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The Conduct of Drug Metabolism Studies Considered Good Practice (II): *In Vitro* Experiments

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Abstract

In vitro drug metabolism studies, which are inexpensive and readily carried out, serve as an adequate screening mechanism to characterize drug metabolites, elucidate their pathways, and make suggestions for further *in vivo* testing. This publication is a sequel to part I in a series and aims at providing a general framework to guide designs and protocols of the *in vitro* drug metabolism studies considered good practice in an efficient manner such that it would help researchers avoid common pitfalls and misleading results. The *in vitro* models include hepatic and non-hepatic microsomes, cDNA-expressed recombinant human CYPs expressed in insect cells or human B lymphoblastoid, chemical P450 inhibitors, S9 fraction, hepatocytes and liver slices. Important conditions for conducting the *in vitro* drug metabolism studies using these models are stated, including relevant concentrations of enzymes, co-factors, inhibitors and test drugs; time of incubation and sampling in order to establish kinetics of reactions; appropriate control settings, buffer selection and method validation. Separate *in vitro* data should be logically integrated to explain results from animal and human studies and to provide insights into the nature and consequences of *in vivo* drug metabolism. This article offers technical information and data and addresses scientific rationales and practical skills related to *in vitro* evaluation of drug metabolism to meet regulatory requirements for drug development.

Keywords

Cytochrome P450 enzymes; drug regulations; hepatocytes; *in vitro* drug metabolism; microsomes

INTRODUCTION

The completion of the human genome sequence and discovery of about 30,000 protein-encoding genes in 2001 raised wide-spread hope for a new era in the prevention and treatment of diseases. The genomic and proteomic gold rush has led to the exploitation of potential disease targets by the new technology for drug intervention [1,2]. This revolution has improved our understanding of the function and molecular architecture of the cytochrome P450 enzyme (CYP) family, and has enriched our knowledge of drug metabolism in this regard [3]. Advances in automated gene and protein sequence analyses as well as the introduction of high performance liquid chromatography coupled with tandem mass spectrometry for separating and identifying new molecules have emerged as new tools in the new century. With these technological advances, publications on *in vitro* drug metabolism have been on the rise in the

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past ten years. According to the data collected by the PubMed, a service of National Institutes of Health which includes citations from MEDLINE and other life science journals for biomedical articles, the total number of publications categorized into the *in vitro* drug metabolism was 8709 in 1996, and increased to 11,729 in 2006— an increase by 35% over the past 10 years (Fig. 1). Amidst the wealth of sequence information, emerging new techniques for monitoring differential gene expression offer valuable assistance exposing regulatory mechanisms of drug metabolic pathways; thus, pinpointing new drug targets and proteins previously not exploited.

These advances not only allow for the exploration of new drug targets but also the re-evaluation of the mechanisms of action of well-established drugs, such as antitubercular drugs isoniazid and ethambutol [4] as an aid to developing new therapeutics, and to better defining the functional genes likely to be critical in drug metabolism and are thus amenable as new drug targets. Nowadays, obtaining pharmacokinetic data for investigational drugs is no longer difficult; discovering drug metabolic pathways and integrating *in vitro* and *in vivo* data into a correct metabolic scheme pose a greater challenge.

The current scientific understanding of the drug development process does not constitute knowledge at the cellular, organ, or whole organism level, and it certainly does not reach a systems understanding of disease treatment. Therefore, additional scientific communications for exchanging ideas and implementing good practice are required to enable us to design study protocols correctly and avoid potential pitfalls early. While there remains a lack of consensus within the pharmaceutical industry on how best to approach *in vitro* drug metabolism studies, this article, a sequel to part I in a series, will address practical and scientifically based approaches to *in vitro* drug metabolism studies and provide an up-to-date, easy-to-use template of protocols to assist researchers with their study designs.

CONDUCTING *IN VITRO* DRUG METABOLISM STUDIES

Assessment of metabolite exposure in regulatory toxicology and pharmacology studies is not routinely required, and it is generally impractical since only preliminary metabolism information is available prior to the first introduction to man [5,6,7]. Noncirculating metabolites (e.g., those excreted in urine or bile, but not found in plasma) are sometimes identified prior to human trials, but they are usually not considered for monitoring. Nevertheless, attempts are generally made to predict metabolites that would be expected to appear in the circulation in humans before the start of clinical trials based on the findings from *in vitro* experiments with human liver microsomes, slices, or hepatocytes. Moreover, researchers at academic centers who are highly motivated to perform hypothesis-driven experiments, often explore the metabolic pathways in order to explain their study findings logically and maximize the benefits of their discovery.

In vitro interspecies metabolism studies are performed to screen for qualitative similarities and differences in metabolism between humans and animal species. This initial screen allows the selection of the most appropriate animal species with a metabolic profile closest to that of humans for use in future toxicity studies. In spite of a lack of correlation observed in some instances between the *in vitro* profile and the *in vivo* metabolism, it is prudent to conduct *in vitro* experiments prior to *in vivo* metabolism studies. With the advent of more sophisticated analytical technologies covered in part I of this sequel, arrangements for early *in vitro* metabolism studies are desirable and commonly accepted, showing important advantages.

