Advances in the Integration of Drug Metabolism into the Lead Optimization Paradigm

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Abstract: The lead optimization paradigm includes a team of experts that has a multitude of parameters to consider when moving from an initial lead compound through the lead optimization phase to the development phase. While in the past the team may have had only a medicinal chemist and a pharmacologist, the current team would often include experts in the areas of drug metabolism and pharmacokinetics (DMPK) as well as chemical toxicity. This review provides an overview of the some of the recent advances in the areas of DMPK screening plus a discussion of some of the assays that can be used to begin to screen for toxicity issues. The focus of this review is the major potential problem areas: oral bioavailability, half-life, drug-drug interactions and metabolism and toxicity issues.

Key Words: Drug metabolism, toxicology, lead optimization, drug discovery, oral bioavailability, half-life, ADME.

INTRODUCTION

There has been a dramatic shift in how discovery lead optimization has been performed in the last twenty years. Previously, lead optimization was primarily an effort that combined the skills of medicinal chemistry and biology to discover novel drug candidates that could be put forward as development compounds. This simple strategy was effective and resulted in the discovery of drugs that are still used today. However, the strategy has changed and now the lead optimization paradigm includes a team of experts that has a multitude of parameters to consider when moving from an initial lead compound through the lead optimization phase. A major part of this shift was the addition of drug metabolism and chemical toxicity as important parameters for consideration in the drug discovery stage [1, 2]

One of the reasons for this paradigm shift was the landmark study reported in 1990 in which it was reported that for 40% of the compounds that failed in clinical trials, the problem was pharmacokinetics (PK) [3-6]. This finding led to the adoption of a new strategy: involve drug metabolism scientists in the lead optimization process. What followed was the rapid expansion of exploratory drug metabolism and pharmacokinetics (DMPK) departments that were given the challenge to develop higher throughput screening assays to test new chemical entities (NCEs) as part of the new lead optimization program. These new screening assays were designed to measure various absorption, distribution, metabolism and excretion (ADME) properties of the NCE as well as the pharmacokinetic (PK) parameters of the molecule [7-20]. One of the reasons that scientists were able to provide these higher throughput assays was the continuing advances in the powerful high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) instrumentation that became available in this same timeframe [21-33]. In many cases, these new screens were inserted into the lead optimization process in a linear, sequential paradigm as shown in Fig. (**1**) [16]. In this paradigm, lower throughput assays were performed later in the optimization process when the number of compounds was lower. This new lead optimization paradigm has already been shown to be very effective. In a recent study of the attrition of compounds during clinical testing, it was found that less than 10% of the compounds failed due to PK reasons [3]. The major reasons for failure in the clinical phase are now lack of efficacy (28%), toxicity (20%), clinical safety (12%) and various commercial concerns (28%) [3, 5]. These more recent findings are adding to the need to find ways to screen for safety and toxicity in the lead optimization phase [34-37]. As the screening throughput has increased, some companies have switched to a highly parallel process as shown in Fig. (**2**). The advantage of the parallel process is that it can shorten the amount of time required for the lead optimization phase [1]. This review will provide an overview of some of the recent advances in the areas of DMPK screening as well as a discussion of some of the assays that can be used to begin to screen for toxicity issues.

This review is not a comprehensive overview of all the various ADME screens, instead the focus of this review will be on four potential problem areas: oral bioavailability, half-life, drug-drug interactions and metabolism and toxicity issues. While these areas are in many ways inter-related, there are some specific assays that are more closely related to each topic.

ORAL BIOAVAILABILITY

As oral dosing is the preferred route of administration for most drugs, oral bioavailability is a key DMPK parameter that needs to be assessed early in the lead optimization regime. As a general rule, low oral bioavailability (F) is not a desirable property for a compound that will be dosed orally. Low oral bioavailability generally leads to higher inter-

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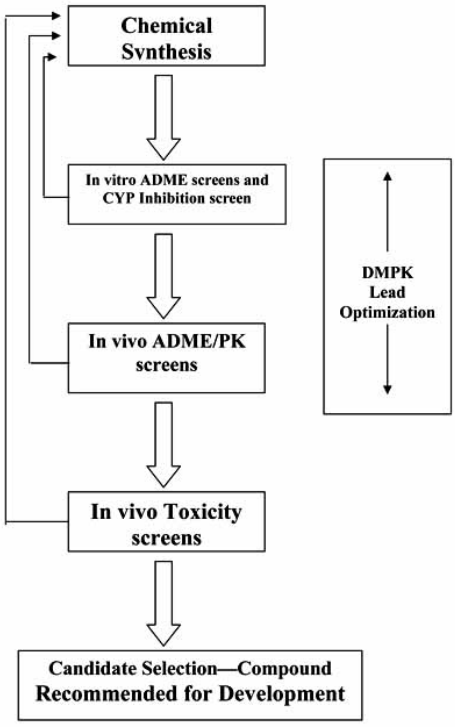

Fig. (1). Figure shows the iterative nature of the lead optimization phase in which higher throughput screens are performed earlier in the process. Adapted, with permission from The Thomson Corporation and Korfmacher W: Lead optimization strategies as part of a drug metabolism environment. Current Opinion in Drug Discovery & Development (2003) 6(4):481-485. Copyright 2008, The Thomson Corporation.

individual variation in systemic exposure [38, 39]. On the other hand, high oral bioavailability leads to smaller dosage and lower API (active pharmaceutical ingredient) cost.

The first *in vivo* measurement of oral bioavailability is usually obtained in the rodent (mouse or rat). The standard measurement of oral bioavailability is typically obtained by performing a "full PK" study in the mouse or rat. In this experiment, the NCE is dosed orally (PO) in three-four rats or mice and is also dosed by the intravenous (IV) route in three-four rats or mice. The percent oral bioavailability (%F) is defined as:

$$\%F = \frac{\text{AUC (infinity) PO * Dose IV}}{\text{AUC (infinity) IV * Dose PO}}$$

Where AUC (infinity) PO is the area under the curve for the concentration time plot for the samples obtained from the PO dosed animals

AUC (infinity) IV is the area under the curve for the concentration time plot for the samples obtained from the IV dosed animals

Dose PO is the oral dose of the NCE

Dose IV is the IV dose of the NCE

As a general rule, the goal for a new drug is to have a minimum %F of 30% in the clinical setting [39]. While the projection of human pharmacokinetics can be done by various methods, the most commonly used techniques rely on some form of allometry-based system which needs the PK data from three preclinical species (typically rat, dog and monkey) in order to make the human PK predictions. Since it is impractical to get the PK from three preclinical species during the lead optimization process, most companies use a screening paradigm to screen out NCEs that have poor oral bioavailability. This %F screening paradigm may use various *in vitro* assays as well as an *in vivo* screen.

Oral bioavailability (F) can be understood by realizing that it is a combination of factors as shown in the following equation:

$$F = Fa \cdot Fg \cdot Fh$$

where Fa is the fraction of the dose absorbed across the GI wall and Fg is the fraction that survives gut metabolism and Fh is the fraction that gets through the liver (avoids hepatic elimination). Fg and Fh together are often referred to as "first pass metabolism". Thus, oral bioavailability can best be characterized as a combination of absorption and metabolism factors. Indeed, one approach for using *in vitro* screening for oral bioavailability is to combine the results of an *in vitro* absorption (permeability) screen with an *in vitro* metabolic

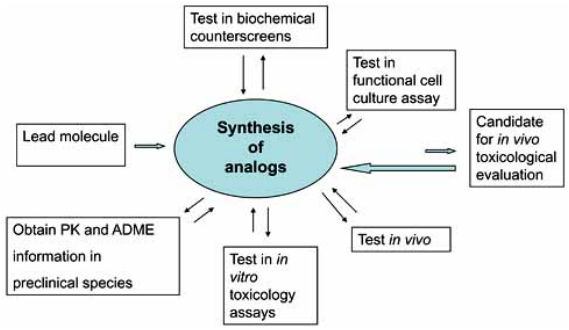

Fig. (2). Non-linear screening paradigm. This paradigm uses multiple screens in a parallel mode to decrease the lead optimisation time. Reprinted from MacCoss and Baillie [1] with permission from AAAS.

stability screen. This approach was described by Mandagere *et al*. [40] and found to be more predictive for those compounds with high permeability and medium or high metabolic stability.

Drug permeability is one of the main factors for predicting the fraction absorbed for a new chemical entity (NCE) [41]. The most common *in vitro* absorption (permeability) screen is still based on the Caco-2 cell (obtained from human adenocarcinoma cells) assay [19, 42-45]. The Caco-2 cell assay has the advantage that it is well understood, it is based on a human cell line and it can also be used to measure the effect of the P-glycoprotein (P-gp) transporter [46]. The disadvantage of Caco-2 cells is that they can take up to three weeks to complete the culture process. There are multiple reports in the literature for how to use Caco-2 cells in a higher throughput manner to support drug discovery screening [47-54].

Another *in vitro* absorption (permeability) screen that has become more popular in recent years is the parallel artificial membrane permeability assay (PAMPA) [19, 55-59]. The PAMPA screen is not a cell-based assay, rather it is based on an artificial membrane that separates two aqueous buffers. The test compound is placed in the donor well and moves by passive diffusion to the acceptor well. PAMPA is performed using 96-well plates and utilizes a UV plate reader for the analysis. Due to the simple design, the throughput is 10-fold higher than a typical Caco-2 assay. Several researchers have shown that both PAMPA and Caco-2 show similar correlations to human oral absorption. Kerns *et al*. [60] showed that PAMPA and Caco-2 correlate well with each other when the predominant permeation mechanism for the compound is passive diffusion. Furthermore, PAMPA permeation values were much less than Caco-2 values when the predominant permeation mechanism for the compound is absorptive transport, while PAMPA permeation values were greater than Caco-2 values when the predominant permeation mechanism for the compound included secretory transport. Due to these differences, Kerns *et al*. [60] proposed that PAMPA would be best used as the first high throughput permeability screen in the earlier discovery phase while the Caco-2 assay would be used as a lower throughput permeability assay later in the lead optimization phase of the study.

More recently, Balani *et al*. [14] have also proposed using PAMPA as the primary screening tool for understanding the structure-permeability relationship, while reserving Caco-2 as the secondary screen to better understand the absorption mechanism and to assess the P-gp interaction potential of the drug candidates. For those compounds that show high PAMPA permeability, but low Caco-2 cell permeability, the difference is often due to the fact that the compounds are P-gp substrates. Many compounds show good oral bioavailability in preclinical animal models even if they are P-gp substrates; this observation is likely due to saturation of intestinal P-gp due to high drug concentration in the gut after oral dosing of the compounds. In a similar manner, P-gp can be saturated in humans so that a compound that is a P-gp substrate may be well absorbed at typical human doses of 100 mg or higher. In addition to P-gp, there are many other transporters including multiple ATP binding cassette (ABC) drug transporters that play a role in drug absorption as well as drug distribution, metabolism, excretion and toxicity [19, 61-63].

While not high throughput, the best tool for assessing oral bioavailability of a NCE is to orally dose the NCE into laboratory animals [13]. Typically for early PK screening purposes, the rodent (mouse or rat) is the most common laboratory animal; mice and rats have the advantage that relatively small amounts of compound are needed for a typical PK screen. Due to the need to assess many NCEs, various models have evolved for measuring the oral bioavailability in rats or mice.

Previously, cassette dosing of rats or mice was viewed as a way to increase the throughput of this oral PK screen. In cassette dosing, multiple compounds (5-10) are formulated into a single dose and given to a single rat or mouse [64-68]. As long as the compounds are not isobaric it is normally possible to differentiate them in a single assay based on high-performance-liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The analytical issue with cassette dosing is that it takes more effort to ensure that the assay will be accurate due to the multiple compounds and their potential (or known) metabolites [69, 70]. In order to get a complete oral PK concentration-time course, blood samples typically need to be taken from the laboratory animals for multiple timepoints out to 24 hours. As described by White and Manitpisitkul [69], the problem with cassette dosing is that it can lead to both false positives and false negatives for various important PK parameters due to drug-drug interactions. Due to these limitations, cassette dosing has been falling out of favor as a PK screening technique [70-72].

