectivity and relative inhibitory potency of tested compounds on different CYPs. However, most selective substrate probes involve analysis by HPLC or LC/MS/MS for metabolite identification, which limits sample throughput [52, 54, 55]. To meet the increased screening demands at early stage of drug discovery process, the need for more efficient methods for routine testing of inhibitors has become obvious.

Recombinant CYP systems are now increasingly used for screening of compounds for favourable metabolic properties in drug discovery [56, 57]. CYP enzymes, heterologously expressed in different cellular systems, show catalytic properties comparable to those of human liver microsomes [58]. These enzymes can be produced in large amounts to

meet the increasing demand of screening assays for drug metabolism research. Methods that combine recombinant CYP with fluorescent or radiometric probes have been developed as HTS to evaluate the inhibitory effect of new drug candidates [59-61]. Among them, assays using microsomes from baculovirus-insect cells heterologously expressing human CYP [48, 61] or intact human cells genetically manipulated to express an individual CYP [50] have been recently described. However, interpretation of kinetic data from recombinant systems requires a cautious analysis [56, 62-64]. Since several enzymes can be involved in the metabolism of a compound, the use of experimental models expressing one single CYP may not properly estimate the inhibitory effects of a given drug. Moreover, relative concentrations of accessory proteins (NADPH P450 reductase and cytochrome b5) or membrane lipid composition may differ in a heterologous expressing system compared to human hepatocytes/human liver and, hence, influence the results.

Another way to optimise the throughput of CYP inhibition screens is the possibility of using fewer concentrations of the potential inhibitor. Different groups have addressed this strategy [33, 60]. Analysis of inhibitory effects on CYP2C9, CYP2D6, and CYP3A4 using selective probes revealed excellent correlations for IC_{50} values generated using the 10-point curve procedure and those obtained using 3-point curves. Moreover, the prediction of the IC_{50} value from the percent inhibition value obtained at a single concentration has been proposed [65, 66]. The use of such a single-point procedure would contribute to reduced amounts of compounds, enzymes, probe substrates, reagents, and analytical instrumentation time, although more studies are needed to ascertain the actual relevance or utility of this assay for *in vitro* drug-drug interaction assessment. During the lead optimisation stage, in which hundreds of compounds are screened, a single concentration inhibition screen using recombinant CYP or HLMs assays would be appropriate. In further assays, full-scale IC_{50} studies are recommended [4].

Several fluorescent probes have been applied for the development of CYP inhibition assays in miniaturized formats (96-, 384-, or 1536-well plates) [59, 61]. Major advantages of performing primary screening assays in microwell plates at < 10 μL volumes are the reduction in costs (smaller amounts of test compounds and reagents used) and the increased compound throughput (>100-fold with respect to conventional assays). Typically assay miniaturization will result in greater data variability; however, the reduced amounts of enzyme used in HTS contribute to minimizing enzyme variability by conducting multiple CYP assays with the same batch of microsomes or recombinant CYP. In combination with fluorescence probes, automated systems have been designed to increase throughput and reliability of *in vitro* inhibition assays [39, 61]. Sample preparation was identified early on as a key bottleneck in screening processes. The application of SAGIAN™ core robotics system for CYP inhibition has improved sample throughput by a factor of eight, with reductions in both sample preparations and data analysis time with respect to manual procedures [39]. The automated assay combines both liquid handlers and an integrated fluorescence plate reader to perform single concentration inhibition assays for 88 compounds. An additional advantage in the automation of sample preparation is the reduction in human errors and, subsequently, in assay variability (i.e. >20% CV for manual assays *vs* <5% for automated methods).