In many cases, the *in vitro* studies serve as an adequate initial screening mechanism that can rule out any inconsequential metabolic pathways and make *in vivo* testing unnecessary [6,7]. The results obtained from those studies also help with the planning or interpretation of toxicological and clinical studies. If *in vitro* CYP reaction phenotype data are obtained early,

the information may indicate whether or not *in vivo* drug interaction studies are necessary, thus guiding toxicologists and clinicians to design their study protocols in a more logical manner. For example, enzymes involved in the metabolism of a drug should be studied for potential induction by that drug in clinical trials. In contrast, enzymes not involved in the metabolism of the drug do not need to be studied in clinical studies [8].

The models most often used *in vitro* include: 1) metabolizing recombinant enzymes (e.g., CYP and UDP-glucuronosyltransferases); 2) subcellular fractions (e.g., microsomes, cytosols or S-9 fractions); and 3) cellular organelles (e.g., hepatocytes and liver slices).

1. METABOLISM BY MICROSOMES

Hepatic elimination occurs in the endoplasmic reticulum, where CYPs play an important role. Omura and Sato were pioneers in characterizing the liver microsomal P450 preparation [9, 10]. Although the CYP superfamily is a dominant group of metabolizing enzymes in humans, other classes play an important role as well, including enzymes responsible for acetylation, methylation, glucuronidation, sulfation, and de-esterification (esterases). N-acetyl and glucuronosyl transferases are other non-CYP enzymes involved in drug metabolism. Lung microsomes that are now commercially available can be used to predict the metabolic possibility of drugs formulated as a nasal spray (personal communications). CYPs found in intestinal epithelial cells have been characterized [11] and shown to significantly affect the amount of drug absorbed into the systemic circulation. For example, drugs susceptible to metabolism via CYP3A4 may exhibit low and/or variable bioavailability.

The recently established CYP phenotyping reaction focuses on determining the CYP isoform involved in the metabolism of a given drug [12]; The list of phenotyped compounds continues to grow [13]. Before conducting CYP phenotyping, however, it is important first to define the predominant clearance mechanisms for the investigational drug in question. This is because if the CYP-mediated reactions play a minor role in the overall clearance (appropriately <30%) of the drug, the CYP phenotyping is unnecessary [8]. CYP3A4 is involved in the biotransformation of the majority of all drugs (>50%) and is expressed at significant levels extrahepatically. Poor oral bioavailability of many drugs can be related to the extensive metabolism by CYP3A4 in the gastrointestinal tract. CYP2C and CYP2D6 are involved in the biotransformation of about 15% and 30% of all drugs, respectively. Table 1 summarizes the relative content of CYP isoforms in human liver and the contribution of each CYP to drug metabolism.

Much emphasis has been put on the liver because this organ has always been regarded as the principal site of drug metabolism. The ratio of milligram of protein per gram of liver appears to be similar among different species (Table 2). Human liver microsomes provide the most convenient way to study CYP-mediated metabolism. Microsomes are a subcellular fraction of tissue obtained by differential high-speed centrifugation [15]. All CYP enzymes can be found in the microsomal fraction. Co-factor requirements for CYP-mediated reactions are well characterized, consisting primarily of a redox-sustaining system, such as NADPH. Additionally, microsomes and recombinant CYPs are the preferred testing systems, as they are more readily available than hepatocytes, and CYP kinetic measurements are not confused with other metabolic processes or cellular uptakes. Nevertheless, microsomes and recombinant CYPs do not encompass all of the Phase II enzymes [6,8], and thus they do not fully represent an endogenous physiological environment. *In vivo* results should always take precedence over *in vitro* results, if a difference arises between the two [6,7].

CYP metabolism can vary widely from one species to another. Interspecies differences in drug metabolism can be predicted by studying the *in vitro* metabolism of a drug via liver microsomes from different species. Comparison of CYP metabolic profiles of different species with those