Several authors have described alternatives to cassette dosing that can still be used for reasonably high throughput oral PK screening [73-76]. Fig. (**3**) provides a simple description of various models that have been used for rodent PK screening [76]. For example, Korfmacher *et al*. [75] have described the use of cassette-accelerated rapid rat screen (CARRS) as a rat oral PK screen. In the CARRS assay, compounds are bundled into cassettes of six compounds, but each compound is dosed into two rats (N = 2 rats/compound). Six samples are taken from each rat at timepoints out to six hours and the samples from the two rats are pooled at each time point to give a total of six plasma samples for each compound. In this way, a total of 36 plasma samples are obtained for the six compounds in one cassette. Furthermore, the standard curve is abbreviated to three levels in duplicate plus a 0 level. The result is that all of the plasma samples and standards for one cassette of six compounds fit in one 96-well plate. The CARRS assay has been used successfully for over 15,000 compounds over the last seven years. In a retrospective study, Mei *et al*. [77] found that the AUC(0-6) that is measured by the CARRS assay was very predictive of the AUC(0-24) that would normally be measured by more traditional PK screens. Therefore, the work of Mei *et al*. [77] provided additional data to support the utility of the CARRS assay.

Another report by Liu *et al*. [76] described an oral PK screen that was similar to the CARRS assay with some minor differences and referred to the assay as "snapshot

Full PK	Cassette Dosing PK	Cassette-Accelerated Rapid Rat Screen (CARRS)	Snapshot PK
Animal: Mouse or Rat One compound per animal IV (n=3)/ PO (n=3) 6-8 animals per compound 6-12 samples per animal over 24 hours Sample volume: 50 – 100 µL	**Animal: Mouse or Rat** Multiple compounds per animal IV (n=3)/ PO (n=3) 6-8 animals per study (multiple compounds) 6-12 samples per animal over 24 hours Sample volume: 50 – 100 µL	**Animal: Rat** One compound per animal PO (n=2) 2 animals per compound 6 samples per animal over 6 hours Sample volume: 100 µL	**Animal: Mouse or Rat** One compound per animal PO (n=2) 2 animals per compound 4 samples per animal over 5 hours Sample volume: 50 µL
Advantages: 1) Full PK parameters 2) No drug-drug interactions	**Advantages:** 1) Full PK parameters 2) Great savings in animal resources	**Advantages:** 1) No drug-drug interactions 2) Good savings in animal resources	**Advantages:** 1) No drug-drug interactions 2) Good savings in animal resources 3) Applicable to rat and mouse
Liabilities: 1) Labor intensive and time consuming	**Liabilities:** 1) Drug-drug interactions 2) Low test dose (mg/kg)	**Liabilities:** 1) Applicable to rat only 2) Partial PK parameters	**Liabilities:** 1) Partial PK parameters

Fig. (3). Figure compares full PK dosing in rats with three higher throughput screening procedures: cassette dosing, CARRS and snapshot PK. Reprinted from Liu *et al*. [76] with permission from Elsevier.

PK". In the snapshot oral PK screen, the rodent species is the mouse and only four timepoints are taken per compound. The advantage of using the mouse is that smaller amounts of compound are needed. The CARRS assay requires about 15 mg of compound for the dosing step, while the snapshot PK model needs a smaller amount (ca. 5 mg) since it is based on mice not rats as the rodent model. Furthermore, the snapshot PK model may have an advantage over CARRS when the efficacy model is based on a mouse model. In another "rapid rat" model, Han *et al*. [70] determined that three timepoints (1, 4 and 8 hours) were sufficient to provide AUC values that agreed well with the traditional seven timepoints that might be taken to measure the AUC(0-8). Han *et al*. [70] also showed that sample pooling could be used to further reduce the number of samples. In their assay, they use two rats/compound and sets of five compounds; this results in six samples per compound and by pooling across compounds (not across rats), they end up with only six samples for the five compounds (using cassette assay strategy). The limitation of their approach is that the five compounds must be at least 5 Da apart from each other in terms of their molecular weight; the authors also stated that pooling dilutes the samples so that assay sensitivity could be an issue in some cases. Finally, they cautioned that metabolites could lead to interferences in the pooled assay. In spite of these potential issues, Han *et al*. [70] stated that their rapid oral PK approach worked well to support various drug discovery programs.

HALF-LIFE

After oral bioavailability, half-life is probably the second most common PK problem area for drug discovery. In most cases, one would like to dose a new drug on a daily basis; in order to have a once a day drug, the human half-life of the compound would need to be in the range of 4-12 hours. For a twice a day drug, the human half-life of the compound would need to be in the range of 2-4 hours. The goal in the discovery setting is to be able to collect sufficient data to estimate the human half-life of the NCE. If the estimated half-life does not meet the requirements for the planned use for the drug, then the medicinal chemist needs to have tools to look for ways to modify the structure of the compound so as to reach the targeted half-life for the program.

Half-life ($t_{1/2}$) can be defined simply as the time it takes for half of the drug level to be cleared from the drug compartment. Half-life is related to two other PK parameters, Vd (apparent volume of distribution) and CLsys (systemic clearance), by the following equation:

$$t_{1/2} = 0.693 \cdot (Vd/CLsys).$$

It is important to understand that for a given compound, Vd is typically very similar across species, while CLsys can vary widely; this is why the $t_{1/2}$ of an NCE can have a wide range of values across species. Once this is understood, then it is apparent that *in vivo* PK studies can be used to obtain the likely human Vd for a NCE, but that an *in vitro* assay will be needed to estimate the human CLsys of a NCE. In this way, it is clear that a combination of *in vivo* and *in vitro* assays will be the best way to predict the human $t_{1/2}$ of a NCE [78-80]. CLsys is a measure of the rate of drug elimination; it is calculated as shown in the following equation:

$$CLsys = Dose\ (iv)/AUC\ (iv)$$

where Dose (iv) is the dose (amount) of the drug administered in the iv route and AUC (iv) is the area under the curve in the resulting concentration-time plot in the PK study. Furthermore, CLsys can be understood as a combination of two parameters as follows:

$$CLsys = CL_H + CL_r$$

where CL_H (hepatic clearance) is the result of liver metabolism and biliary clearance and

CL_r is simply renal clearance. For many compounds, CL_r is a small part of CLsys and can be ignored in the human PK predictions; when CL_r is significant, *in vivo* PK studies in

laboratory animal can be used to estimate the importance of this in the clinical setting. When CL_r is minor (this is typical), then CLsys is largely defined as CL_H. CL_H can be estimated from either *in vivo* PK studies or various *in vitro* assays. The simplest way to get an *in vitro* estimate of CL_H is by measuring the metabolic stability of the NCE (or series of NCEs) [81, 82]. Both liver microsomes and hepatocytes have been used for *in vitro* assays for metabolic stability; microsomes have been commonly used in the past, but most researchers would state that hepatocytes are preferred in terms of their value for improved *in vitro* predictivity [83-89]. Hepatocytes provide the capability of producing both Phase I (oxidation plus hydrolysis of esters and amides) and Phase II (conjugative metabolism). However, because liver microsomes tend to be cheaper and more robust than hepatocytes, it is still common to use microsomes for a metabolic stability screening assay for NCEs [83].

Whether one chooses to use liver microsomes or hepatocytes, the analytical challenges are the same for setting up a higher throughput metabolic stability screen for NCEs. Typical higher throughput metabolic stability assays rely on some combination of robotics and HPLC-MS/MS in order to be able to handle the large number of samples that are generated and need to be assayed [19, 27, 90-104]. The basic procedure is to incubate the NCE in the microsomes or hepatocytes at 37 °C for a specified period of time and then to stop the reaction and assay the sample. There are two common ways to express the metabolic stability: single timepoint and multiple timepoints. In the single timepoint method, the % NCE remaining at a set timepoint (e.g., 20 minutes) can be used—this is very useful for ranking compounds. In the multiple timepoint method, a series of sample timepoints are taken (e.g., 5, 10, 20, 40 minutes) after the incubation is started and these are assayed for the NCE—this is useful for calculating the *in vitro* intrinsic clearance (Clint) of the NCE [81]. In either process, the percent of the NCE remaining in the sample can be determined by simply obtaining the peak area ratio of the timepoint sample over the time zero sample [8, 81, 82].

Janiszewski *et al*. [105] described a high-throughput screen for metabolic stability based on automation and HPLC-MS assays. The system made use of multiple injectors as well as a dual-column chromatography system to speed up the analytical step which was reported to be 30 seconds per sample. The described system was stated to have a capacity of over 2,000 samples per instrument per day. More recently, Chovan *et al*. [91] reported the use of a fast gradient HPLC-MS/MS system combined with a robotic system for handling the *in vitro* incubation step for use in a high-throughput metabolic stability screen. This screen also took advantage of commercially available MS method development software tools in order to be able to handle the large number of compounds that need to be assayed in this type of system. Very recently, Plumb *et al*. [106] described the utility of ultra-performance liquid chromatography (UPLC)-MS/MS for use in a microsomal metabolic stability assay. These authors point out the importance of having a high-resolution chromatographic system (such as UPLC) as part of the analytical step in order to ensure that the results are a true measurement of the NCE and not a combination of the NCE and some metabolite that may have been formed in the incubation step [106]. The UPLC system produced very sharp chromatographic peaks (peaks widths of 2-3 s).

DRUG-DRUG INTERACTIONS

Drug-drug interactions (DDIs) are a continuing concern for any NCE. The potential for drug-drug interactions is now well understood and there are multiple reports on the utility of various *in vitro* assays that can be used to assess the potential of a NCE for either producing drug-drug interactions in the clinic or being affected by drug-drug interactions in the clinic [7, 78, 107-122]. Typically, drug-drug interactions are defined as the effect of one drug on the PK profile of a second drug; these effects can either increase or decrease the concentration of the second drug. Currently, the US Food and Drug Administration (FDA) defines it as a clinically relevant DDI when a drug coadministration increases the AUC or Cmax (maximal plasma concentration) by greater than two-fold [112, 123]. DDIs have been estimated to be the cause of at least 500,000 adverse drug reactions annually in the US alone [123]. Multiple drugs that were once on the market have been recalled due to their potential to cause fatal DDIs [119]. For these reasons, pharmaceutical companies realize the importance of trying to screen out NCEs that have the potential to cause DDIs and to do this in the early discovery phase when possible.

The most common assay to check for DDI potential is the cytochrome P450 (CYP) inhibition assay based on human liver microsomes (HLM) [109, 123]. While there are multiple human CYP isozymes, there are a smaller number that account for most of the drug metabolizing activity (80% of drugs are metabolized by either CYP3A4 or CYP2D6) [19]. The CYP inhibition assays that are based on HLM, use a standard incubation procedure (typically in a 96-well format) with various probe reactions for individual CYP enzymes (e.g., midazolam for CYP 3A4). Because the extents of each of the probe reactions are monitored, the assay is independent of the test compound. This feature allows one to set up very high speed assays to measure the various probe compounds. These assays are predominantly based on HPLC-MS/MS techniques for the analytical step.

There have been multiple reports in recent years describing how HPLC-MS/MS can be used for providing higher throughput assays to support various CYP enzyme inhibition assays [24, 92, 106, 113, 117, 124-130]. For example, Peng *et al*. [43] described a high throughput assay based on HPLC-MS/MS to screen for five important CYP isozymes—CYP 3A4, CYP2D6, CYP2C9, CYP2C19 and CYP1A2. Their method was based on HLM incubation of six CYP-specific probe substrates. The samples were analyzed using HPLC-MS/MS with a monolithic silica rod HPLC column that allowed for a ballistic gradient and a mobile phase flow rate of 5 mL/min, resulting in a total sample run time of 24 seconds. Testino and Patony [129] describe the use of an *in vitro* substrate cocktail incubated with human liver microsomes and analyzed by LC-MS/MS to provide high throughput inhibition screening for five CYP enzymes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4). Kim *et al*. [117] have reported a high-throughput CYP inhibition assay based on HPLC-MS/MS with a fast gradient; their system

was designed to measure nine human CYP isozymes. Youdim *et al*. [130] recently described the use of a miniaturized 384-well assay format as part of their high-throughput CYP inhibition assay. The 384-well assay used human liver microsomes in conjunction with a cocktail of probe substrates metabolized by five major CYP enzymes. Recently, Rainville *et al*. [128] described a high-throughput CYP inhibition assay for six major CYP enzymes that utilized UPLC-MS/MS to provide a chromatographic run time of less than one minute per sample.

