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Chapter

In Vitro Drug Metabolism Studies Using Human Liver Microsomes

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Abstract

Metabolism of most pharmaceutical drugs occurs in the liver. In drug metabolism, enzymes convert drugs to highly water-soluble metabolites to facilitate excretion from the body. Thus, *in vitro* models for studying drug metabolism usually target hepatocytes or subcellular liver fractions like microsomes, cytosols, or S9 fractions with high concentrations of specific enzymes. The most popular subcellular fraction used during drug discovery tends to be the microsomes, as these are easy to prepare and store, are amenable to high throughput screening, and are a relatively low-cost option. Understanding the metabolic stability and kinetics of glucuronidation of an investigational drug is crucial for predicting the pharmacokinetic parameters that support dosing and dose frequency. This chapter provides detailed information about metabolite profiling, metabolic stability, glucuronidation kinetics, reactive metabolites identification, CYP enzyme inhibition, and general protocols using human liver microsomes.

Keywords: metabolism, metabolite profiling, metabolic stability, glucuronidation, reactive metabolites, drug–drug interaction

1. Introduction

The evolution of a new drug entity proceeds through a preclinical screening stage, during which the pharmacological and toxicological properties are scrutinized [1]. After oral administration, the drug gets absorbed and reaches the liver through the portal circulation for its metabolism. Cytochrome P450 (CYP450) enzymes are responsible for metabolizing most of the drugs in Phase I metabolism. However, flavin-containing monooxygenase (FMO) and enzymatic or nonenzymatic hydrolysis are also involved in the drug's metabolism but to a lesser extent. Phase II metabolism results in the production of metabolites conjugated to different chemical moieties like glucuronide, sulfate, glutathione, glycine, and acetate [2].

In 2016, the U.S. Food and Drug Administration (FDA) issued regulations for determining the safety and evaluation of drug metabolites for their toxicity in nonclinical species. These guidelines also provide a recommendation for identifying and characterizing drug metabolites. *In vivo* clinical metabolism studies involve the screening of biological matrices such as serum, urine, feces, and hair for identification

of metabolites, whereas *in vitro* drug metabolism studies using human liver microsomes (HLM), human hepatocytes (either fresh or cryopreserved), and recombinant expression of cytochrome P450 enzymes (supersomes) in determining the human metabolic pathways [3, 4]. Comprehensive studies for Phase I and Phase II metabolism involve extensive HLM and human hepatocytes [5]. HLM helps determine the activity of drug metabolizing enzymes, CYPs and UGTs, present in the liver conveniently and straightforwardly [6, 7]. Further, it offers numerous advantages: high throughput screening, ease of storage, economic, repeatable, and simple usage with higher chances of clinical success [8]. Characterization of drug metabolic properties, assessing metabolic stability, and identifying metabolites are essential in determining the safety and success of clinical development [9].

2. Drug metabolism: a brief background

The concept of drug metabolism emerged around the mid-19th century but flourished in the 20th century [10]. Metabolism of most pharmaceutical drugs occurs in the liver. In drug metabolism, enzymes convert drugs to highly polar metabolites to facilitate excretion from the body. Drug metabolism helps assess the oral bioavailability, elimination half-life, and clearance of the body's drug substance. The deduced parameters help decide the dose adjustment and the drug substance's administration frequency [11]. The drug concentration should always reside within the therapeutic window, i.e., between the minimum effective concentration (MEC) and the maximum safety concentration (MSC), to avoid therapeutic failure and adverse effects [12]. CYPs being abundant in the liver, metabolize the majority of drugs [12]. Furthermore, CYPs regulate the biotransformation of endogenous as well as exogenous compounds [13]. Among all the CYP isoforms, CYP3A4 contributes to the metabolism of more than 50% of the marketed drugs [14, 15].