of human liver microsomes helps to identify the most relevant animal model for pharmacokinetic and toxicological studies. Commercial microsomes can be purchased from the following companies: XenoTech LLC (www.xenotechllc.com), Human Biologics (www.humanbiologics.com), Cedra, Co. (www.cedracorp.com), BD Gentest (www.bdbiosciences.com) and Celsis International (www.celsis.com). However, it has been shown that the activity of the commercial microsomes varied significantly from batch to batch, and from vendor to vendor. For instance, the rat liver microsomes from two vendors showed great activity in metabolizing drugs buspirone and loperamide, while those from the third vendor exhibited no activity at all; and three different batches of rat liver microsomes from the same vendor resulted in different activity in metabolizing buspirone and loperamide [18]. The differences in the microsomal activity may be due to inherent animal-to-animal variation and differences in preparation processes among the vendors. For example, some vendor used phenylmethylsulfonylfluoride to prepare liver microsomes because phenylmethylsulfonylfluoride can inhibit trypsin-like proteases that might otherwise cause proteolytic degradation of microsomes. Whereas, others applied ethylene diamine tetraacetic acid (EDTA) for the same purpose. Phenylmethylsulfonylfluoride is also an inhibitor of certain carboxylesterases, and EDTA chelates calcium and iron, resulting in inhibition of calcium-dependent phospholipases and lipid peroxidation, respectively. It is a good practice to check microsomal characterization data of each batch provided by the vendor with respect to cytochrome b5 and cytochrome P450 content, and NADPH-cytochrome c reductase activity, so that comparisons and justification of different study results can be made among batches. A quick and simple method to check the quality of different batches of either new or re-used microsomes is to run a UV spectrum for carbon monoxide binding difference [19,20], and if a peak at 420 nm appears, instead at 450 nm, it is likely that the CYP has started to degrade to P420, or the microsomes have been contaminated with hemoglobin that is sometimes found in the microsomes and produces a peak at 420 nm [21].

The concentrated microsome solution needs to be diluted with either 250 mM sucrose [15] or potassium phosphate buffer (PPB, 50–100 mM, pH 7.4) to a protein concentration of 0.5 mg/mL [12,22]. It is desirable to incubate the test drugs with low amounts of microsomal protein to readily quantify the necessary metabolites. A concentration of microsomal protein <0.5 mg/mL is recommended [8,23], and a microsomal protein concentration of 0.2 mg/mL has even been used [24]. The low protein concentration helps to minimize the proportion of protein bound to drugs. The final protein concentration should be validated by a standard protein assay using bovine serum albumin as standard.

The CYP enzymes retain their activity for many years in microsomes or whole liver stored at low temperature (e.g., -80°C) [8]. Microsomes that are thawed and maintained on ice for less than 2 h can be re-frozen at -80°C and re-used without significant loss of enzyme activity [20]. This thawing-restoring flexibility allows us to use microsomal aliquots from a single batch for different experiments to facilitate normalization or comparison of different experiment data, and thus avoid confusing results introduced from different batches and vendors. Di *et al.* [18] examined activity of different microsomal aliquots from the same commercial batch every week for 50 days for their activity inhibited by midazolam, buspirone and loperamide, and found good reproducibility of the microsomal activity inhibited by the drugs across all aliquots. However, microsomes that have been incubated at 37°C should not be re-used or refrozen.

The *in vitro* experimental concentration of a drug should be a little higher than the maximal blood concentration achieved in the animal study. If the concentrations have not been determined, a range of 1–10 μM of the final drug concentration can generally be used to closely mimic *in vivo* levels of the drug. However, literature reported that the incubating concentrations of the test drugs (0.5–15 μM) affected the stability results of these drugs in the same microsomal

preparation [18]. In general, a test drug at its higher concentration (e.g., 10 μM) shows more stable in the microsomes than at its lower concentration. For example, the percent remaining of propranolol at 10 μM was 76-fold higher than that at 1 μM . Some drugs considered CYP substrates did not show the concentration-related stability under the same condition [18]. Drugs that are not the substrates of CYPs are usually stable in microsomal incubation and their stability will not be affected by their incubating concentrations [25]. It is prudent to set up at least two concentrations of a test drug (e.g., 1 and 10 μM) to determine if the drug's concentration has any effect on its own stability in microsomes and other biomatrices. Reasons for recommending the use of low concentration ($\sim 1 \mu\text{M}$) of a test drug in the *in vitro* study include 1) at the low concentration, the drug-enzyme reaction presumably follows a first-order process, i.e., $[S] < K_m$, and hence, the reaction rate is proportional to the drug concentration; and 2) drugs with poor aqueous solubility tend to precipitate out when their concentrations are relatively high (e.g., 10 μM) in microsomal preparation where concentrations of organic solvents must be kept low ($< 1\%$) to avoid any effects of the organic solvents on the microsomal activity. As a result, an artificial good stability represented by high % remaining of the drug may be seen after adding an organic solvent (e.g., acetonitrile) to the microsomal preparation (a process that may completely solubilize the drug) to dissolve and extract the drug from the preparation for quantitative analysis.

Control incubations should be carried out by excluding either the substrate, NADPH (or an NADPH generating system consisting of 1 mM NADP, 5 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase), or microsomes from the incubation mixture. Table 3 exemplifies an easy-to-use template that sets proper control groups for different purposes to exclude potential interfering factors from the microsomal study and helps us reach a correct conclusion.