One area of some continuing discussion is the best way to use the CYP inhibition potential data [16, 112]. Both Bertrand *et al*. [111] and Masimirembwa *et al*. [131] show logic diagrams for how to use these data. A typical use for these data is described by Yan and Caldwell [132] who classify a compound's potential for inhibition based on the measured IC50 value; a strong inhibitor has an IC50< 1 μM, a moderate inhibitor has an IC50 from 1-10 μM, and a weak inhibitor has an IC50 > 10 μM. Using this classification scheme, Yan and Caldwell [132] describe a multi-step approach (Fig. **5** in [132]) for obtaining and utilizing CYP inhibition data.

A second area of research is the development of various *in vitro* assays for the measurement of a compound's potential for enzyme induction when dosed in humans [8, 133-136]. The typical example of a drug that is an enzyme inducer is the drug rifamicin (often used as a positive control in enzyme induction assays). Rifampicin is a strong inducer of CYP3A4; it can have a strong effect on coadministered drugs that are metabolized by CYP3A4. For example, if rifampicin is coadministered with cyclosporine, then the plasma levels of cyclosporine would be reduced significantly resulting in therapeutic failure for cyclosporine [8]. The number of marketed drugs that are reported to cause enzyme induction is small; Masimirembwa *et al*. [131] show a table listing six drugs that show clinically relevant *in vivo* induction. Yan and Caldwell [132] describe various assays that have been developed for assessing enzyme induction potential. Both Masimirembwa *et al*. [131] and Yan and Caldwell [132] discuss the use of reverse transcriptase polymerase chain reaction (RT-PCR) as an *in vitro* method for assessing the induction potential for a discovery compound. Worboys and Carlile [137] provide a good discussion on the challenges involved with properly assessing the clinical induction potential of a compound that is tested using the RT-PCR assay while still in the discovery phase. In a recent report, Sinz *et al*. [136] describe the current best practices for enzyme induction assays. According to Sinz *et al*. [136], high throughput screening models utilize various nuclear hormone receptors (e.g., PXR) in ligand binding or cell-based transactivation/reporter assays. For example, a high-throughput induction assay has been reported that is based on a genetically engineered cell line that expresses a PXRE-luciferase reporter gene [8, 138, 139]. In addition, Sinz *et al*. state that while immortalized hepatocyte cell lines can be used to assess enzyme induction of specific drug metabolizing enzymes, cultured primary human hepatocytes are the best established *in vitro* model for predicting enzyme induction and most accepted by various regulatory agencies [136]. Indeed, Youdim *et al*. [140] have described a higher throughput cocktail assay based on human hepatocytes to assess the CYP induction potential of NCEs; specifically, their assay measured the induction of the major CYPs (CYP3A4, CYP1A2, CYP2C9, CYP2C19 and CYP2D6).

It should be noted that while not a high-throughput assay, it is important to understand the importance of the various CYP isozymes in the metabolism of a candidate compound (this is often referred to as CYP reaction phenotyping) [122, 141]. There are at least two *in vitro* ways to determine the CYP enzyme that metabolizes a compound: one way is to measure substrate depletion and the other way is to assess the rate of metabolite formation [141]. For the latter process, one needs to have some knowledge of the major routes of metabolism for the test compound. It is important to understand the CYP enzyme metabolic profile in order to assess the likelihood that the compound will be prone to DDIs when in the clinic. The significance of the CYP enzyme profile is largely program dependant. One needs to consider other drugs that may be co-dosed with the test compound—are any of these drugs inducers of the CYP enzyme that metabolizes the test compound? As a general rule, the likelihood of DDIs is greater when a test compound is predominantly metabolized by a single CYP enzyme compared to a compound that is metabolized by several CYP enzymes [141].

METABOLISM AND TOXICITY ISSUES

While the understanding of the metabolism of a drug in clinical testing has clearly been an important topic over the last two decades, the recently released draft guidelines on the issue of metabolites in safety testing (MIST) have put a spotlight on the need to have an understanding of the metabolism of a candidate drug much earlier in the development phase [142-147]. These guidelines mandate a need to not only understand the structure of the human circulating metabolites, but also to have a quantitative estimate of their concentrations at steady-state. This requirement will increase the pressure on both discovery and development scientists to further understand the metabolism of their candidate drugs in both humans and laboratory animals that are used for toxicological evaluation of the candidate drug. This will also put further emphasis on the need to be able to screen for metabolism issues while still in the drug discovery setting.

The identification of drug metabolites is a topic of continuing interest and there are multiple articles that have described various techniques that can be used for metabolite identification in various *in vivo* and *in vitro* samples [148-171]. Most of these procedures are based on using various types of mass spectrometers to both discover and characterize the metabolites. For example, Yao *et al*. [168] describe the use of multiple ion monitoring to look for potential metabolites and to trigger the acquisition of enhanced product ion scans; in this case, the authors made use of the unique capabilities of the hybrid quadrupole-linear ion trap mass spectrometer (QTRAP MS). Tiller *et al*. [148] discuss the utility of using a quadrupole-time-of-flight mass spectrometer (QTOF MS) to allow for a semi-automated high throughput identification strategy; in this case the use of higher mass resolution capabilities of the system allow them to get accurate mass data that can be used to help with the

metabolite identification. Tiller *et al*. [148] further reported that this technology allowed them to provide *in vitro* metabolite profiling data for 21 NCEs in one day. Recently, Ruan *et al*. [151] have described the utility of the new hybrid linear ion trap/Orbitrap mass spectrometer (a high mass resolution instrument) and multiple post-acquisition data mining techniques for the detection and characterization of *in vitro* metabolites. As shown in Fig. (**4**), Lim *et al*. [169] show the utility of using high mass resolution for distinguishing a metabolite from background components. One of these post-acquisition data mining techniques is the use of the mass defect filtering technique [151, 152, 161, 172]. Mass defect filtering takes advantage of the fact that most drugs have a mass defect that is significantly different from most endogenous compounds so that by using a high mass resolution mass spectrometer, one can easily separate most of the background compounds from the data which makes it much easier to locate metabolites [152, 172].

While many metabolites are inactive and cleared rapidly, some metabolites are pharmacologically active ("active metabolites") and other metabolites are reactive and toxic. The formation of reactive or toxic metabolites is referred to as bioactivation or metabolic activation and is a topic that has received a lot of attention in recent years [173-180]. This has led to an increased need to detect reactive metabolites in the drug discovery stage [147, 160, 173, 174, 181-183]. A typical example of metabolic activation of a drug to a reactive metabolite is acetaminophen. Acetaminophen is metabolized to the reactive metabolite, NAPQI, which is then conjugated by glutathione (GSH) to form a stable metabolite that can be easily detected [173]. Due to the fact that many reactive metabolites form adducts with nucleophiles such as GSH, multiple research efforts have led to various ways to detect and identify GSH metabolites [184-193]. These methods can be useful when analyzing samples from *in vitro* incubations (liver microsomes or hepatocytes) in which glutathione has been added or *in vivo* samples (plasma, urine or bile) from laboratory animals dosed with the NCE. For example, Wen *et al*. [189] described the use of the QTrap MS system for the detection and characterization of glutathione metabolites by utilizing the negative precursor ion mode (set for *m/z* 272) for detecting potential glutathione metabolites. When a potential glutathione metabolite was found, the MS system was switched to the positive ion mode and an enhanced product ion (EPI) mass spectrum was obtained in order to get product ion information which could be used for characterizing the compound. This system was used to find several GSH metabolites of acetaminophen and clozapine after subjecting the test compounds to HLM incubations (supplemented with 1 mM GSH).

Mutlib *et al*. [187] described the utility of a stable isotope labeled glutathione for making it easier to detect glutathione adducts. By using a +3 Da (Dalton) labeled GSH compound in combination with unlabelled GSH (1:1 mixture), the authors demonstrate the improved ability to screen for GSH adducts formed from reactive metabolites in an *in vitro* incubation. The authors also combined the capabilities of a QTrap MS system to more quickly detect and characterize the glutathione adducts.

Zhu *et al*. [192] described the use of high-resolution mass spectrometry and mass defect filtering as another tool for detecting glutathione adducts of reactive metabolites; in this case, the technique was applied to *in vitro* samples as well as rat bile samples. Finally, Mahajan and Evans [188] recently reported on the use of a dual negative precursor ion approach

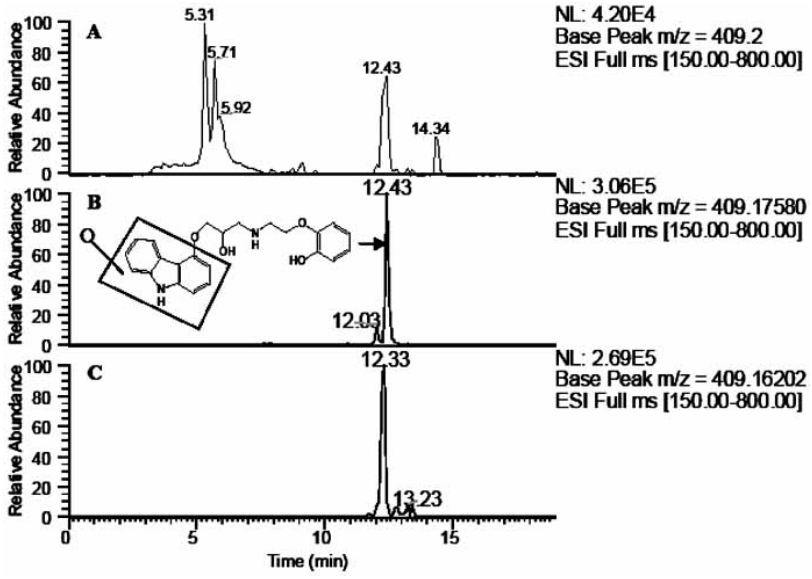

Fig. (4). High mass resolution provided by the Orbitrap MS allows for the separation of a metabolite and a co-eluting isobaric matrix component. Trace A shows the extracted mass chromatogram using nominal mass resolution (*m/z* 409). Trace B shows the exact mass chromatogram for the metabolite (*m/z* 409.17580). Trace C shows the exact mass chromatogram for the coeluting endogenous compound (*m/z* 409.16202). Adapted from Lim *et al*. [169] Copyright © 2007, used with permission of John Wiley and Sons.

as an improved procedure for detecting glutathione adducts. In this report, they described the use of *m/z* 272 and 254 as precursor ions when scanning in the negative ionization mode and they stated that this resulted in improved selectivity over the more common use of *m/z* 129 as the precursor ion when scanning in the positive ionization mode [188].

Acyl glucuronides are another class of potentially reactive metabolites that one should consider when testing a NCE in an *in vitro* or *in vivo* metabolite identification assay [194-197]. Acyl glucuronides have been considered to be potentially toxic metabolites and a possible cause of at least some cases of idiosyncratic toxicity [198-203]. Furthermore, acyl glucuronides have the potential to react covalently with proteins and these reactions have been implicated in the toxicity that resulted in the withdrawal of several drugs from the US market [202]. In order to assay samples for acyl glucuronides, special sample collection and processing techniques need to be observed as well as careful use of HPLC-MS/MS conditions [195-197]. For example, under atmospheric pressure chemical ionization (APCI) conditions, an acyl glucuronide may be unstable in the MS source, while the same compound may be stable when assayed using electrospray ionization (ESI) conditions [196].

While *in vitro* studies are an efficient way to screen for metabolites, perhaps the best way to collect relevant data on biotransformation pathways is to make use of bile-duct-cannulated (BDC) laboratory animals [202]. Studies with BDC animals can provide an important perspective in terms of the extent that a compound is metabolized down pathways that result in either glutathione metabolites or acyl glucuronide metabolites. While not commonly available during the lead optimization process, the ideal way to perform these studies is to use a radiolabelled version of the NCE. Use of the radiolabelled version of the NCE provides a facile way to quantify the various metabolites by quantifying the radioactivity. Indeed if a radiolabelled version of the NCE is available, one can also perform a mass balance study which can provide some assurance that the drug related materials are excreted in a timely manner. It should be noted that there are some differing perspectives within the industry as to what constitutes a positive mass balance study *vs* one that should be repeated due to poor recovery [202, 204].