Competitive inhibition is the most common mechanism involved in CYP-dependent drug-drug interactions and this is the reason why most of HTS inhibition assays are designed on the assumption of this type of inhibition. With the application of such protocols, most mechanism-based (irreversible) inhibitors will be missed while uncompetitive, non-competitive and mixed-type inhibitors will be analysed as competitive inhibitors. Drug-drug interactions due to irreversible inhibitors are much less favourable and often cause more serious side effects; because the inhibitory effect remains after elimination of the inhibitor from blood and tissue. Obviously, a modification of the assays is needed to easily screen irreversible inhibitors. In irreversible inhibition, enzymes progressively lost activity when more reactive metabolites were generated during incubation and, as a consequence, IC_{50} values decrease with incubation time [67]. In contrast, the IC_{50} value of a reversible inhibitor which follows simple Michaelis-Menten kinetics should not change significantly with incubation time. Based on this strategy, fluorimetric kinetic assays have been proposed as a reliable method for rapidly distinguishing reversible and irreversible CYP inhibitors [67, 68]. Fluorescence is continuously measured at short time intervals (i.e. 2 min) without stopping enzyme reactions by means of a microplate fluorescence reader, and the time-dependent pattern of IC_{50} values is analysed. A different approach is based on the observation that pre-incubation of the enzyme with the test compound differently affects the magnitude of effects produced by competitive or irreversible inhibitors. After pre-incubation, the IC_{50} value of mechanism-based inhibitors substantially decreased compared with those in co-incubation assays (inhibitor and substrate added simultaneously), whereas this difference was not observed for competitive inhibitors [48, 69]. For HTS, IC_{50} shift is often conducted by incubating recombinant human CYPs with fluorescent probes, however, these assays present certain limitations. Fluorescence-based assays are susceptible to interference by fluorescent test compounds (or their metabolites) and, moreover, the effects of metabolites generated by one enzyme on other CYP cannot be tested in recombinant single-enzyme systems. Alternatively, a new strategy has been proposed to profile compounds for irreversible inhibition of CYP3A4, CYP2 C19, CYP2C9, CYP2D6, and CYP1A2 in HLM [70]. The method is based on automated screening of the apparent partition ratio, followed by confirmation of potent positive from the screen by time-dependent and concentration-dependent inactivation assays and, finally, evaluation of reversibility of inactivation to differentiate quasi-irreversible and mechanisms-based inactivators. This three-step screening procedure has been validated with acceptable accuracy and precision for detection and confirmation of mechanism-based inhibitors in drug discovery.

In vitro inhibition parameters (Ki, IC_{50}) only become useful when they can successfully predict *in vivo* effects. Most inhibition assays are based on the use of appropriate

probe substrates in combination with recombinant CYPs or HLMs [4, 43, 59]. A major limitation in making conclusive statements from these assays is that ultimately *in vivo* metabolism is complicated by the role of processes missed in subcellular models (drug transport across membranes, further metabolism by cytosolic enzymes, or binding to intracellular proteins) which can all be determinant in the actual concentration of substrate and inhibitor available to the enzyme [71, 72]. Assays performed in intact cells are, in some aspects, more predictive than subcellular models; however, there are few reports of CYP inhibition studies in living cells [50, 73-75].

HIGH THROUGHPUT BIOANALYSIS

In parallel to the development of biological throughput models for *in vitro* absorption and metabolic screening, automated high throughput methods of sample analysis should be implemented. In the past few years, great progress has been made in the analytical area concerning HTS, by the use of detection instruments with high sensitivity and which are able to screen a large number of compounds in a short period of time. Two main analytical methodologies have been used in medium/high-throughput screening: fluorescence based methods, and liquid chromatography coupled to different mass spectrometry analyzers.

Fluorimetric Methodology

Fluorescence based assays are highly sensitive and allow the simultaneous measurement of a large number of samples, making use of plate readers, thus enhancing sample throughput [59, 61]. Fluorimetric methods are particularly used to measure CYP inhibition and have considerably reduced the effort needed to detect possible drug-drug interactions. These assays are based on the identification of the formation rates of highly fluorescent metabolites produced from non- or low- fluorescent CYP substrates.

Fluorescent assays can be performed in 96 well plate format culture plates. The use of microtiter plate readers increases sample throughput remarkably in comparison with conventional analytical methods. A major limitation is that most fluorimetric probes are not selective for particular CYP enzyme. Obviously, non-selective substrates cannot be used for assays models showing several CYPs, such as microsomes or hepatocytes, and their application is limited to recombinant models expressing an individual enzyme. Moreover, inhibitory effects of fluorescent compounds or those metabolized to fluorescent products cannot be tested.