Drug metabolism reactions are divided into Phase I, Phase II, and Phase III reactions. Phase I reactions result in oxidation, reduction, and hydrolysis. The Phase I enzyme families include the CYP superfamily, flavin-containing monooxygenases (FMO), monoamine oxidases, alcohol or aldehyde dehydrogenases, reductases, esterases, amidases, and epoxide hydrolases. Phase II reactions lead to the addition or conjugation of highly polar groups to the drug molecule after Phase I reactions. Occasionally, direct Phase II reactions occur when susceptible functional groups are present on the molecule without being preceded by Phase I reactions. Common Phase II reactions include glucuronidation, sulphation, methylation, N-acetylation, and glutathione conjugation [16]. Phase III metabolism occurs through the elimination of drug molecules through the efflux pump [12]. The primary objective of drug metabolism is to eliminate the drug from the body by converting the lipophilic centers to hydrophilic centers, thus making them water-soluble for easy elimination through the kidney [17, 18]. Sometimes, metabolism may result in the conversion of a drug into a toxic metabolite. On the contrary, metabolism also converts an inactive drug (prodrug) to its active metabolite for achieving the desired medicinal results [18]. Many metabolites of known drugs like desloratadine (parent drug- loratadine), oxazepam (parent drug- diazepam), and cetirizine (parent drug- hydroxyzine) have been found to possess equivalent or enhanced therapeutic activity than the parent drug [19]. Similarly, the discovery of paracetamol was precious as it replaced the use of phenacetin, a toxic parent moiety. Hence, the metabolite's activity plays a significant part in bioequivalence studies [20].

First-pass metabolism explains metabolism before a drug reaches systemic circulation. This term refers to orally administered drugs that undergo metabolism in the gut or the liver before reaching the systemic circulation. **Figure 1** illustrates the various barriers to the drug reaching systemic circulation by the first-pass metabolism. During the drug discovery and development phases, the drug's metabolic fate should be kept in mind. Several approaches are in use ranging from empirical data-driven approaches to mechanistic models to predict drug metabolism. The empirical data-driven approaches, such as machine learning, involve approximations and assumptions, thereby providing high-speed predictions with low precision. In contrast, the mechanistic models involve quantum mechanics or molecular dynamics for providing significantly high accuracy; however, they consume time and effort [21].

Factors affecting drug metabolism are categorized into inter-individual factors and intra-individual factors [22, 23]. Inter-individual factors such as genetic factors, species differences, health conditions, enzyme induction/inhibition by xenobiotics or environmental factors, nutritional differences, and behavioral and cultural differences vary across individuals. However, they are uniform throughout the life of the organism [22]. Intra-individual factors can change throughout the lifetime, and different