Another way to assess the potential for reactive metabolites to bind with proteins is the covalent-binding assay that is an attempt to put a quantitative value on the extent of the protein-binding potential of the reactive metabolites [202]. The details for this protein-binding assay can be found in the report by Evans *et al*. [205]. The protein-binding assay requires a radiolabelled version of the NCE. For the *in vitro* assay, the radiolabelled NCE is incubated at 10 μM in liver microsomes with added GSH for enough time so that the metabolism can occur to a significant degree. Then the protein is precipitated and the pellet is washed and dissolved for scintillation counting. Using a similar procedure, one can also dose the radiolabelled compound in rats (PO 20 mg/kg) and collect liver and plasma samples after various time intervals out to 24 hours [205]. In this report, a binding level of 50 pmol /mg protein was the proposed maximum level for covalent binding of a NCE [202, 205]. It should be noted that this test should be viewed in proper prospective. Other relevant parameters, such as the proposed drug's dose, indication and duration of treatment, should be part of the decision making process.

It should be noted that the mere detection of one or more GSH metabolites or acyl glucuronide metabolites would not by itself preclude a compound from being considered for nomination for development. One would want to assess the percentage of the drug candidate that is metabolized to these compounds. Furthermore, one would want to consider the expected use of the drug; clearly a drug compound that would be used as a decongestant would need to have lower risks than a drug compound that might be effective against liver cancer, for example. For these considerations, it is important for the medicinal chemists to work closely with their DMPK colleagues as well as the pharmacology scientists in understanding the significance of any potentially negative findings during the lead optimization phase of new drug discovery.

In addition to the assays that have been described above, there are other *in vitro* and *in vivo* toxicology assays that have been used by various companies [8, 19, 37, 206-208]. One type of assay that is now well accepted is the hERG assay; indeed, most regulatory agencies now expect to have experimental hERG data as part of the drug development package. In the drug discovery stage, various *in vitro* assays to measure hERG activity are now available [19, 208-210]. The hERG patch clamp assay is considered the definitive *in vitro* assay to measure hERG activity. For compounds that show an undesirable hERG signal in the *in vitro* assay, various *in vivo* assays can be used to provide a more definitive measure of the compounds potential to cause cardiovascular problems such as lengthening of the QT interval [208, 211].

In the past, *in vivo* toxicology studies were typically delayed until after a compound had been recommended for development. This is starting to change as companies are searching for ways to provide additional toxicity screens as part of the lead optimization paradigm. One *in vivo* model that has shown promise as an early toxicity screen is the zebrafish model [19, 212-216]. Zebrafish have the advantage that they are small, easy to grow and the embryo is optically transparent [216]. The zebrafish is rapidly becoming an accepted model for toxicology studies [216, 217]. Due to their small size, it is possible to use zebrafish in a 96-well plate format as an early toxicity screen as part of the lead optimization effort [216].

CONCLUSIONS

Various ADME screens are now routinely used as part of the drug discovery process in the lead optimization phase. The fact that many of these ADME screens are relatively high throughput has allowed them to be used in parallel in order to provide useful data that can be used by the medicinal chemist to synthesize compounds that have acceptable ADME properties. At the same time, various safety/toxicity assays are now being utilized earlier in the drug discovery process. The results of this extra screening will be to produce NCEs that have improved PK and safety profiles, which

should help to reduce the attrition rate as compounds move forward from discovery to development.

REFERENCES

[1] MacCoss, M.; Baillie T.A. Organic chemistry in drug discovery. *Science,* **2004**, *303*(5665), 1810-3.

[2] Lombardino, J.G.; Lowe J.A. 3rd. The role of the medicinal chemist in drug discovery--then and now. *Nat. Rev. Drug Discov.,* **2004**, *3*(10), 853-62.

[3] Kola, I.; Landis J. Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discov.,* **2004**, *3*(8), 711-5.

[4] Thompson, T.N. Early ADME in support of drug discovery: the role of metabolic stability studies. *Curr. Drug Metab.,* **2000**, *1*(3), 215-41.

[5] Yengi, L.G.; Leung L.; Kao J. The evolving role of drug metabolism in drug discovery and development. *Pharm. Res.,* **2007**, *24*(5), 842-58.

[6] Kennedy, T. Managing the drug discovery/developmement interface. *Drug Discov. Today,* **1997**, *2*(10), 436-444.

[7] Kumar, G.N.; Surapaneni S. Role of drug metabolism in drug discovery and development. *Med. Res. Rev.,* **2001**, *21*(5), 397-411.

[8] Li, A.P. Screening for human ADME/Tox drug properties in drug discovery. *Drug Discov. Today,* **2001**, *6*(7), 357-366.

[9] Eddershaw, P.J.; Beresford A.P.; Bayliss M.K. ADME/PK as part of a rational approach to drug discovery. *Drug Discov. Today,* **2000**, *5*(9), 409-414.

[10] Caldwell, G.W.; Ritchie D.M.; Masucci J.A.; Hageman W.; Yan Z. The new pre-preclinical paradigm: compound optimization in early and late phase drug discovery. *Curr. Top. Med. Chem.,* **2001**, *1*(5), 353-66.

[11] Riley, R.J.; Martin I.J.; Cooper A.E. The influence of DMPK as an integrated partner in modern drug discovery. *Curr. Drug Metab.,* **2002**, *3*(5), 527-50.

[12] Li, A.P. *In vitro* approaches to evaluate ADMET drug properties. *Curr. Top. Med. Chem.,* **2004**, *4*(7), 701-6.

[13] Singh, S.S. Preclinical pharmacokinetics: an approach towards safer and efficacious drugs. *Curr. Drug Metab.,* **2006**, *7*(2), 165-82.

[14] Balani, S.K.; Miwa G.T.; Gan L.S.; Wu J.T.; Lee F.W. Strategy of utilizing *in vitro* and *in vivo* ADME tools for lead optimization and drug candidate selection. *Curr. Top. Med. Chem.,* **2005**, *5*(11), 1033-8.

[15] Chaturvedi, P.R.; Decker C.J.; Odinecs A. Prediction of pharmacokinetic properties using experimental approaches during early drug discovery. *Curr. Opin. Chem. Biol.,* **2001**, *5*(4), 452-63.

[16] Korfmacher, W.A. Lead optimization strategies as part of a drug metabolism environment. *Curr. Opin. Drug Discov. Dev.,* **2003**, *6*(4), 481-5.

[17] Wang, J.; Urban L.; Bojanic D. Maximising use of *in vitro* ADMET tools to predict *in vivo* bioavailability and safety. *Expert Opin. Drug Metab. Toxicol.,* **2007**, *3*(5), 641-65.

[18] Lin, J.; Sahakian D.C.; de Morais S.M.; Xu J.J.; Polzer R.J.; Winter S.M. The role of absorption, distribution, metabolism, excretion and toxicity in drug discovery. *Curr. Top. Med. Chem.,* **2003**, *3*(10), 1125-54.

[19] Kerns, E.; Di L. *Drug-like Properties: Concepts, Structure Design and Methods*. Academic Press, Burlington, MA, **2008**.

[20] Tarbit, M.H.; Berman J. High-throughput approaches for evaluating absorption, metabolism and excretion properties of lead compounds. *Curr. Opin. Chem. Biol.,* **1998**, *2*, 411-416.

[21] Korfmacher, W.A.; Cox K.A.; Bryant M.S.; Veals J.; Ng K.; Lin C.C. HPLC-API/MS/MS: a powerful tool for integrating drug metabolism into the drug discovery process. *Drug Discov. Today,* **1997**, *2*, 532-537.

[22] Korfmacher, W., Mass Spectrometry in Early Pharmacokinetic Investigations. In: *Mass Spectrometry in Medicinal Chemistry*, Wanner, K.; Hofner, G. Eds. Wiley-VCH, Weinheim, Federal Republic of Germany, **2007**, Vol. *36*, pp. 401-428.

[23] Ackermann, B.L.; Berna M.J.; Murphy A.T. Recent advances in use of LC/MS/MS for quantitative high-throughput bioanalytical support of drug discovery. *Curr. Top. Med. Chem.,* **2002**, *2*(1), 53-66.

[24] Korfmacher, W.A. Principles and applications of LC-MS in new drug discovery. *Drug Discov. Today,* **2005**, *10*(20), 1357-1367.

[25] Hopfgartner, G.; Bourgogne E. Quantitative high-throughput analysis of drugs in biological matrices by mass spectrometry. *Mass Spectrom. Rev.,* **2003**, *22*(3), 195-214.

[26] Xu, X.; Lan J.; Korfmacher W. Rapid LC/MS/MS Method Development for Drug Discovery. *Anal. Chem.,* **2005**, *77*(October 1), 389 A -394 A.

[27] Korfmacher, W., *Using Mass Spectrometry for Drug Metabolism Studies*. CRC Press, Boca Raton, FL, **2005**, p. 370.

[28] Jemal, M.; Xia Y.Q. LC-MS Development strategies for quantitative bioanalysis. *Curr. Drug Metab.,* **2006**, *7*(5), 491-502.

[29] Korfmacher, W. New Strategies for the implementation and Support of Bioanalysis in a Drug Metabolism Environment. In: *Integrated Strategies for Drug Discovery using Mass Spectrometry* Lee, M.S. Ed., John Wiley & Sons, Inc., Hoboken, NJ, **2005**, pp. 359-378.

[30] Korfmacher, W. Strategies and Techniques for for Higher Throughput ADME/PK Assays. In: *High-Throughput Analysis in the Pharmaceutical Industry* Wang, P.G. Ed. CRC Press, Boca Raton, FL, **2008**, pp. 205-232.

[31] Xu, X.; Lan J.; Zhou Q.; Tucker G.; Jean J.; Korfmacher W. Designing LC-MS/MS methods that are good enough to provide bioanalytical support for new drug discovery assays. *Am. Drug Discov.,* **2007**, *2*(3), 6-11.

[32] Ackermann, B.; Berna M.; Eckstein J.A.; Ott L.W.; Chaudhary A.G. Current applications of liquid chromatography/mass spectrometry in pharmaceutical discovery after a decade of innovation. *Annu. Rev. Anal. Chem.,* **2008**, *1*, 357-396.

[33] Espada, A.; Molina-Martin M.; Dage J.; Kuo M.S. Application of LC/MS and related techniques to high-throughput drug discovery. *Drug Discov. Today,* **2008**, *13*(9-10), 417-23.

[34] Leeson, P.D.; Springthorpe B. The influence of drug-like concepts on decision-making in medicinal chemistry. *Nat. Rev. Drug Discov.,* **2007**, *6*(11), 881-90.

[35] Bender, A.; Scheiber J.; Glick M.; Davies J.W.; Azzaoui K.; Hamon J.; Urban L.; Whitebread S.; Jenkins J.L. Analysis of pharmacology data and the prediction of adverse drug reactions and off-target effects from chemical structure. *Chem. Med. Chem.,* **2007**, *2*(6), 861-73.

[36] Walker, D.K. The use of pharmacokinetic and pharmacodynamic data in the assessment of drug safety in early drug development. *Br. J. Clin. Pharmacol.,* **2004**, *58*(6), 601-8.

[37] Guengerich, F.P.; MacDonald J.S. Applying mechanisms of chemical toxicity to predict drug safety. *Chem. Res. Toxicol.,* **2007**, *20*(3), 344-69.

[38] Hellriegel, E.T.; Bjornsson T.D.; Hauck W.W. Interpatient variability in bioavailability is related to the extent of absorption: implications for bioavailability and bioequivalence studies. *Clin. Pharmacol. Ther.,* **1996**, *60*(6), 601-7.

[39] Stoner, C.L.; Cleton A.; Johnson K.; Oh D.M.; Hallak H.; Brodfuehrer J.; Surendran N.; Han H.K. Integrated oral bioavailability projection using *in vitro* screening data as a selection tool in drug discovery. *Int. J. Pharm.,* **2004**, *269*(1), 241-9.

[40] Mandagere, A.K.; Thompson T.N.; Hwang K.K. Graphical model for estimating oral bioavailability of drugs in humans and other species from their Caco-2 permeability and *in vitro* liver enzyme metabolic stability rates. *J. Med. Chem.,* **2002**, *45*(2), 304-11.

[41] Fagerholm, U. The role of permeability in drug ADME/PK, interactions and toxicity--presentation of a permeability-based classification system (PCS) for prediction of ADME/PK in humans. *Pharm. Res.,* **2008**, *25*(3), 625-38.