Active and control groups should be prepared and incubated under the same conditions. Samples need to be aliquoted at various time points (e.g., 0, 10, 20, 40 and 80 min) and added to an equal volume of ice-cold extraction solvent (e.g., acetonitrile or dichloromethane) to end the reaction. A longer incubation time is not necessary [8,24]. Some vendors do not recommend for an incubation time longer than 2 h when conducting drug stability study in microsomes at 37°C [20]. If extended incubations have to be carried out for reasons, additional controls should be performed in parallel to verify that the enzyme activity is kept and no thermal degradation occurs to the test drug during the incubation period. The mixtures should be centrifuged at 14,000 g for 20 min at 4°C. The supernatants should either be analyzed immediately or frozen at -80°C until analysis. Triplication of the sample number for the additional data set is desirable at this time to calculate the mean. If the standard deviation is required, the number of samples should be increased to > 3 to justify its statistical significance. If metabolite(s) can not be easily identified, the remaining percentage of the parent drug versus time should be noted.

Organic solvents used for dissolving lipophilic drugs usually have inhibitory effects on the CYP activity. DMSO, even at concentrations as low as 0.2, 0.5, or 1%, was found to inhibit *in vitro* microsomal activity and result in fault stabilization of the drugs incubated in microsomal preparation [18]. Other organic solvents such as methanol, ethanol, acetonitrile, and PEG 400 were also found to inhibit the activity by 15–25% of CYP2E1, CYP3A4, CYP2D6, CYP2C9, and CYP2C19 [26,27]. It needs to mention that DMSO potently inhibits CYP2E1 activity. Therefore, DMSO is not recommended for the CYP2E1-related studies (www.xenotechllc.com). The following final concentrations of organic solvents are recommended for water insoluble drugs while sustaining the CYP activity: methanol, $< 0.2\%$; acetonitrile, $< 0.2\%$; and PEG 400 $< 1.0\%$.

2. REACTION PHENOTYPING STUDY

Establishing whether a drug is a CYP substrate and, if so, which CYP enzymes are involved in its biotransformation can help predict and avoid pharmacokinetic variability and minimize adverse drug reactions. Reaction phenotyping is the process of determining which human CYP isoform participates in the metabolism of a drug, and whether the CYP isoform is polymorphically expressed (such as CYP2D6) or is highly inducible (such as CYP2B6). Reaction phenotyping can not proceed unless an analytical method for the drug and its metabolite(s) has been well-established for quantification, which includes available authentic standards and the internal standards. The latter are used for correcting sample-to-sample variations. Reaction phenotyping generally involves four types of analysis: correlation analysis, K_m and V_{max} value determination, recombinant human CYPs activity, and specific chemical and antibody inhibition.

2.1. Correlation Analysis

Correlation analysis involves determining the rate of drug metabolism catalyzed by several human liver microsomal samples, followed by correlating reaction rate with the sample-to-sample variation of CYP isoform activity in each individual sample of human liver microsomes. Metabolite formation velocity (nmol/min/mg protein) should be plotted on the Y-axis as a function of the activity (or concentrations) of a specific CYP isoform on the X-axis to assess the relationship between them. Correlation coefficients (r) and regression coefficients (r^2) are usually determined by Pearson's regression analysis after the relationship is established. Pearson's r and r^2 values must exceed the assigned levels and be close to 0.99 to demonstrate statistically significant activity of the CYP isoform on the drug's metabolism.

2.2. Michaelis-Menten Kinetic Parameters K_m and V_{max}

When a metabolite is identified, its metabolism kinetics in liver microsomes is expected to be illustrated by apparent first-order Michaelis-Menten kinetics utilizing apparent K_m (μM) and V_{max} (nmol/min/mg protein). To establish the Michaelis-Menten kinetics, varying concentrations (0, 1, 10, 50, 100, 200, 500, and 1000 μM) of the drug should be incubated with a given concentration of liver microsomes (e.g., 0.5 mg/mL) for 60 min. Formation of metabolite(s) by liver microsomes (or recombinant CYPs) through the 60-min incubation interval is determined by quantitatively monitoring chromatographic peak areas of metabolites. It is recommended that concentrations of CYP probe substrate (or test drug) be used at or below its Michaelis-Menten constant (K_m) [8,23] although many studies have used concentrations of test drugs far higher than those that can be reasonably achieved in humans and animals without compromising the guidelines for good practice.

When the drug concentration is much higher than the K_m (a plateau level), the rate of the reaction becomes constant for a given enzyme concentration. Under these conditions, the enzyme is saturated with the drug, and the reaction is proceeding at maximal velocity. In other words, a further increase in drug concentration will not alter the velocity of the reaction (zero-order reaction). When the drug concentration is smaller than the K_m , the rate is proportional to the substrate concentration and a first-order reaction is obtained [28]. Metabolite formation velocity (nmol/min/mg protein) should be plotted on Y-axis as a function of the drug concentration on the X-axis to estimate the K_m . In addition, the metabolite formation velocity (V , nmol/min/mg protein) can be plotted on the Y-axis against the ratio of metabolite formation velocity to drug concentrations on the X-axis [24]. The enzyme kinetic parameters (V_{max} , K_m) can be obtained by nonlinear least-squares analysis using the Win-Nonlin program. The most commonly used Michaelis-Menten equations include the following:

$$V = V_{\max} [S] / \{K_m + [S]\} \quad (1)$$

$$[S]/V = [S]/V_{\max} + K_m/V_{\max} \quad (2)$$

2.3. cDNA-Expressed Recombinant Human CYP Activity

The cDNA for the common CYPs have been cloned, and the recombinant human enzymatic proteins have been expressed in a variety of cells. The use of these recombinant enzymes provides an effective way to further confirm results obtained for the apparent metabolic pathways using microsomes [29]. However, the rationale for performing the CYP reaction phenotyping for an investigational drug is viable only if CYPs are a major contributor in the overall clearance (>30%) [8,30].