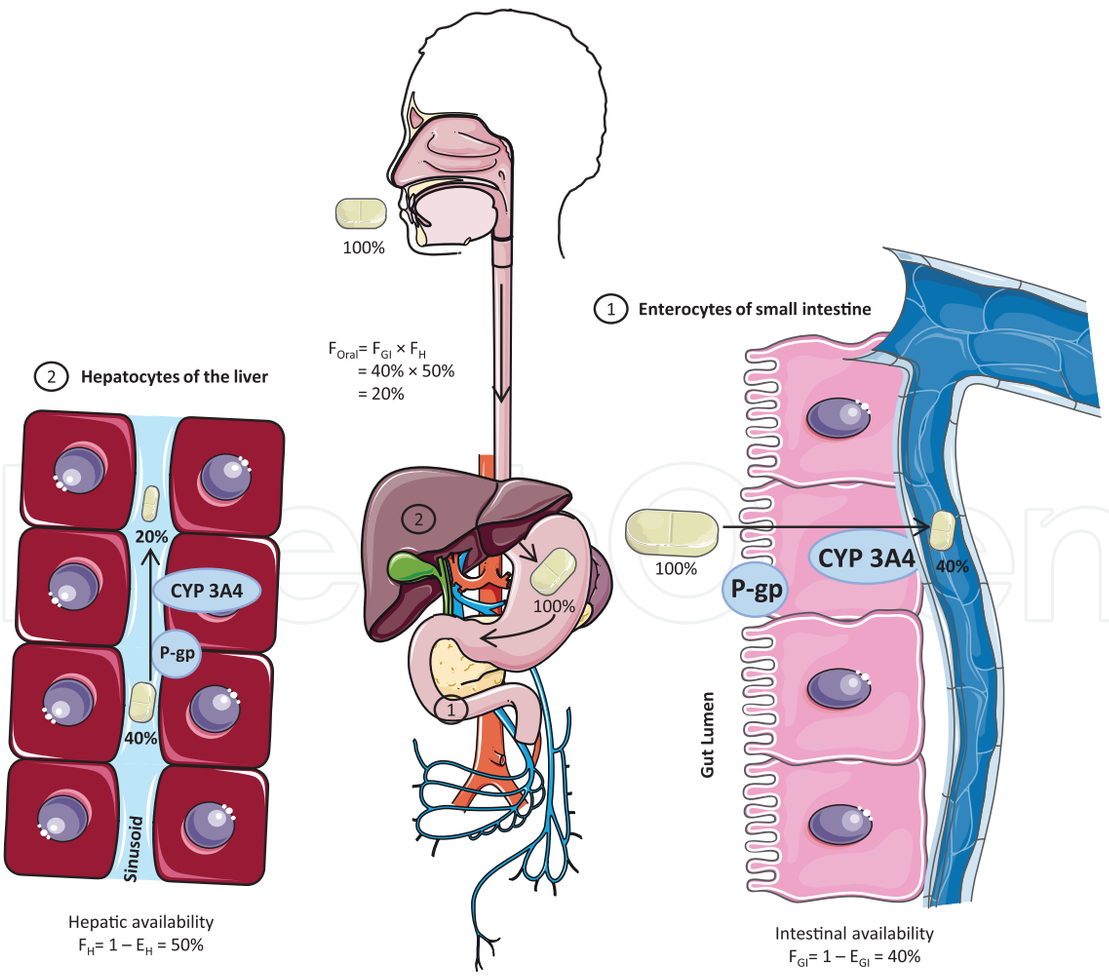

Figure 1.
First-pass metabolism by CYP3A4 and/or transport by P-glycoprotein (P-gp) in the enterocytes of small intestine wall and then hepatocytes of the liver before reaching the systemic circulation. FGI: Intestinal availability, F_H: Hepatic availability, F_{oral}: Oral bioavailability, E_{GI}: Intestinal extraction ratio, E_H: Hepatic extraction ratio.

endogenous and exogenous conditions control these factors, but the effect may be more significant under genetic influence. These can occur through the interaction of xenobiotics with transcription factors or xenobiotics with the drug-metabolizing enzymes. This direct interaction of xenobiotics with the drug-metabolizing enzymes causes the induction or inhibition of those enzymes [23]. Internal factors include age, pregnancy, hormones, sex, diseased state, genetics, and species. In contrast, external factors comprise the environment and diet (alcohol, tobacco, chemicals, and drugs) [24, 25]. Several studies reported that the reduction of rate and efficiency of the drug metabolism in the aging population is due to changes in the drug-metabolizing enzyme activity, variation in plasma protein binding, hepatic blood flow, and decrease in the liver mass, leading to the slowing down of excretion of few metabolized drugs [26]. The results of fasting on drug biotransformation are around 10–20%. This factor becomes crucial when a drug with a narrow therapeutic range is administered or when fasting produces an effect in combination with other factors [27].

In vitro systems help in mimicking and understanding the *in vivo* metabolism process. Of these, liver microsomes and hepatocytes are utilized to predict hepatic clearance [28]. HLM and suspended hepatocytes are the most common *in vitro* methods for determining metabolic stability [29]. They generate metabolites on a large scale for determining metabolic stability and profiling for comparison. The technological advancements have led to the generation of recombinantly expressed CYPs, slicing of the tissues, isolation of hepatocytes, and purification of the enzymes in a reproducible manner [30]. Immobilizing HLM on magnetizable beads coated with silica (HLM-MGBS) showed increased *in-vitro* metabolic efficiency [31]. The cellular or tissue models are used to assess the toxicity of the drug substance and its metabolites in cells or tissues [32, 33]. The placental toxicity of anticancer drugs was elucidated using placental tissue explants and trophoblast cell lines [34].