[42] Hubatsch, I.; Ragnarsson E.G.; Artursson P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat. Protoc.,* **2007**, *2*(9), 2111-9.

[43] Shah, P.; Jogani V.; Bagchi T.; Misra A. Role of Caco-2 cell monolayers in prediction of intestinal drug absorption. *Biotechnol. Prog.,* **2006**, *22*(1), 186-98.

[44] van Breemen, R.B.; Li Y. Caco-2 cell permeability assays to measure drug absorption. *Expert Opin. Drug Metab. Toxicol.,* **2005**, *1*(2), 175-85.

[45] Polli, J.E.; Ginski M.J. Human drug absorption kinetics and comparison to Caco-2 monolayer permeabilities. *Pharm. Res.,* **1998**, *15*(1), 47-52.

[46] Balimane, P.V.; Patel K.; Marino A.; Chong S. Utility of 96 well Caco-2 cell system for increased throughput of P-gp screening in drug discovery. *Eur. J. Pharm. Biopharm.,* **2004**, *58*(1), 99-105.

[47] Markowska, M.; Oberle R.; Juzwin S.; Hsu C.P.; Gryszkiewicz M.; Streeter A.J. Optimizing Caco-2 cell monolayers to increase

throughput in drug intestinal absorption analysis. *J. Pharmacol. Toxicol. Methods,* **2001**, *46*(1), 51-5.

[48] Tannergren, C.; Langguth P.; Hoffmann K.J. Compound mixtures in Caco-2 cell permeability screens as a means to increase screening capacity. *Pharmazie,* **2001**, *56*(4), 337-42.

[49] Larger, P.; Altamura M.; Catalioto R.M.; Giuliani S.; Maggi C.A.; Valenti C.; Triolo A. Simultaneous LC-MS/MS determination of reference pharmaceuticals as a method for the characterization of the Caco-2 cell monolayer absorption properties. *Anal. Chem.,* **2002**, *74*(20), 5273-81.

[50] Wong, A.K.; Ross B.P.; Chan Y.N.; Artursson P.; Lazorova L.; Jones A.; Toth I. Determination of transport in the Caco-2 cell assay of compounds varying in lipophilicity using LC-MS: enhanced transport of Leu-enkephalin analogues. *Eur. J. Pharm. Sci.,* **2002**, *16*(3), 113-8.

[51] Fung, E.N.; Chu I.; Li C.; Liu T.; Soares A.; Morrison R.; Nomeir A.A. Higher-throughput screening for Caco-2 permeability utilizing a multiple sprayer liquid chromatography/tandem mass spectrometry system. *Rapid Commun. Mass Spectrom.,* **2003**, *17*(18), 2147-52.

[52] Hakala, K.S.; Laitinen L.; Kaukonen A.M.; Hirvonen J.; Kostiainen R.; Kotiaho T. Development of LC/MS/MS methods for cocktail dosed Caco-2 samples using atmospheric pressure photoionization and electrospray ionization. *Anal. Chem.,* **2003**, *75*(21), 5969-77.

[53] Laitinen, L.; Kangas H.; Kaukonen A.M.; Hakala K.; Kotiaho T.; Kostiainen R.; Hirvonen J. N-in-one permeability studies of heterogeneous sets of compounds across Caco-2 cell monolayers. *Pharm. Res.,* **2003**, *20*(2), 187-97.

[54] Li, Y.; Shin Y.G.; Yu C.; Kosmeder J.W.; Hirschelman W.H.; Pezzuto J.M.; van Breemen R.B. Increasing the throughput and productivity of Caco-2 cell permeability assays using liquid chromatography-mass spectrometry: application to resveratrol absorption and metabolism. *Comb. Chem. High Throughput Screen.,* **2003**, *6*(8), 757-67.

[55] Hidalgo, I.J. Assessing the absorption of new pharmaceuticals. *Curr. Top. Med. Chem.,* **2001**, *1*(5), 385-401.

[56] Bermejo, M.; Avdeef A.; Ruiz A.; Nalda R.; Ruell J.A.; Tsinman O.; Gonzalez I.; Fernandez C.; Sanchez G.; Garrigues T.M.; Merino V. PAMPA--a drug absorption *in vitro* model 7. Comparing rat *in situ*, Caco-2, and PAMPA permeability of fluoroquinolones. *Eur. J. Pharm. Sci.,* **2004**, *21*(4), 429-41.

[57] Verma, R.P.; Hansch C.; Selassie C.D. Comparative QSAR studies on PAMPA/modified PAMPA for high throughput profiling of drug absorption potential with respect to Caco-2 cells and human intestinal absorption. *J. Comput. Aided Mol. Des.,* **2007**, *21*(1-3), 3-22.

[58] Avdeef, A. The rise of PAMPA. *Expert Opin. Drug Metab. Toxicol.,* **2005**, *1*(2), 325-42.

[59] Avdeef, A.; Bendels S.; Di L.; Faller B.; Kansy M.; Sugano K.; Yamauchi Y. PAMPA--critical factors for better predictions of absorption. *J. Pharm. Sci.,* **2007**, *96*(11), 2893-909.

[60] Kerns, E.H.; Di L.; Petusky S.; Farris M.; Ley R.; Jupp P. Combined application of parallel artificial membrane permeability assay and Caco-2 permeability assays in drug discovery. *J. Pharm. Sci.,* **2004**, *93*(6), 1440-53.

[61] Szakacs, G.; Varadi A.; Ozvegy-Laczka C.; Sarkadi B. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). *Drug Discov. Today,* **2008**, *13*(9-10), 379-93.

[62] Glavinas, H.; Krajcsi P.; Cserepes J.; Sarkadi B. The role of ABC transporters in drug resistance, metabolism and toxicity. *Curr. Drug Deliv.,* **2004**, *1*(1), 27-42.

[63] Bodo, A.; Bakos E.; Szeri F.; Varadi A.; Sarkadi B. The role of multidrug transporters in drug availability, metabolism and toxicity. *Toxicol. Lett.,* **2003**, *140-141*, 133-43.

[64] Beaudry, F.; Le Blanc J.C.; Coutu M.; Brown N.K. *In vivo* pharmacokinetic screening in cassette dosing experiments; the use of on-line Pprospekt liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry technology in drug discovery. *Rapid Commun. Mass Spectrom.,* **1998**, *12*(17), 1216-22.

[65] Smith, N.F.; Hayes A.; Nutley B.P.; Raynaud F.I.; Workman P. Evaluation of the cassette dosing approach for assessing the pharmacokinetics of geldanamycin analogues in mice. *Cancer Chemother. Pharmacol.,* **2004**, *54*(6), 475-86.

[66] Zhang, M.Y.; Kerns E.; McConnell O.; Sonnenberg-Reines J.; Zaleska M.M.; Steven Jacobsen J.; Butera J.; Kreft A. Brain and plasma exposure profiling in early drug discovery using cassette administration and fast liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.,* **2004**, *34*(2), 359-68.

[67] Ohkawa, T.; Ishida Y.; Kanaoka E.; Takahashi K.; Okabe H.; Matsumoto T.; Nakamoto S.; Tamada J.; Koike M.; Yoshikawa T. A new generic column switching system for quantitation in cassette dosing using LC/MS/MS. *J. Pharm. Biomed. Anal.,* **2003**, *31*(6), 1089-99.

[68] Olah, T.V.; McLoughlin D.A.; Gilbert J.D. The simultaneous determination of mixtures of drug candidates by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry as an *in vivo* drug screening procedure. *Rapid Commun. Mass Spectrom.,* **1997**, *11*(1), 17-23.

[69] White, R.E.; Manitpisitkul P. Pharmacokinetic theory of cassette dosing in drug discovery screening. *Drug Metab. Dispos.,* **2001**, *29*(7), 957-66.

[70] Han, H.K.; Sadagopan N.; Reichard G.A.; Yapa U.; Zhu T.; Hubbel A.; Johnson K.; Brodfuehrer J. An efficient approach for the rapid assessment of oral rat exposures for new chemical entities in drug discovery. *J. Pharm. Sci.,* **2006**, *95*(8), 1684-92.

[71] Manitpisitkul, P.; White R.E. Whatever happened to cassette-dosing pharmacokinetics? *Drug Discov. Today,* **2004**, *9*(15), 652-8.

[72] Christ, D.D. Cassette dosing pharmacokinetics: valuable tool or flawed science? *Drug Metab. Dispos.,* **2001**, *29*(7), 935.

[73] Kassel, D.B. Applications of high-throughput ADME in drug discovery. *Curr. Opin. Chem. Biol.,* **2004**, *8*(3), 339-45.

[74] Briem, S.; Martinsson S.; Bueters T.; Skoglund E. Combined approach for high-throughput preparation and analysis of plasma samples from exposure studies. *Rapid Commun. Mass Spectrom.,* **2007**, *21*(13), 1965-72.

[75] Korfmacher, W.A.; Cox K.A.; Ng K.J.; Veals J.; Hsieh Y.; Wainhaus S.; Broske L.; Prelusky D.; Nomeir A.; White R.E. Cassette-accelerated rapid rat screen: a systematic procedure for the dosing and liquid chromatography/atmospheric pressure ionization tandem mass spectrometric analysis of new chemical entities as part of new drug discovery. *Rapid Commun. Mass Spectrom.,* **2001**, *15*(5), 335-40.

[76] Liu, B.; Chang J.; Gordon W.P.; Isbell J.; Zhou Y.; Tuntland T. Snapshot PK: a rapid rodent *in vivo* preclinical screening approach. *Drug Discov. Today,* **2008**, *13*(7-8), 360-7.

[77] Mei, H.; Korfmacher W.; Morrison R. Rapid *in vivo* oral screening in rats: reliability, acceptance criteria, and filtering efficiency. *AAPS J.,* **2006**, *8*(3), E493-500.

[78] Obach, R.S.; Baxter J.G.; Liston T.E.; Silber B.M.; Jones B.C.; MacIntyre F.; Rance D.J.; Wastall P. The prediction of human pharmacokinetic parameters from preclinical and *in vitro* metabolism data. *J. Pharmacol. Exp. Ther.,* **1997**, *283*(1), 46-58.

[79] Barter, Z.E.; Bayliss M.K.; Beaune P.H.; Boobis A.R.; Carlile D.J.; Edwards R.J.; Houston J.B.; Lake B.G.; Lipscomb J.C.; Pelkonen O.R.; Tucker G.T.; Rostami-Hodjegan A. Scaling factors for the extrapolation of *in vivo* metabolic drug clearance from *in vitro* data: reaching a consensus on values of human microsomal protein and hepatocellularity per gram of liver. *Curr. Drug Metab.,* **2007**, *8*(1), 33-45.

[80] Houston, J.B.; Galetin A. Progress towards prediction of human pharmacokinetic parameters from *in vitro* technologies. *Drug Metab. Rev.,* **2003**, *35*(4), 393-415.

[81] Baranczewski, P.; Stanczak A.; Sundberg K.; Svensson R.; Wallin A.; Jansson J.; Garberg P.; Postlind H. Introduction to *in vitro* estimation of metabolic stability and drug interactions of new chemical entities in drug discovery and development. *Pharmacol. Rep.,* **2006**, *58*(4), 453-72.

[82] Riley, R.J.; Grime K. Metabolic screening *in vitro*: metabolic stability, CYP inhibition and induction. *Drug Discov. Today: Technol.,* **2004**, *1*, 365-372.

[83] Faller, B.; Wang J.; Zimmerlin A.; Bell L.; Hamon J.; Whitebread S.; Azzaoui K.; Bojanic D.; Urban L. High-throughput *in vitro* profiling assays: lessons learnt from experiences at Novartis. *Expert Opin. Drug Metab. Toxicol.,* **2006**, *2*(6), 823-33.

[84] Reddy, A.; Heimbach T.; Freiwald S.; Smith D.; Winters R.; Michael S.; Surendran N.; Cai H. Validation of a semi-automated human hepatocyte assay for the determination and prediction of

intrinsic clearance in discovery. *J. Pharm. Biomed. Anal.,* **2005**, *37*(2), 319-26.

[85] Griffin, S.J.; Houston J.B. Comparison of fresh and cryopreserved rat hepatocyte suspensions for the prediction of *in vitro* intrinsic clearance. *Drug Metab. Dispos.,* **2004**, *32*(5), 552-8.