Mass Spectrometry Methods

Because of the need for higher throughput in ADME assays, high performance liquid chromatography (HPLC) coupled to different mass spectrometry analyzers such as triple quadrupole (QQQ), ion traps (IT) and time of flight (TOF) has been widespread in medium/high-throughput screening routines. Nowadays, most of the *in vitro* HTS systems described above rely on mass spectrometry. This technology has been habitually incorporated in drug stability throughput, inhibition screenings, and in metabolite identification. The advantages of mass spectrometry are; high sensitivity, selectivity, ease of automation relative to traditional analytical methods, the possibility to analyze endogenous and non-fluorescent substrates in inhibition assays, and that it enables rapid permeability (Caco-2) assessment (Fig. **2**).

Over the past few years, the growing concern about the number of samples to be analyzed in ADME screening has led several groups to investigate different approaches to the problem. Breemen *et al.* [76] and Zhao *et al.* [37] initially developed a pulsed ultrafiltration mass-spectrometry system which employed liver microsomes incubation in an on-line mass quadruple analyzer equipped with an electro spray ionization source (ESI). To get throughput, different compounds are incubated with microsomes in multiple ultrafiltration chambers arranged in parallel with a HPLC injector and a quadrupole mass analyzer. Constant flow of incubation buffer is maintained through the chambers but only one chamber at time is connected to the mass spectrometer and individual mass spectra are recorded after each injection. Therefore, as larger numbers of chambers are used higher throughput is achieved. After recording mass spectra the chambers can be reloaded with fresh microsomes and new compounds. Other authors incorporate automated data processing strategies to improve throughput [34]. Ming *et al.* [77] have developed a system which put together an HPLC coupled to a quadrupole mass spectrometer with an intelligent data acquisition device. This scheme allows the simultaneous screening of microsomal stability and metabolite profiling. The intelligent data acquisition device permits the automatic determination of the mass to charge ratio *(m/z)* of an unknown compound, and the utilization of the molecular information to perform selective ion monitoring (SIR) experiments for quantification. Such novelty enables the experiment to be modified during data acquisition and considerably increases the throughput. A different approach incorporates pooling techniques to increase speed in sample throughput. Zongwei *et al.* [78] reported a system where an ion trap mass analyzer is used in combination with simultaneous cassette analysis of the parent compound. Such a method enables the detection of the generated metabolites by using full scan ion trap spectra and provides not only high throughput but also the full scan mass spectra data for each analyte. The fingerprint matching between the metabolite and the parent drug provide conclusive confirmation of the metabolite detection and identification.

Among the most used mass spectrometry based approaches is liquid chromatography tandem mass spectrometry (MS/MS) with fast HPLC gradient. This strategy has emerged as the most suitable for supporting *in vitro* throughput in drug screening [79-81]. Samples can be analyzed one at time or as mixtures by applying fast gradients and short columns which significantly decrease the HPLC run time and enhance throughput [82, 83]. One of the most time-consuming steps in the analytical process is method development. To overcome this problem an automated system which integrates different components, including an *in vitro* incubation device, software for automatic MS/MS method development and generic fast liquid chromatography for sample analysis was developed [84]. This system was found to be efficient in early metabolic stability testing and is capable of assaying 96 compounds/day.