3. HLM: a best *in vitro* model to conduct high-throughput drug metabolism studies

HLM are the subcellular fractions derived from the liver's endoplasmic reticulum obtained by differential high-speed centrifugation. **Figure 2** displays the steps involved in the preparation of HLM from the human liver. HLM contains various enzymes such as CYPs, flavin-monooxygenase (FMO), carboxylesterases, epoxide hydrolase, and UGTs, making it a preferred *in vitro* model for drug metabolism studies. Assessing interindividual variability is possible with HLM as the activity of the microsomes differs in different individuals. This interindividual variability can be minimized while performing general metabolism studies by pooling the microsomes from different individuals. Microsomes from other human organs (intestine, kidney, lung) are also available and are utilized to evaluate extra-hepatic metabolism [35]. HLM aids in various studies like metabolite identification and profiling, assessment of interspecies variations, estimation of *in vivo* clearance, reaction phenotyping, and elucidation of the metabolic pathways [5, 8, 30, 36–38]. Furthermore, gender-specific microsomes are utilized in studying gender-based disparities in drug metabolism studies.

NADPH or NADPH regenerating system (NRS) is essential for the incubation process, and while determining the UGT activity, UDGPA and alamethicin are the

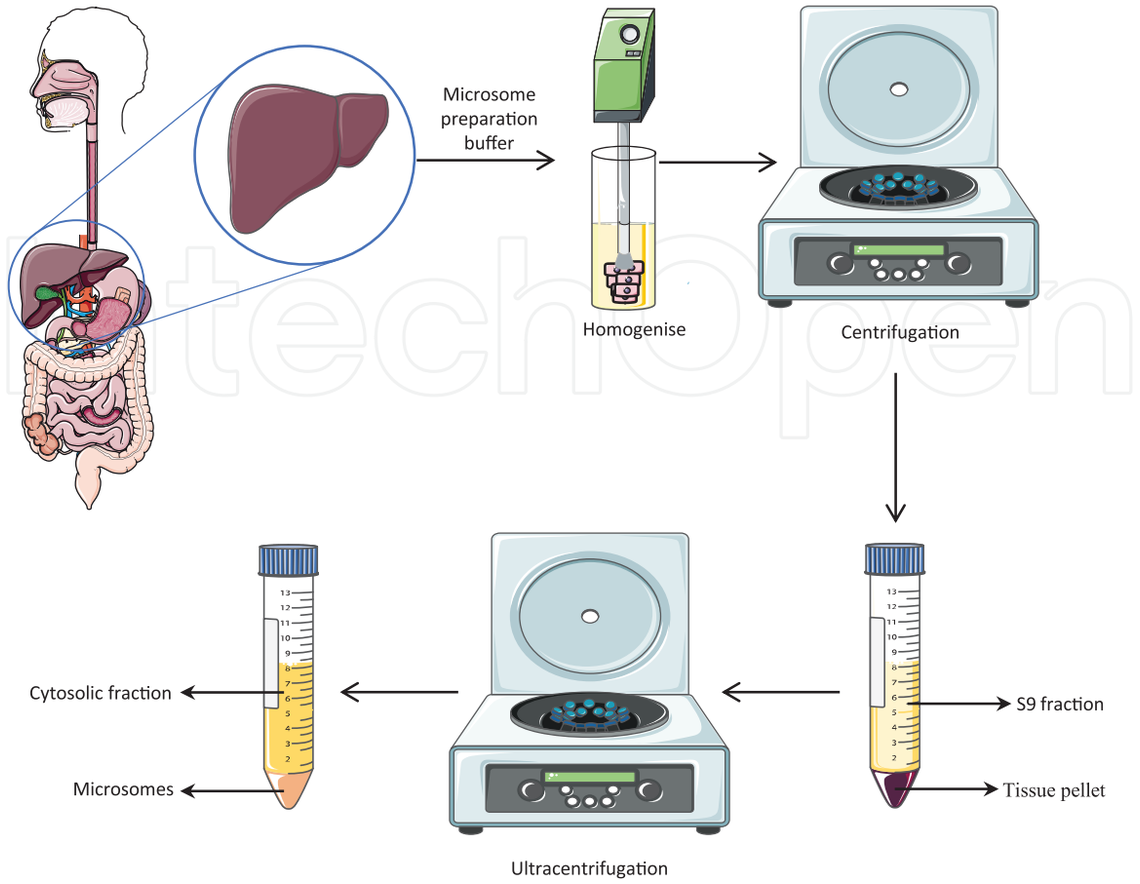

Figure 2.
Preparation of human liver microsomes. Microsome preparation buffer composition: 10 mM potassium phosphate buffer, pH 7.4, with 1.15% (w/v) potassium chloride. Homogenize the liver twice at 20,500 rpm, each time for 30 s, with a 30 s cooling period between bursts. Centrifuge the homogenate for 5 min at 1000 × g, 4°C, then increase to 10,000 × g for a further 10 min. Ultracentrifuge the S9 fraction for 60 min at 105,000 × g, 4°C.

prerequisites [5, 8, 37]. HLM are preferred as they are simple, economical, easy to store for long-term usage, and offer high throughput screening. Nevertheless, HLM has few drawbacks as it is unsuitable for quantitative assessments in drug metabolism studies since it lacks *N*-acetyltransferase (NAT), glutathione-S-transferase (GST), sulfotransferase (SULT) enzymes, and other cofactors. This drawback restricts the competitiveness in metabolism as well as limits the generation of a few metabolites. It also fails to consider the percentage of drugs bound to plasma proteins instead of microsomes crucial for *in vivo* metabolism studies [5, 8, 36, 37].

4. Drug metabolism studies by HLM