[86] McGinnity, D.F.; Soars M.G.; Urbanowicz R.A.; Riley R.J. Evaluation of fresh and cryopreserved hepatocytes as *in vitro* drug metabolism tools for the prediction of metabolic clearance. *Drug Metab. Dispos.,* **2004**, *32*(11), 1247-53.

[87] Soars, M.G.; Grime K.; Sproston J.L.; Webborn P.J.; Riley R.J. Use of hepatocytes to assess the contribution of hepatic uptake to clearance *in vivo*. *Drug Metab. Dispos.,* **2007**, *35*(6), 859-65.

[88] Soars, M.G.; McGinnity D.F.; Grime K.; Riley R.J. The pivotal role of hepatocytes in drug discovery. *Chem. Biol. Interact.,* **2007**, *168*(1), 2-15.

[89] Hewitt, N.J.; Lechon M.J.; Houston J.B.; Hallifax D.; Brown H.S.; Maurel P.; Kenna J.G.; Gustavsson L.; Lohmann C.; Skonberg C.; Guillouzo A.; Tuschl G.; Li A.P.; LeCluyse E.; Groothuis G.M.; Hengstler J.G. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab. Rev.,* **2007**, *39*(1), 159-234.

[90] Drexler, D.M.; Belcastro J.V.; Dickinson K.E.; Edinger K.J.; Hnatyshyn S.Y.; Josephs J.L.; Langish R.A.; McNaney C.A.; Santone K.S.; Shipkova P.A.; Tymiak A.A.; Zvyaga T.A.; Sanders M. An automated high throughput liquid chromatography-mass spectrometry process to assess the metabolic stability of drug candidates. *Assay Drug Dev. Technol.,* **2007**, *5*(2), 247-64.

[91] Chovan, L.E.; Black-Schaefer C.; Dandliker P.J.; Lau Y.Y. Automatic mass spectrometry method development for drug discovery: application in metabolic stability assays. *Rapid Commun. Mass Spectrom.,* **2004**, *18*(24), 3105-12.

[92] Jenkins, K.M.; Angeles R.; Quintos M.T.; Xu R.; Kassel D.B.; Rourick R.A. Automated high throughput ADME assays for metabolic stability and cytochrome P450 inhibition profiling of combinatorial libraries. *J. Pharm. Biomed. Anal.,* **2004**, *34*(5), 989-1004.

[93] Kantharaj, E.; Tuytelaars A.; Proost P.E.; Ongel Z.; Van Assouw H.P.; Gilissen R.A. Simultaneous measurement of drug metabolic stability and identification of metabolites using ion-trap mass spectrometry. *Rapid Commun. Mass Spectrom.,* **2003**, *17*(23), 2661-8.

[94] Korfmacher, W.A.; Palmer C.A.; Nardo C.; Dunn-Meynell K.; Grotz D.; Cox K.; Lin C.C.; Elicone C.; Liu C.; Duchoslav E. Development of an automated mass spectrometry system for the quantitative analysis of liver microsomal incubation samples: a tool for rapid screening of new compounds for metabolic stability. *Rapid Commun. Mass Spectrom.,* **1999**, *13*(10), 901-7.

[95] McNaney, C.A.; Drexler D.M.; Hnatyshyn S.Y.; Zvyaga T.A.; Knipe J.O.; Belcastro J.V.; Sanders M. An automated liquid chromatography-mass spectrometry process to determine metabolic stability half-life and intrinsic clearance of drug candidates by substrate depletion. *Assay Drug Dev. Technol.,* **2008**, *6*(1), 121-9.

[96] Shou, W.Z.; Magis L.; Li A.C.; Naidong W.; Bryant M.S. A novel approach to perform metabolite screening during the quantitative LC-MS/MS analyses of *in vitro* metabolic stability samples using a hybrid triple-quadrupole linear ion trap mass spectrometer. *J. Mass Spectrom.,* **2005**, *40*(10), 1347-56.

[97] Thompson, T.N. Optimization of metabolic stability as a goal of modern drug design. *Med. Res. Rev.,* **2001**, *21*(5), 412-49.

[98] Tong, X.S.; Xu S.; Zheng S.; Pivnichny J.V.; Martin J.; Dufresne C. High throughput metabolic stability screen for lead optimization in drug discovery. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.,* **2006**, *833*(2), 165-73.

[99] Zhao, S.X.; Forman D.; Wallace N.; Smith B.J.; Meyer D.; Kazolias D.; Gao F.; Soglia J.; Cole M.; Nettleton D. Simple strategies for reducing sample loads in *in vitro* metabolic stability high-throughput screening experiments: A comparison between traditional, two-time-point and pooled sample analyses. *J. Pharm. Sci.,* **2005**, *94*(1), 38-45.

[100] Hsieh, Y.; Favreau L.; Cheng K.C.; Chen J. Chiral supercritical fluid chromatography/tandem mass spectrometry for the simultaneous determination of pindolol and propranolol in metabolic stability samples. *Rapid Commun. Mass Spectrom.,* **2005**, *19*(21), 3037-41.

[101] Hsieh, Y.; Favreau L.; Schwerdt J.; Cheng K.C. Supercritical fluid chromatography/tandem mass spectrometric method for analysis of pharmaceutical compounds in metabolic stability samples. *J. Pharm. Biomed. Anal.,* **2006**, *40*(3), 799-804.

[102] Kassel, D.B., High Throughput Strategies for *In Vitro* ADME Assays: How Fast Can We Go? In: *Using Mass Spectrometry for Drug Metabolism Studies* (Ed. Korfmacher W), pp. 83-102. CRC Press, Boca Raton, FL, 2005.

[103] Saunders, K.C. Automation and robotics in ADME screening. *Drug Discov. Today:Technologies,* **2004**, *1*(4), 373-380.

[104] Di, L.; Kerns E.H.; Ma X.J.; Huang Y.; Carter G.T. Applications of high throughput microsomal stability assay in drug discovery. *Comb. Chem. High Throughput Screen,* **2008**, *11*(6), 469-76.

[105] Janiszewski, J.S.; Rogers K.J.; Whalen K.M.; Cole M.J.; Liston T.E.; Duchoslav E.; Fouda H.G. A high-capacity LC/MS system for the bioanalysis of samples generated from plate-based metabolic screening. *Anal. Chem.,* **2001**, *73*(7), 1495-501.

[106] Plumb, R.S.; Potts W.B., 3rd; Rainville P.D.; Alden P.G.; Shave D.H.; Baynham G.; Mazzeo J.R. Addressing the analytical throughput challenges in ADME screening using rapid ultra-performance liquid chromatography/tandem mass spectrometry methodologies. *Rapid Commun. Mass Spectrom.,* **2008**, *22*(14), 2139-52.

[107] Obach, R.S. Drug-drug interactions: an important negative attribute in drugs. *Drugs Today (Barc),* **2003**, *39*(5), 301-38.

[108] Obach, R.S.; Walsky R.L.; Venkatakrishnan K. Mechanism-based inactivation of human cytochrome p450 enzymes and the prediction of drug-drug interactions. *Drug Metab. Dispos.,* **2007**, *35*(2), 246-55.

[109] Obach, R.S.; Walsky R.L.; Venkatakrishnan K.; Gaman E.A.; Houston J.B.; Tremaine L.M. The utility of *in vitro* cytochrome P450 inhibition data in the prediction of drug-drug interactions. *J. Pharmacol. Exp. Ther.,* **2006**, *316*(1), 336-48.

[110] Obach, R.S.; Walsky R.L.; Venkatakrishnan K.; Houston J.B.; Tremaine L.M. *In vitro* cytochrome P450 inhibition data and the prediction of drug-drug interactions: qualitative relationships, quantitative predictions, and the rank-order approach. *Clin. Pharmacol. Ther.,* **2005**, *78*(6), 582-92.

[111] Bertrand, M.; Jackson P.; Walther B. Rapid assessment of drug metabolism in the drug discovery process. *Eur. J. Pharm. Sci.,* **2000**, *11* (Suppl. 2), S61-72.

[112] Bjornsson, T.D.; Callaghan J.T.; Einolf H.J.; Fischer V.; Gan L.; Grimm S.; Kao J.; King S.P.; Miwa G.; Ni L.; Kumar G.; McLeod J.; Obach R.S.; Roberts S.; Roe A.; Shah A.; Snikeris F.; Sullivan J.T.; Tweedie D.; Vega J.M.; Walsh J.; Wrighton S.A. The conduct of *in vitro* and *in vivo* drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *Drug Metab. Dispos.,* **2003**, *31*(7), 815-32.

[113] Chu, I.; Nomeir A.A. Utility of mass spectrometry for *in vitro* ADME assays. *Curr. Drug Metab.,* **2006**, *7*(5), 467-77.

[114] Di, L.; Kerns E.H. Profiling drug-like properties in discovery research. *Curr. Opin. Chem. Biol.,* **2003**, *7*(3), 402-8.

[115] Di, L.; Kerns E.H.; Li S.Q.; Carter G.T. Comparison of cytochrome P450 inhibition assays for drug discovery using human liver microsomes with LC-MS, rhCYP450 isozymes with fluorescence, and double cocktail with LC-MS. *Int. J. Pharm.,* **2007**, *335*(1-2), 1-11.

[116] Gao, F.; Johnson D.L.; Ekins S.; Janiszewski J.; Kelly K.G.; Meyer R.D.; West M. Optimizing higher throughput methods to assess drug-drug interactions for CYP1A2, CYP2C9, CYP2C19, CYP2D6, rCYP2D6, and CYP3A4 *in vitro* using a single point IC(50). *J. Biomol. Screen.,* **2002**, *7*(4), 373-82.

[117] Kim, M.J.; Kim H.; Cha I.J.; Park J.S.; Shon J.H.; Liu K.H.; Shin J.G. High-throughput screening of inhibitory potential of nine cytochrome P450 enzymes *in vitro* using liquid chromatography/ tandem mass spectrometry. *Rapid Commun. Mass Spectrom.,* **2005**, *19*(18), 2651-8.

[118] Riley, R.J.; Grime K.; Weaver R. Time-dependent CYP inhibition. *Expert Opin. Drug Metab. Toxicol.,* **2007**, *3*(1), 51-66.

[119] Wienkers, L.C.; Heath T.G. Predicting *in vivo* drug interactions from *in vitro* drug discovery data. *Nat. Rev. Drug Discov.,* **2005**, *4*(10), 825-33.

[120] Hutzler, M.; Messing D.M.; Wienkers L.C. Predicting drug-drug interactions in drug discovery: where are we now and where are we going? *Curr. Opin. Drug Discov. Dev.,* **2005**, *8*(1), 51-8.

[121] Shou, M. Prediction of pharmacokinetics and drug-drug interactions from *in vitro* metabolism data. *Curr. Opin. Drug Discov. Dev.,* **2005**, *8*(1), 66-77.

[122] McGinnity, D.F.; Waters N.J.; Tucker J.; Riley R.J. Integrated *in vitro* analysis for the *in vivo* prediction of cytochrome P450-mediated drug-drug interactions. *Drug Metab. Dispos.,* **2008**, *36*(6), 1126-34.

[123] Bell, L.; Bickford S.; Nguyen P.H.; Wang J.; He T.; Zhang B.; Friche Y.; Zimmerlin A.; Urban L.; Bojanic D. Evaluation of fluorescence- and mass spectrometry-based CYP inhibition assays for use in drug discovery. *J. Biomol. Screen.,* **2008**, *13*(5), 343-53.

[124] Chu, I.; Favreau L.; Soares T.; Lin C.; Nomeir A.A. Validation of higher-throughput high-performance liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry assays to conduct cytochrome P450s CYP2D6 and CYP3A4 enzyme inhibition studies in human liver microsomes. *Rapid Commun. Mass Spectrom.,* **2000**, *14*(4), 207-14.

[125] Bu, H.Z.; Magis L.; Knuth K.; Teitelbaum P. High-throughput cytochrome P450 (CYP) inhibition screening *via* a cassette probe-dosing strategy. VI. Simultaneous evaluation of inhibition potential of drugs on human hepatic isozymes CYP2A6, 3A4, 2C9, 2D6 and 2E1. *Rapid Commun. Mass Spectrom.,* **2001**, *15*(10), 741-8.