Supersomes provided by BD Gentest (www.bdbiosciences.com) contain various recombinant human CYPs expressed in insect cells. Human Biologics (www.humanbiologics.com) is another company that provides CYPs expressed in human B lymphoblastoid. Recombinant human CYPs should be incubated individually with a drug under the same conditions as the liver microsomal studies to assess the ability of a particular CYP (such as CYP3A, CYP2D6, or CYP2C) to metabolize the drug. All incubations containing Supersomes (100 pmol CYP/ml) [17,31], the test drug and Sorensen buffer (5 mM, pH 7.4) need to be conducted at least in duplicate at 37°C. Incubations should start by adding NADPH to a final concentration of 1–10 mM after 5 min of pre-incubation. Reactions stop 30 min later (or longer, depending on the metabolite formation rate) by adding an equal volume of ice-cold extraction solvent.

Metabolism should be evaluated in microsomes prepared from insect cells transfected with cDNAs encoding for various human CYP isoforms. The recombinant CYP isoforms and microsomes from untransfected insect cells should be used in parallel as controls. The mean metabolite formation in microsomes from untransfected cells is then subtracted from the mean metabolite formation in microsomes from cells transfected with corresponding CYP isoforms to validate the specific isoforms involved in metabolism. The metabolite formation rate (pmol/min/pmol P450) following incubation of the parent drug at a given concentration (e.g., 10 µM) with individual recombinant CYP isoforms should be assessed, and the major isoforms involved identified. The enzyme kinetic parameters (K_m , $V_{\max}$) should be calculated for the most active isoform. To accomplish this, a series of concentrations of the test drug should be incubated with the isoform in order to obtain the K_m and $V_{\max}$. If only negligible amount of the metabolite is found in the incubations with the isoform, it is unlikely that the enzyme is involved in the metabolite formation.

2.4. Chemical and Antibody Inhibition

Chemical and antibody inhibition experiments involve an evaluation of the effect of known CYP enzyme inhibitors or inhibitory antibodies on the metabolism of a drug by either pooled human liver microsomes or individual CYP isoforms. Chemical inhibition can be non-selective if the concentrations of the inhibitor are used inappropriately high. However, the concern arising with chemical inhibitors is not a factor with those antibodies that have been shown to be selective CYP inhibitors.

Using selective chemical inhibitors for each major pathway, the metabolic pathways for a drug can be readily demonstrated or ruled out. Careful consideration of the incubating concentrations for the inhibitor and the drug is essential to reach a selective approach whilst avoiding misleading results. For example, quinidine and ketoconazole are relatively selective inhibitors of CYP2D6 and CYP3A4, respectively, at a concentration below 1 µM; however,

at higher concentrations, both drugs inhibit other CYP isoforms, though the inhibition is not known to be clinically pertinent.

In theory, the chemical inhibition of CYPs can be reversible (competitive or non-competitive) or irreversible. Irreversible inhibition usually derives from activation of a drug by CYPs into a reactive metabolite, which tightly binds to the enzyme active site, leading to a long lasting inactivation. This process is called “mechanism based inhibition” or “suicide inhibition”. Fontana *et al.* presented a general and systematic review with this regard [13].

Chemical inhibition experiments can be performed before or after the above cDNA-expressed recombinant CYPs studies. These inhibition studies provide either additional evidence to support the results from the cDNA-expressed recombinant CYPs studies, or information on how to direct such studies to identify the active isoforms. The protocol requires the test drug to be incubated with liver microsomes (0.5 mg/mL) at 37°C for 30 min (or 60 min if necessary) in the presence and absence of selective inhibitors [8]. The following inhibitors against the isoforms and their concentrations are recommended [6,8,23,31]: Furafylline (CYP1A2; 0.1, 1, 10 µM), 8-Methoxypsoralen (CYP2A6; 0.1, 1, 10 µM), Glitazones or quercetin (CYP2C8; 0.5, 1, 10 µM), Sulphaphenazole (CYP2C19; 5, 20, 100 µM), Quinidine (CYP2D6; 0.5, 1, 10 µM), Clomethiazole (CYP2E1; 0.1, 1, 10 µM), Troleandomycin (TAO; CYP3A; 0.5, 1, 10 µM). Inhibitors are usually dissolved in methanol that is then added to the microsomal incubation mixture. The volume of methanol should be < 1% (v/v) of the mixture. All inhibitors should be pre-incubated in the presence of NADPH (final concentration of 10 mM) and microsomes at 37°C before adding the test drug. Appropriate controls for the inhibitors include an incubation mixture (1% methanol) in the presence (positive) and absence (blank) of the test drug. Therefore, the percent inhibition of the metabolite formation can be quantitatively expressed based on the control values.