CYP activity changes in different species and this interspecies variation in drug metabolism can be estimated by investigating the *in vitro* drug metabolism in liver microsomes obtained from various species. The appropriate animal model for pharmacokinetics and toxicological studies can be determined by comparing the CYP metabolic profiles obtained from different species with the HLM. Microsomes can be acquired from the below-stated corporations: 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 is to be noted that the activity of the microsomes fluctuates among different batches and vendors. For instance, rat liver microsomes obtained from two different vendors demonstrated significant activity differences in the biotransformation of buspirone and loperamide. In contrast, the one obtained from the third vendor showed no activity. Furthermore, three batches obtained from the same supplier exhibited different activities in the biotransformation of buspirone and loperamide [39]. These differences were observed because of the innate differences in animals and varying preparation methods chosen by the vendors. Few vendors prepare liver microsomes by using phenylmethylsulfonylfluoride, while others use ethylenediaminetetraacetic acid (EDTA). Phenylmethylsulfonylfluoride inhibits trypsin-like proteases that are responsible for microsomal proteolytic degradation and certain carboxylesterases. On the other hand, EDTA chelates calcium and iron inhibiting both calcium-dependent phospholipases and lipid peroxidation. For each fresh batch, it is vital to examine the microsomal characterization data given by the vendor to check the CYP content, cytochrome b5, and NADPH-cytochrome c reductase activity.

For quantifying metabolites, drugs are incubated with microsomes with a low microsomal protein concentration, i.e., ≤0.5 mg/mL [40, 41]. This low concentration reduces the extent of protein binding to the drug. The final protein concentration of preparation is assessed by a Bradford protein assay or Lowry protein assay with bovine serum albumin as a standard. Storage of HLM at low temperatures (−80°C) maintains the activity of CYP enzymes for an extended period [40]. Microsomes thawed and kept on ice for less than 2 hours can be re-frozen at −80°C for reuse as there will be insignificant loss of enzyme activity [42].

The drug concentration used *in vitro* studies is higher than that observed in blood in an animal study. When the *in vivo* drug concentrations are unknown, the final drug concentration is chosen from a range of 1–10 μM to simulate the *in vivo* conditions. Drug's incubating concentrations (0.5–15 μM) have a significant influence on the drug's stability consequences: higher concentrations of the drug are more stable in microsomes [39]. Thus, it is recommended to work with two different concentrations of a drug for understanding the concentration impact on its stability within the microsomes and additional matrices. Low concentrations of the drug are proposed for *in vitro* studies due to the following reasons: 1) The reaction between the test drug and enzyme follows a first-order reaction, i.e., the rate of the reaction is directly proportional to the concentration of the drug 2) Concentration of organic solvents always should be low as it reduces the microsomal activity.