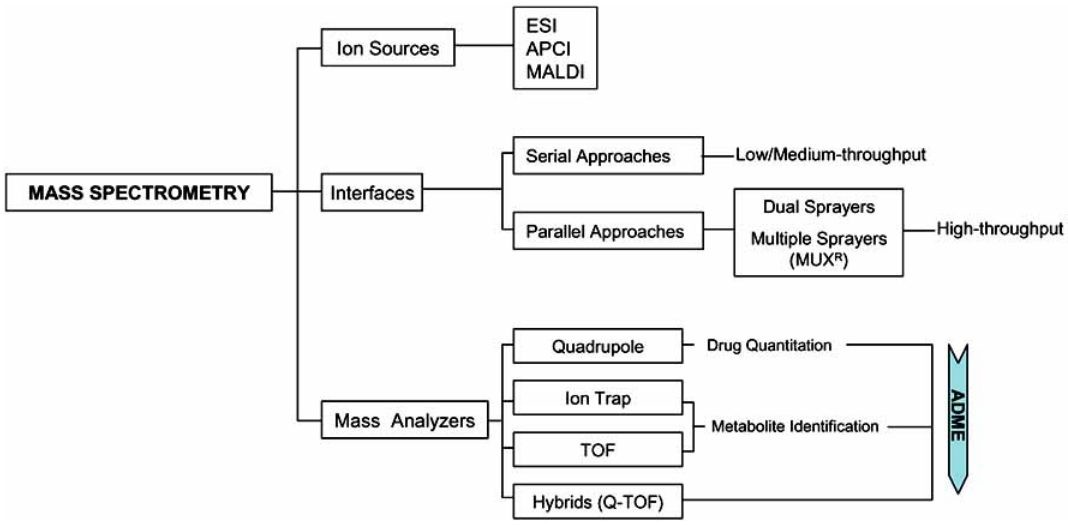

Fig. (2). General scheme of common mass spectrometry techniques used in ADME assays. Quantitative analysis of parent drug is needed in the early drug discovery phase, in the fast screening of metabolic s, enzyme activities and drug-drug interactions studies with cytochrome P450 enzymes. Electro spray ionization (ESI), atmospheric pressures chemical ionization (APCI), matrix assisted laser desorption ionization, time of flight (TOF).

In order to increase throughput, interesting alternatives to the traditional serial HPLC-MS system are currently being developed, among them, parallel liquid chromatography tandem mass spectrometry [85]. In this system, two or more HPLC columns are run in parallel coupled to one mass spectrometer. Recently, a system with eight parallel HPLC channels-mass spectrometer in combination with a custom automated data processing application capable of analyzing up to 240 samples per hour has been proposed [86]. After sample separation in the columns, flows can be introduced onto a mass spectrometer interface either in a serial mode with a valve selector or with parallel sprayers. Yang *et al.* [87] described a four-channel multiplexed electrospray interface (MUX, Micromass) coupled to a triple quadrupole mass spectrometer. This system allows the continuous introduction onto the mass spectrometer of the effluent of four different HPLC columns. The sampling rotor permits only one spray at a time to be admitted to the sampling cone of the mass spectrometer. This novelty increased the throughput fourfold. However the technique needs further development as interference from spray to spray and the lower sensitivity than that of a single sprayer interface are two of the technique's drawbacks.

Different efforts to automate the process and reduce the time required by the investigator for data handling, method development and other time-consuming tasks have been made. King *et al.* [85] reported the development of a system which consists of four fully independent binary HPLC pumps, an autosampler, and a series of switching and selector valves. All the systems are controlled and synchronized by Aria software. In industry different software has been implemented for the application and management of data from *in vitro* studies of the prediction of intestinal absorption performed with Caco-2 (NorayBio).

Traditionally improvements to optimize throughput have relied on mass spectrometry or software development, while few improvements in liquid chromatography have been achieved. A system which uses a novel ultra-performance liquid chromatography (UPCL) coupled to a hybrid quadrupole/time-of-flight mass spectrometer (Q-TOF) has been recently described [88]. High mass resolution and exact mass measurements can be achieved, avoiding false positives and non-trivial metabolites. Furthermore, the chromato-graphic enhancement obtained with the UPCL system allows a net reduction of ion suppression leading to an improvement in the MS sensitivity.

CONCLUSIONS

In vitro ADME screenings are being applied earlier and earlier in drug development since they are essential for identifying compounds likely to present unfavorable ADME parameters. Absorption and metabolism are major determinants governing both pharmacokinetics and pharmacological response. Progress during the past years, in *in vitro* screening throughput together with innovations in analytical chemistry and the widespread use of mass spectrometry have provided several suitable tools for early absorption and metabolic assessment. Although each of these tools has specific limitations, it is reasonable to suggest that an integrated screening procedure that is able to exploit the strengths of each particular model could be the best strategy for general metabolic screening of new molecules in drug development. Assays with microsomes or recombinant systems could be very useful for preliminary screening, but after selecting a few compounds, further studies in a fully metabolic competent model (i.e. human hepatocytes) are recommended. The simpler the metabolic tests, the more adapted they are for ranking large series of compounds (HTS). According to this assumption high throughput less reliable methods could be used at the early stages of drug discovery leaving lower throughput more accurate methods for optimization of lead compound absorption.

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