3. HEPATOCYTES AND LIVER SLICES

Hepatocytes and liver slices are the most physiologically relevant samples used for qualitative and quantitative measurement of hepatic phase I and II metabolism of drugs, since these contain the full complement of enzymes and cofactors that a drug is likely to encounter during first pass metabolism. The cofactors are self-sufficient and the natural orientation for linked enzymes is preserved [6]. Additionally, interactions with transporter proteins present in hepatocyte membranes can be important determinants of hepatic clearance. Isolated hepatocytes and precision-cut liver slices can be used to predict *in vivo* metabolism more precisely, including potentials of hydrolysis, oxidation, reduction, and glucuronidation. Although microsomes continue to be the first-line screening model for high throughput assays, the number of publications in the last 5 years citing metabolism in hepatocytes has increased by approximately 30% [13], partially due to commercial availability of the cryopreserved cells. Preparation of hepatocytes has been reported in detail by Soars *et al.* [32], while Edwards *et al.* [30] and Lake *et al.* [33] have reported on the preparation of precision-cut human liver slices.

Hepatocytes (cryopreserved or plated) from different species are now commercially available from www.celsis.com, www.xenotechllc.com or www.bdbiosciences.com. One of the major problems with these preparations is that enzymatic activities are not stable [6], and cell viability ought to be checked by trypan blue exclusion or lactate dehydrogenase assay during the metabolism experiments. Also some transporters may be rapidly down-regulated after isolation of hepatocytes, and support matrices may introduce artifacts (additional collagen diffusion barrier or loss of enzyme activity) [23]. Prior to culturing, hepatocytes should be thawed and suspended in isotonic Percoll solution. After centrifugation, hepatocytes must be re-suspended in modified Krebs Henseleit Buffer (KHB), followed by viability testing. It is worth noting

that viability of hepatocytes drops substantially during the early incubation period (personal communications). Control incubations should include the test drug in the culture medium without hepatocytes and hepatocytes alone in the absence of the test drug to clarify the effects of hepatocytes on metabolism.

Factors having some influence on the morphology and functions of hepatocytes in culture include the following: 1) medium formulation; 2) extracellular matrix; 3) initial cell suspension and density; and 4) drug concentrations.

Medium formulation affects the preservation of hepatocyte-specific functions and morphology. Cells are generally seeded in a medium containing fetal calf serum to enhance surface attachment of the cells (including hepatocytes). However, formulation of the medium with serum is not recommended for culturing hepatocytes for the following reasons. Serum seems to be capable of inhibiting the formation of bile canaliculi, a special morphology of hepatocytes, and may lead to fibroblast overgrowth after several days of culturing, regardless of whether the cells are cultured in a conventional or sandwich configuration [34]. In serum-containing medium, hepatocytes tend to spread out and form fibroblast-like protrusions. As a result, the nuclear volume would increase, the cytoplasm appears granulated, and the bile canaliculi-like structure would disappear. Whereas, in the serum-free collagen sandwich cultures, primary hepatocytes stay morphologically unchanged for a few weeks, which allows us to investigate alterations in hepatocytes induced by the drugs [13]. Addition of the glucocorticoid hormone, dexamethasone, to the medium is recommended for the long-term preservation of hepatocyte-specific functions, polygonal hepatocyte morphology, structural integrity such as cytoplasmic membranes and bile canaliculi-like feature [35,36,37].

Hepatocytes cultured in a sandwich configuration between two layers of gelled extracellular matrix (collagen I and matrigel being the most commonly used) show their specific functions for a longer period [38]. The use of collagen I gel as a component of the extracellular matrix for the hepatocyte sandwich culture is appropriate for long-term culturing. The morphological distinction between hepatocytes seeded onto collagen-coated plates without a collagen gel overlay (conventional monolayer) and those with a collagen gel overlay (sandwich) is visible a few hours after seeding. Hepatocytes on the conventional monolayer appear more flattened than those on the sandwich culture because the monolayer lacks a three-dimensional extracellular matrix environment, and hepatocytes have to quickly adopt their polygonal shape to establish cell-cell contacts. Whereas, in the sandwich culture, the cell-cell contact process takes longer, and most cells still show the spherical and singular shape after hours of seeding. The morphological stability of hepatocytes on the conventional monolayer can be improved by using collagen gel.

Initial cell suspension seems to have an effect on the quality of human hepatocytes, but not on the rat hepatocytes. Appropriate cell density is also important. Hepatocytes seeded at 100% confluence can not spread out. Rather, they display hepatocyte-like morphology for 1–2 days and then start to detach from the surface. Cell density should be kept at confluence about 90%.