[126] Peng, S.X.; Barbone A.G.; Ritchie D.M. High-throughput cytochrome p450 inhibition assays by ultrafast gradient liquid chromatography with tandem mass spectrometry using monolithic columns. *Rapid Commun. Mass Spectrom.,* **2003**, *17*(6), 509-18.

[127] Bhoopathy, S.; Xin B.; Unger S.E.; Karnes H.T. A novel incubation direct injection LC/MS/MS technique for *in vitro* drug metabolism screening studies involving the CYP 2D6 and the CYP 3A4 isozymes. *J. Pharm. Biomed. Anal.,* **2005**, *37*(4), 739-49.

[128] Rainville, P.D.; Wheaton J.P.; Alden P.G.; Plumb R.S. Sub one minute inhibition assays for the major cytochrome P450 enzymes utilizing ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.,* **2008**, *22*(9), 1345-50.

[129] Testino, S.A., Jr.; Patonay G. High-throughput inhibition screening of major human cytochrome P450 enzymes using an *in vitro* cocktail and liquid chromatography-tandem mass spectrometry. *J. Pharm. Biomed. Anal.,* **2003**, *30*(5), 1459-67.

[130] Youdim, K.A.; Lyons R.; Payne L.; Jones B.C.; Saunders K. An automated, high-throughput, 384 well Cytochrome P450 cocktail IC(50) assay using a rapid resolution LC-MS/MS end-point. *J. Pharm. Biomed. Anal.,* **2008**, *48*(1), 92-9.

[131] Masimirembwa, C.M.; Thompson R.; Andersson T.B. *In vitro* high throughput screening of compounds for favorable metabolic properties in drug discovery. *Comb. Chem. High Throughput Screen,* **2001**, *4*(3), 245-63.

[132] Yan, Z.; Caldwell G.W. Metabolism profiling, and cytochrome P450 inhibition & induction in drug discovery. *Curr. Top. Med. Chem.,* **2001**, *1*(5), 403-25.

[133] Vermeir, M.; Annaert P.; Mamidi R.N.; Roymans D.; Meuldermans W.; Mannens G. Cell-based models to study hepatic drug metabolism and enzyme induction in humans. *Expert Opin. Drug Metab. Toxicol.,* **2005**, *1*(1), 75-90.

[134] Mankowski, D.C.; Ekins S. Prediction of human drug metabolizing enzyme induction. *Curr. Drug Metab.,* **2003**, *4*(5), 381-91.

[135] Kato, M.; Chiba K.; Horikawa M.; Sugiyama Y. The quantitative prediction of *in vivo* enzyme-induction caused by drug exposure from *in vitro* information on human hepatocytes. *Drug Metab. Pharmacokinet.,* **2005**, *20*(4), 236-43.

[136] Sinz, M.; Wallace G.; Sahi J. Current industrial practices in assessing cyp450 enzyme induction: Preclinical and clinical. *AAPS J,* **2008**, *10*(2), 391-400.

[137] Worboys, P.D.; Carlile D.J. Implications and consequences of enzyme induction on preclinical and clinical drug development. *Xenobiotica,* **2001**, *31*(8-9), 539-56.

[138] Lehmann, J.M.; McKee D.D.; Watson M.A.; Willson T.M.; Moore J.T.; Kliewer S.A. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J. Clin. Invest.,* **1998**, *102*(5), 1016-23.

[139] Tippin, T.K.; Hamilton G.; Moore L.; Beaudet E.J.; Jolley S.; Brodie T.A.; Andrews R.C.; Becherer J.D.; McDougald D.L.; Gaul M.D.; Hoivik D.J.; Mellon-Kusibab K.; Lehmann J.; Kliewer S.; Novick S.; Laethem R.; Zhao Z.; LeCluyse E.L. CYP3A induction by N-hydroxyformamide tumor necrosis factor-alpha converting enzyme/matrix metalloproteinase inhibitors use of a pregname X receptor activation assay and primary hepatocyte culture for assessing induction potential in humans. *Drug Metab. Dispos.,* **2003**, *31*(7), 870-7.

[140] Youdim, K.A.; Tyman C.A.; Jones B.C.; Hyland R. Induction of cytochrome P450: assessment in an immortalized human hepatocyte cell line (Fa2N4) using a novel higher throughput cocktail assay. *Drug Metab. Dispos.,* **2007**, *35*(2), 275-82.

[141] Youdim, K.A.; Zayed A.; Dickins M.; Phipps A.; Griffiths M.; Darekar A.; Hyland R.; Fahmi O.; Hurst S.; Plowchalk D.R.; Cook J.; Guo F.; Obach R.S. Application of CYP3A4 *in vitro* data to predict clinical drug-drug interactions; predictions of compounds as objects of interaction. *Br. J. Clin. Pharmacol.,* **2008**, *65*(5), 680-92.

[142] Smith, D.A.; Obach R.S. Seeing through the mist: abundance versus percentage. Commentary on metabolites in safety testing. *Drug Metab. Dispos.,* **2005**, *33*(10), 1409-17.

[143] Humphreys, W.G.; Unger S.E. Safety assessment of drug metabolites: characterization of chemically stable metabolites. *Chem. Res. Toxicol.,* **2006**, *19*(12), 1564-1569.

[144] Smith, D.A.; Obach R.S. Metabolites and safety: what are the concerns, and how should we address them? *Chem. Res. Toxicol.,* **2006**, *19*(12), 1570-1579.

[145] USFDA, Safety Testing of Drug Metabolites. 2008, http://www.fda.gov/cder/guidance/689fnl.pdf.

[146] Baillie, T.A.; Cayen M.N.; Fouda H.; Gerson R.J.; Green J.D.; Grossman S.J.; Klunk L.J.; LeBlanc B.; Perkins D.G.; Shipley L.A. Drug metabolites in safety testing. *Toxicol. Appl. Pharmacol.,* **2002**, *182*(3), 188-96.

[147] Baillie, T.A. Metabolism and toxicity of drugs. Two decades of progress in industrial drug metabolism. *Chem. Res. Toxicol.,* **2008**, *21*(1), 129-37.

[148] Tiller, P.R.; Yu S.; Castro-Perez J.; Fillgrove K.L.; Baillie T.A. High-throughput, accurate mass liquid chromatography/tandem mass spectrometry on a quadrupole time-of-flight system as a 'first-line' approach for metabolite identification studies. *Rapid Commun. Mass Spectrom.,* **2008**, *22*(7), 1053-61.

[149] Hop, C.E.; Tiller P.R.; Romanyshyn L. *In vitro* metabolite identification using fast gradient high performance liquid chromatography combined with tandem mass spectrometry. *Rapid Commun. Mass Spectrom.,* **2002**, *16*(3), 212-9.

[150] Tiller, P.R.; Romanyshyn L.A. Liquid chromatography/tandem mass spectrometric quantification with metabolite screening as a strategy to enhance the early drug discovery process. *Rapid Commun. Mass Spectrom.,* **2002**, *16*(12), 1225-31.

[151] Ruan, Q.; Peterman S.; Szewc M.A.; Ma L.; Cui D.; Humphreys W.G.; Zhu M. An integrated method for metabolite detection and identification using a linear ion trap/Orbitrap mass spectrometer and multiple data processing techniques: application to indinavir metabolite detection. *J. Mass Spectrom.,* **2008**, *43*(2), 251-61.

[152] Zhang, H.; Zhu M.; Ray K.L.; Ma L.; Zhang D. Mass defect profiles of biological matrices and the general applicability of mass defect filtering for metabolite detection. *Rapid Commun. Mass Spectrom.,* **2008**, *22*(13), 2082-8.

[153] Jemal, M.; Ouyang Z.; Zhao W.; Zhu M.; Wu W.W. A strategy for metabolite identification using triple-quadrupole mass spectrometry with enhanced resolution and accurate mass capability. *Rapid Commun. Mass Spectrom.,* **2003**, *17*(24), 2732-40.

[154] Nassar, A.E.; Adams P.E. Metabolite characterization in drug discovery utilizing robotic liquid-handling, quadrupole time-of-flight mass spectrometry and in-silico prediction. *Curr. Drug Metab.,* **2003**, *4*(4), 259-71.

[155] Nassar, A.E.; Lee D.Y. Novel approach to performing metabolite identification in drug metabolism. *J. Chromatogr. Sci.,* **2007**, *45*(3), 113-9.

[156] Nassar, A.E.; Parmentier Y.; Martinet M.; Lee D.Y. Liquid chromatography-accurate radioisotope counting and microplate scintillation counter technologies in drug metabolism studies. *J. Chromatogr. Sci.,* **2004**, *42*(7), 348-53.

[157] Nassar, A.E.; Talaat R. Strategies for dealing with metabolite elucidation in drug discovery and development. *Drug Discov. Today,* **2004**, *9*(7), 317-327.

[158] Castro-Perez, J.M. Current and future trends in the application of HPLC-MS to metabolite-identification studies. *Drug Discov. Today,* **2007**, *12*(5-6), 249-56.