The control groups should exclude the substrate, microsomes, NADPH, or the NRS from the incubation solution. Ice-cold organic solvent (e.g., acetonitrile or methanol) is used to quench the reaction. Incubation time of less than 2 h at 37°C is suggested for performing a stability study using microsomes [42]. In the extended incubation period, additional control group incubations need to be included to ensure the enzyme's activity and thermal degradation of the drug. When metabolite identification is difficult, the % of unchanged parent drug versus time will be recorded. Organic solvents employed for solubilizing lipophilic drugs inhibit CYP activity. DMSO concentrations of 0.2, 0.5, or 1% inhibit the CYP activity resulting in erroneous stability data of incubated drugs. DMSO specifically inhibits CYP2E1, and hence, it should be avoided for studies involving the CYP2E1 enzyme. Organic solvents like methanol, ethanol, acetonitrile, and PEG 400 also inhibit about 15–25% of CYP2E1, CYP3A4, CYP2D6, CYP2C9, and CYP2C19 activity. The permissible limits for the organic solvents in solubilizing the drugs while retaining the CYP activity are methanol <1.0%, acetonitrile <1.0% and DMSO <0.2% [11].

4.1 Reaction phenotyping studies

Reaction phenotyping, also known as enzyme mapping, helps determine the enzymes involved in the metabolism of a specific drug. The data from these studies are essential in identifying potential drug interactions with common co-medications. Further, these studies help in anticipating possible pharmacokinetic changes caused by genetic polymorphisms in certain enzymes. Understanding the role of a specific enzyme involved in the metabolism of a drug is vital in the following conditions: 1) Identifying potential DDI with concomitant medications that may be inhibitors or inducers of the same enzymes [43]. 2) Establishing the metabolism of a drug by an enzyme that exhibits genetic polymorphism may result in significant inter-individual variability [44]. 3) Determining the formation of pharmacologically active metabolites [45]. 4) Deducing the extent of drug metabolism and the generation of significant metabolites [45].

In general, *in vitro* reaction phenotyping studies helps identify and characterize the formation of significant metabolites in drug metabolism at the preclinical stage. Toxicological studies assess the safety of these metabolites. In addition to toxicological considerations, detecting any pharmacological effects of major metabolites is also essential. Human radiolabel mass balance studies before phase III trials unravel main elimination pathways and systemic metabolite exposure. Data from the human radiolabel mass balance and the *in vitro* studies confirm metabolic pathways and the enzymes responsible for the drug metabolism. The CYP and non-CYP enzymes that contribute to ≥25% of drug elimination should be uncovered. The *in vivo* contribution is assessed by interaction with a potent, selective inhibitor or pharmacogenetic studies to decipher elimination pathways [46]. Assessment of reaction phenotyping uses approaches such as recombinantly expressed enzymes and correlation analysis. Recombinantly expressed enzymes use scaling methods like the relative activity factor (RAF) or the intersystem extrapolation factor (ISEF) for interpreting the relative contribution of the individual enzymes. The correlation analysis utilizes pooled HLM obtained from at least 10 donors for testing the activity toward respective probe substrates [47].

4.2 Enzyme inhibition studies

Enzyme inhibition experiments evaluate known CYP enzyme inhibitors on the metabolism of a drug by either pooled HLM or individual CYP isoforms. The usage of selective chemical inhibitors allows easy illustration of the metabolic pathways. To prevent false results, careful estimation of the drug and inhibitor concentrations for incubation is a must. Higher inhibitor concentrations exhibit non-selective chemical inhibition. For instance, quinidine and ketoconazole at <1 μM concentration act as selective CYP2D6 and CYP3A4 inhibitors. Although, at higher concentrations, these drugs inhibit other CYP isoforms as well. Chemical CYP inhibition is categorized into two types: reversible (could be competitive inhibition or non-competitive inhibition) and irreversible inhibition. In irreversible inhibition ("mechanism-based inhibition" or "suicide inhibition"), the CYP enzyme metabolizes the drug into a reactive metabolite that firmly binds to the enzyme's active site leading to a prolonged inactivation [48, 49]. These studies can be conducted before or after carrying out the cDNA-expressed recombinant CYP enzyme studies. They impart extra proof to assist the cDNA-expressed recombinant CYPs study results. Further, they may also provide a direction to these studies for the active isoform identification.