Concentrations of a drug added to hepatocytes for the metabolism study should be carefully calculated, especially if the drug is classified as a cytotoxic agent. At a high concentration, the drug may produce apoptosis of the cells and non-specific effects on the CYP activity of the hepatocytes. In contrast, at a low concentration, one can not distinguish inhibition from activation by the drug on the CYPs, nor can one identify the related metabolite.

4. S9 FRACTION

The S9 fraction (post-mitochondrial supernatant fraction) is a mixture of microsomes and cytosol. Accordingly, it contains a wide variety of phase I and phase II enzymes including

CYPs, flavin-monooxygenases, carboxylesterases, epoxide hydrolase, UDP-glucuronosyltransferases, sulfotransferases, methyltransferases, acetyltransferases, glutathione S-transferases and other drug metabolism enzymes. Microsomes are derived from S9 by an ultracentrifugation (100,000 g for 1 h; Table 4) process which separates the cytosolic fraction from the microsomal fraction. CYP activity in the S9 fraction is approximately 20–25% of those in the corresponding microsomal fraction.

Cytosolic (soluble) enzymes, therefore, are not contained in the microsomal fraction. Instead, they are present in the S9 fraction, which is the post-mitochondrial supernatant fraction prepared by subjecting tissue homogenate to centrifugation at 12,000 g for 10 min. This subcellular fraction contains both cytosol and microsomes, and is well suited for determining the profile of metabolism of drugs in humans or animals because it contains enzymes involved in both phase I and II biotransformations. Whereas, microsomes are vesicles derived from the endoplasmic reticulum, which contain CYP enzymes mainly responsible for phase I biotransformation reactions. *In vitro* metabolism studies are sometimes conducted in the presence and absence (control) of an induced liver S9 or intestinal S9 fraction to evaluate the effects of cytosolic enzymes on drug metabolism. This type of study would be carried out to amplify the metabolic reaction of the test drug and quickly confirm the existence of the reaction. If the metabolic reaction exists in the liver microsomes, it would be confirmed in the S9 preparation as well. However, if a reaction occurs in the S9 fraction, it does not necessarily mean that the reaction will also occur in the microsomes because the latter does not contain cytosolic enzymes. One may consider confirming the presence of this reaction in the S9 fraction before deciding whether any additional tests are needed [39]. It is worth noting that the use of the human liver S9 fraction for *in vitro* studies may be complicated by marked intersubject differences in drug-metabolizing enzyme activities [40].

5. CONCLUDING THOUGHTS

Although *in vitro* experimental models can never resemble the complexity of a whole organism, their simplicity provides the ability to specifically manipulate and analyze single parameters. Indeed, the *in vitro* and *in vivo* CYP induction studies are helpful in interpreting initial animal pharmacokinetic studies, but the complexity in extrapolation of metabolic *in vitro* data to animal pharmacokinetic studies is obvious. An induction study with the compound DPC 681 is a perfect example [41]: the Western blot analysis clearly showed that DPC 681 increased the level of CYP3A4 protein in primary culture of human hepatocytes. Oral administration of the compound to rats and human volunteers for consecutive 12 (rats) and 13 (humans) days, respectively, also reduced the plasma concentrations of the compound probably due to its induction of CYP3A4. However, the compound failed to demonstrate induction of microsomal CYP3A4 activity in primary culture of human hepatocytes in a dose-dependent manner. This story revealed the difficulty in obtaining *in vivo* results in consistent with *in vitro* results. When a difference arises between *in vitro* and *in vivo* findings, the *in vivo* results should always take precedence over *in vitro* studies [6]. It is worth mentioning that the animal induction studies should not be used to predict induction responses in humans because there are differences between humans and animal species in CYP isoforms, substrate specificity, and the optimal CYP inducers.

In general, non-clinical studies of the major human metabolites, which are defined as metabolites identified in human plasma accounting for greater than 10 percent of drug related material (administered dose or systemic exposure whichever is less) that are not present at sufficient levels to permit adequate evaluation during standard nonclinical animal studies [5], should precede large scale clinical trials. Studies may need to be conducted earlier, if the metabolite: 1) belongs to a chemical class with known toxicity; 2) has a positive structural alert for genotoxicity, carcinogenicity or reproductive toxicity; or 3) is potentially associated with

a novel toxicity not observed with the parent drug. Four kinds of safety studies can be performed to assess the safety of a unique or major metabolite. These studies include general toxicity studies, genotoxicity studies, embryo-fetal development studies, and carcinogenicity studies [5,43].

A metabolite may be more active (e.g., codeine to morphine), equal active (e.g., aspirin to salicylic acid), less active (e.g., glucuronide conjugates of the parent drug; [29]), or possess different activity (phenylbutazone to oxyphenbutazone) compared with that of the parent molecule. An active metabolite may not only interact with the therapeutic target receptors and disease pathways, but it may also cause undesirable effects. This is particularly important when such a metabolite is produced in humans and not in animals (defined as a unique metabolite [5]). It would be prudent to make a distinction between metabolites that are major versus minor, those that are pharmacologically/toxicologically active versus inactive, those that are oxidative or conjugated, or formed from reactive intermediates. Chemically reactive intermediates are rarely detectable because of their short half-lives [42]. But if stable intermediates are formed (e.g., glutathione conjugates), some indication of exposure to these stable intermediates may be required, which would eliminate the need for further qualification.