[159] Hsieh, Y. HPLC-MS/MS in drug metabolism and pharmacokinetic screening. *Expert Opin. Drug Metab. Toxicol.,* **2008**, *4*(1), 93-101.
[160] Staack, R.F.; Hopfgartner G. New analytical strategies in studying drug metabolism. *Anal. Bioanal. Chem.,* **2007**, *388*(7), 1365-80.
[161] Bateman, K.P.; Castro-Perez J.; Wrona M.; Shockcor J.P.; Yu K.; Oballa R.; Nicoll-Griffith D.A. MSE with mass defect filtering for *in vitro* and *in vivo* metabolite identification. *Rapid Commun. Mass Spectrom.,* **2007**, *21*(9), 1485-96.
[162] Chen, Y.; Monshouwer M.; Fitch W.L. Analytical tools and approaches for metabolite identification in early drug discovery. *Pharm. Res.,* **2007**, *24*(2), 248-57.
[163] Hopfgartner, G.; Zell M., Q Trap MS: A New Tool for Metabolite Identification. In: *Using Mass Spectrometry for Drug Metabolism Studies.* Korfmacher, W. Ed. CRC Press, Boca Raton, FL, **2005**, pp. 277-304.
[164] Li, A.C.; Gohdes M.A.; Shou W.Z. 'N-in-one' strategy for metabolite identification using a liquid chromatography/hybrid triple quadrupole linear ion trap instrument using multiple dependent product ion scans triggered with full mass scan. *Rapid Commun. Mass Spectrom.,* **2007**, *21*(8), 1421-30.
[165] Ma, S.; Chowdhury S.K.; Alton K.B. Application of mass spectrometry for metabolite identification. *Curr. Drug Metab.,* **2006**, *7*(5), 503-23.
[166] Xia, Y.Q.; Miller J.D.; Bakhtiar R.; Franklin R.B.; Liu D.Q. Use of a quadrupole linear ion trap mass spectrometer in metabolite identification and bioanalysis. *Rapid Commun. Mass Spectrom.,* **2003**, *17*(11), 1137-45.
[167] Cox, K., Special Requirements for Metabolite Characterization. In: *Using Mass Spectrometry for Drug Metabolism Studies.* Korfmacher, W. Ed. CRC Press, Boca Raton, FL, **2005**, pp. 229-252.
[168] Yao, M.; Ma L.; Humphreys W.G.; Zhu M. Rapid screening and characterization of drug metabolites using a multiple ion monitoring-dependent MS/MS acquisition method on a hybrid triple quadrupole-linear ion trap mass spectrometer. *J. Mass Spectrom.,* **2008**, *43*(10), 1364-75.
[169] Lim, H.K.; Chen J.; Sensenhauser C.; Cook K.; Subrahmanyam V. Metabolite identification by data-dependent accurate mass spectrometric analysis at resolving power of 60,000 in external calibration mode using an LTQ/Orbitrap. *Rapid Commun. Mass Spectrom.,* **2007**, *21*(12), 1821-32.
[170] Peterman, S.M.; Duczak N., Jr.; Kalgutkar A.S.; Lame M.E.; Soglia J.R. Application of a linear ion trap/orbitrap mass spectrometer in metabolite characterization studies: examination of the human liver microsomal metabolism of the non-tricyclic antidepressant nefazodone using data-dependent accurate mass measurements. *J. Am. Soc. Mass Spectrom.,* **2006**, *17*(3), 363-75.
[171] Gao, H.; Materne O.L.; Howe D.L.; Brummel C.L. Method for rapid metabolite profiling of drug candidates in fresh hepatocytes using liquid chromatography coupled with a hybrid quadrupole linear ion trap. *Rapid Commun. Mass Spectrom.,* **2007**, *21*(22), 3683-93.
[172] Zhu, M.; Ma L.; Zhang D.; Ray K.; Zhao W.; Humphreys W.G.; Skiles G.; Sanders M.; Zhang H. Detection and characterization of metabolites in biological matrices using mass defect filtering of liquid chromatography/high resolution mass spectrometry data. *Drug Metab. Dispos.,* **2006**, *34*(10), 1722-33.
[173] Kalgutkar, A.S.; Soglia J.R. Minimising the potential for metabolic activation in drug discovery. *Expert Opin. Drug Metab. Toxicol.,* **2005**, *1*(1), 91-142.
[174] Kumar, S.; Kassahun K.; Tschirret-Guth R.A.; Mitra K.; Baillie T.A. Minimizing metabolic activation during pharmaceutical lead optimization: progress, knowledge gaps and future directions. *Curr. Opin. Drug Discov. Dev.,* **2008**, *11*(1), 43-52.
[175] Evans, D.C.; Baillie T.A. Minimizing the potential for metabolic activation as an integral part of drug design. *Curr. Opin. Drug Discov. Dev.,* **2005**, *8*(1), 44-50.
[176] Doss, G.A.; Baillie T.A. Addressing metabolic activation as an integral component of drug design. *Drug Metab. Rev.,* **2006**, *38*(4), 641-9.
[177] Baillie, T.A. Future of toxicology-metabolic activation and drug design: challenges and opportunities in chemical toxicology. *Chem. Res. Toxicol.,* **2006**, *19*(7), 889-93.
[178] Takakusa, H.; Masumoto H.; Yukinaga H.; Makino C.; Nakayama S.; Okazaki O.; Sudo K. Covalent binding and tissue distribution/retention assessment of drugs associated with idiosyncratic drug toxicity. *Drug Metab. Dispos.,* **2008**, *36*(9), 1770-9.
[179] Masubuchi, N.; Makino C.; Murayama N. Prediction of *in vivo* potential for metabolic activation of drugs into chemically reactive intermediate: correlation of *in vitro* and *in vivo* generation of reactive intermediates and *in vitro* glutathione conjugate formation in rats and humans. *Chem Res. Toxicol.,* **2007**, *20*(3), 455-64.
[180] Samuel, K.; Yin W.; Stearns R.A.; Tang Y.S.; Chaudhary A.G.; Jewell J.P.; Lanza T., Jr.; Lin L.S.; Hagmann W.K.; Evans D.C.; Kumar S. Addressing the metabolic activation potential of new leads in drug discovery: a case study using ion trap mass spectrometry and tritium labeling techniques. *J. Mass Spectrom.,* **2003**, *38*(2), 211-21.
[181] Obach, R.S.; Kalgutkar A.S.; Soglia J.R.; Zhao S.X. Can *in vitro* metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. *Chem. Res. Toxicol.,* **2008**, *21*(9), 1814-22.
[182] Kalgutkar, A.S.; Driscoll J.; Zhao S.X.; Walker G.S.; Shepard R.M.; Soglia J.R.; Atherton J.; Yu L.; Mutlib A.E.; Munchhof M.J.; Reiter L.A.; Jones C.S.; Doty J.L.; Trevena K.A.; Shaffer C.L.; Ripp S.L. A rational chemical intervention strategy to circumvent bioactivation liabilities associated with a nonpeptidyl thrombopoietin receptor agonist containing a 2-amino-4-arylthiazole motif. *Chem. Res. Toxicol.,* **2007**, *20*(12), 1954-65.
[183] Baillie, T.A.; Kassahun K. Biological reactive intermediates in drug discovery and development: a perspective from the pharmaceutical industry. *Adv. Exp. Med. Biol.,* **2001**, *500*, 45-51.
[184] Dieckhaus, C.M.; Fernandez-Metzler C.L.; King R.; Krolikowski P.H.; Baillie T.A. Negative ion tandem mass spectrometry for the detection of glutathione conjugates. *Chem. Res. Toxicol.,* **2005**, *18*(4), 630-8.
[185] Ma, L.; Wen B.; Ruan Q.; Zhu M. Rapid screening of glutathione-trapped reactive metabolites by linear ion trap mass spectrometry with isotope pattern-dependent scanning and postacquisition data mining. *Chem. Res. Toxicol.,* **2008**, *21*(7), 1477-83.
[186] Zheng, J.; Ma L.; Xin B.; Olah T.; Humphreys W.G.; Zhu M. Screening and identification of GSH-trapped reactive metabolites using hybrid triple quadruple linear ion trap mass spectrometry. *Chem. Res. Toxicol.,* **2007**, *20*(5), 757-66.
[187] Mutlib, A.; Lam W.; Atherton J.; Chen H.; Galatsis P.; Stolle W. Application of stable isotope labeled glutathione and rapid scanning mass spectrometers in detecting and characterizing reactive metabolites. *Rapid Commun. Mass Spectrom.,* **2005**, *19*(23), 3482-92.
[188] Mahajan, M.K.; Evans C.A. Dual negative precursor ion scan approach for rapid detection of glutathione conjugates using liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.,* **2008**, *22*(7), 1032-40.
[189] Wen, B.; Ma L.; Nelson S.D.; Zhu M. High-throughput screening and characterization of reactive metabolites using polarity switching of hybrid triple quadrupole linear ion trap mass spectrometry. *Anal. Chem.,* **2008**, *80*(5), 1788-99.
[190] Yan, Z.; Caldwell G.W. Stable-isotope trapping and high-throughput screenings of reactive metabolites using the isotope MS signature. *Anal. Chem.,* **2004**, *76*(23), 6835-47.
[191] Castro-Perez, J.; Plumb R.; Liang L.; Yang E. A high-throughput liquid chromatography/tandem mass spectrometry method for screening glutathione conjugates using exact mass neutral loss acquisition. *Rapid Commun. Mass Spectrom.,* **2005**, *19*(6), 798-804.
[192] Zhu, M.; Ma L.; Zhang H.; Humphreys W.G. Detection and structural characterization of glutathione-trapped reactive metabolites using liquid chromatography-high-resolution mass spectrometry and mass defect filtering. *Anal. Chem.,* **2007**, *79*(21), 8333-41.
[193] Yan, Z.; Caldwell G.W.; Maher N. Unbiased high-throughput screening of reactive metabolites on the linear ion trap mass spectrometer using polarity switch and mass tag triggered data-dependent acquisition. *Anal. Chem.,* **2008**, *80*(16), 6410-22.
[194] Plumb, R.S.; Rainville P.D.; Potts W.B., 3rd; Castro-Perez J.M.; Johnson K.A.; Wilson I.D. High-temperature ultra-performance liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry applied to ibuprofen metabolites in human urine. *Rapid Commun. Mass Spectrom.,* **2007**, *21*(24), 4079-85.

[195] Wainhaus, S.B.; White R.E.; Dunn-Meynell K.; Grotz D.E.; Weston D.J.; Veals J.; Korfmacher W.A. Semi-quantitation of acyl glucuronides in early drug discovery by LC-MS/MS. *Am. Pharm. Rev.,* **2002**, *5*(2), 86-93.

[196] Wainhaus, S.B., Acyl Glucuronides: Assays and Issues. In: *Using Mass Spectrometry for Drug Metabolism Studies.* Korfmacher, W. Ed. CRC Press, Boca Raton, FL, **2005**, pp. 175-202.

[197] Rindgen, D.; Grotz D.; Clarke N.J.; Cox K.A. The application of HPLC/tandem mass spectrometry for the assessment of acyl glucuronide formation in *in vitro* and *in vivo* systems in a drug discovery environment. *Am. Pharm. Rev.,* **2001**, *4*(4), 52-58.

[198] Wang, J.; Davis M.; Li F.; Azam F.; Scatina J.; Talaat R. A novel approach for predicting acyl glucuronide reactivity *via* Schiff base formation: development of rapidly formed peptide adducts for LC/MS/MS measurements. *Chem. Res. Toxicol.,* **2004**, *17*(9), 1206-16.

[199] Bailey, M.J.; Dickinson R.G. Acyl glucuronide reactivity in perspective: biological consequences. *Chem. Biol. Interact.,* **2003**, *145*(2), 117-37.

[200] Shipkova, M.; Armstrong V.W.; Oellerich M.; Wieland E. Acyl glucuronide drug metabolites: toxicological and analytical implications. *Ther. Drug Monit.,* **2003**, *25*(1), 1-16.

[201] Williams, D.P.; Park B.K. Idiosyncratic toxicity: the role of toxicophores and bioactivation. *Drug Discov. Today,* **2003**, *8*(22), 1044-50.

[202] Shu, Y.Z.; Johnson B.M.; Yang T.J. Role of biotransformation studies in minimizing metabolism-related liabilities in drug discovery. *AAPS J.,* **2008**, *10*(1), 178-92.

[203] Walgren, J.L.; Mitchell M.D.; Thompson D.C. Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Crit. Rev. Toxicol.,* **2005**, *35*(4), 325-61.

[204] Roffey, S.J.; Obach R.S.; Gedge J.I.; Smith D.A. What is the objective of the mass balance study? A retrospective analysis of data in animal and human excretion studies employing radiolabeled drugs. *Drug Metab. Rev.,* **2007**, *39*(1), 17-43.

[205] Evans, D.C.; Watt A.P.; Nicoll-Griffith D.A.; Baillie T.A. Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chem. Res. Toxicol.,* **2004**, *17*(1), 3-16.

Received: 30 January, 2009 Revised: 15 March, 2009 Accepted: 09 Apirl, 2009

[206] Kramer, J.A.; Sagartz J.E.; Morris D.L. The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates. *Nat. Rev. Drug Discov.,* **2007**, *6*(8), 636-49.

[207] Li, A.P.; Segall M. Early ADME/Tox studies and in silico screening. *Drug Discov. Today,* **2002**, *7*(1), 25-7.

[208] Netzer, R.; Ebneth A.; Bischoff U.; Pongs O. Screening lead compounds for QT interval prolongation. *Drug Discov. Today,* **2001**, *6*(2), 78-84.

[209] Netzer, R.; Bischoff U.; Ebneth A. HTS techniques to investigate the potential effects of compounds on cardiac ion channels at early-stages of drug discovery. *Curr. Opin. Drug Discov. Dev.,* **2003**, *6*(4), 462-9.

[210] Shamovsky, I.; Connolly S.; David L.; Ivanova S.; Norden B.; Springthorpe B.; Urbahns K. Overcoming undesirable HERG potency of chemokine receptor antagonists using baseline lipophilicity relationships. *J. Med. Chem.,* **2008**, *51*(5), 1162-78.

[211] Kettenhofen, R.; Bohlen H. Preclinical assessment of cardiac toxicity. *Drug Discov. Today,* **2008**, *13*(15-16), 702-7.

[212] Milan, D.J.; Jones I.L.; Ellinor P.T.; MacRae C.A. *In vivo* recording of adult zebrafish electrocardiogram and assessment of drug-induced QT prolongation. *Am. J. Physiol. Heart Circ. Physiol.,* **2006**, *291*(1), H269-73.

[213] Rubinstein, A.L. Zebrafish assays for drug toxicity screening. *Expert Opin. Drug Metab. Toxicol.,* **2006**, *2*(2), 231-40.

[214] Barros, T.P.; Alderton W.K.; Reynolds H.M.; Roach A.G.; Berghmans S. Zebrafish: an emerging technology for *in vivo* pharmacological assessment to identify potential safety liabilities in early drug discovery. *Br. J. Pharmacol.,* **2008**, *154*(7), 1400-13.

[215] McGrath, P.; Li C.Q. Zebrafish: a predictive model for assessing drug-induced toxicity. *Drug Discov. Today,* **2008**, *13*(9-10), 394-401.

[216] Zon, L.I.; Peterson R.T. *In vivo* drug discovery in the zebrafish. *Nat. Rev. Drug Discov.,* **2005**, *4*(1), 35-44.

[217] Parng, C.; Seng W.L.; Semino C.; McGrath P. Zebrafish: a preclinical model for drug screening. *Assay Drug Dev. Technol.,* **2002**, *1*(1 Pt 1), 41-8.