Metabolic pathways are divided into phase I and phase II reactions, and both phases often occur in parallel for a particular compound. In phase I reactions, enzymes modify the parent drugs via hydrolysis, oxidation and reduction, increasing the polarity and also the excretion of the drug. The resulting phase I metabolites are not necessarily inactive at this stage, as is the case with codeine, which is demethylated to the more potent morphine. Most often, phase I reactions are preparative stages for further reactions exposing reactive sites to the molecule structure for the subsequent phase II processes (i.e., conjugation reactions). Phase II reactions are often considered “true” detoxifying reactions, as the conjugation of bulky and polar groups most often terminates the activity of the substrate and enhances elimination. Conjugated metabolites (or phase II products) are generally excluded from the safety evaluation due to their inactivity, unless there is a reason to believe that they are chemically reactive (e.g., acyl glucuronides). Only in rare cases, such as in the case of morphine-6-O-glucuronide, is the conjugated metabolite more potent than the parent drug itself. Glucuronidation is the main phase II reaction in all mammals, with the other important pathways being sulfation, methylation, acetylation and conjugation with amino acids or glutathione.

In summary, this article addressed the complexity in extrapolation of *in vitro* metabolic data to *in vivo* pharmacokinetics and the techniques associated with *in vitro* evaluation of drug metabolism. It pointed out the advantages and disadvantages of these techniques and appealed for data-sharing between laboratories to reach consistent results and conclusions and avoid common pitfalls.

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ABBREVIATIONS

ADME	Absorption distribution metabolism elimination
AUC	Area under curve
CYP	Cytochrome P450 enzyme

DMSO

Dimethyl sulfoxide

EPA

Envirmental Protection Agency

FDA

Food and Drug Administration

LC/MS

Liquid chromatography/mass spectrometry

PPB

Potassium phosphate buffer

NADPH

Reduced nicotinamide-adenine dinucleotide phosphate

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Fig. (1). Increasing number of publications per year related to *in vitro* drug metabolism during the past 10 years. The data were obtained from PubMed, a service of the US National Institutes of Health.

Table 1

Relative Abundance of Human CYP Isoforms and their Contribution to Drug Metabolism [14]

CYP isoform	Content in liver (% total CYP)	% of drugs metabolized by the CYP
3A4/5	29–30	52
2D6	1.5–4	30
2C8	7	
2C9	12	10–11
2C19	0.2	4
1A2	12–13	4–6
2E1	7	2–5
2B6	0.2–5	25

Table 2Liver Characteristics of Different Species (mean $\pm$ SE, n=4) [16]

Measurements	Human	Rat (S-D)	Dog (beagle)	Monkey (macaca)
Body weight (kg)	70	0.29 $\pm$ 0.0	8.6 $\pm$ 0.4	2.8
Liver weight (g)	1700	10.6 $\pm$ 0.3	360 $\pm$ 20	62.5
mg protein/g liver	131 $\pm$ 7	164 $\pm$ 14	120 $\pm$ 5	126 $\pm$ 3
P450 content* (pmol/mg protein)	307 $\pm$ 160	673 $\pm$ 50	385 $\pm$ 36	1030 $\pm$ 106

* Data (mean $\pm$ SD, n=3–18) were obtained from Shimada *et al.* [17], and the monkey strain was cynomolgus, instead.

Table 3
Experimental Designs for Conducting Drug Metabolism Studies Using Liver Microsomes

Groups	Components added				Purposes
	Drug	PPB	Microsomes (5 mg/mL) in PPB	NADPH (100 mM) in PPB	
Test	100 μ L	700 μ L	100 μ L	100 μ L	determine full reaction
Control 1	100 μ L	800 μ L	100 μ L		determine if reaction is energy dependent
Control 2	100 μ L	800 μ L		100 μ L	determine if reaction is protein dependent
Control 3	100 μ L	900 μ L			determine stability of the test drug in PPB

Table 4
Subcellular Fractions Contributing to Drug Metabolism*

Centrifugation rate (gravity) and spin time	Sediment	Supernatant
12,000 g, 10 min	Unbroken cells, mitochondria, nuclei, lysosomes, peroxisomes, and others	S9
100,000 g, 60 min [15]	Plasma membrane, endoplasmic reticulum containing microsomes	Cytosol
200,000 g, 3 h	Ribosomes	—

* Liver homogenate is subjected to centrifugation at 800 g for 10 min to obtain suspension of broken cells. The suspension is then centrifuged to obtain the sediment and supernatant (S9), respectively. The supernatant obtained at different speeds is sequentially re-centrifuged at the next higher speed and time to obtain different subcellular fractions. Microsomes have a reddish brown color, due to the presence of the iron-containing co-factor, heme, in the P450s. P450s are highly abundant in livers of rats, mice and humans.