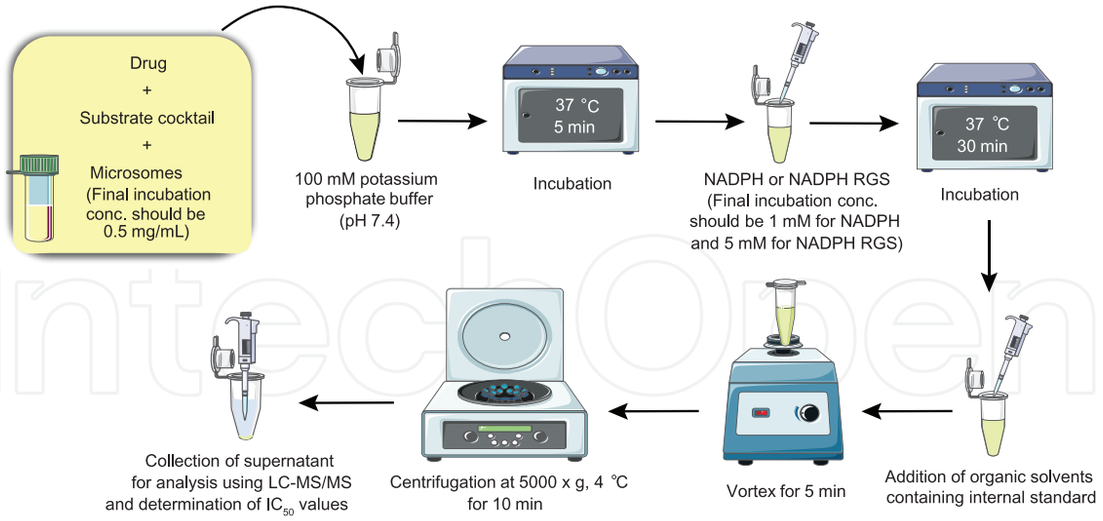

Figure 3.
Workflow to assess enzyme inhibition using human liver microsomes.

Figure 3 demonstrates the protocol for the CYP inhibition study. The procedure involves incubating the drug with liver microsomes in the presence and absence of selective inhibitors at 37°C for 30 min [40]. The following inhibitors against the isoforms and their concentrations are recommended: furafylline (CYP1A2; 0.1, 1, 10 μM), 8- glitazones or quercetin (CYP2C8; 0.5, 1, 10 μM), quinidine (CYP2D6, 0.5, 1, 10 μM), sulphaphenazole (CYP2C19; 5, 20, 100 μM), methoxypsoralen (CYP2A6; 0.1, 1, 10 μM), troleandomycin (TAO; CYP3A, 0.5, 1, 10 μM), clomethiazole (CYP2E1, 0.1,1,10 μM) [40, 50]. Methanol (< 1% (v/v) of the entire mixture) is used to dissolve the inhibitors before adding them to the incubation mixture. Prior to drug addition, inhibitors undergo preincubation at 37°C, with NADPH and microsomes, reaching a final concentration of 10 mM. A positive control is carried out in the presence of the drug with 1% methanol in the incubation mixture, whereas a blank control lacks the drug. Thus, the control values are employed to successfully determine the percentage of inhibition observed in the metabolite generation.

Conventionally, *in vitro* CYP enzyme inhibition studies were conducted using HLM, for which isoform-specific substrates were incubated along with the investigational drug. At the end of the incubation, the formation of the metabolite is monitored by analytical techniques like high-performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrometry (LC-/MS), or fluorescence and this procedure is repeated for at least three different concentrations of that drug [51, 52]. Identification of many drug molecules in the drug discovery process is possible using combinatorial chemistry approaches and high throughput screening techniques.

4.3 Drug metabolite profiling

Metabolite profiling refers to the relative quantification, identification, and characterization of the number of metabolites formed in the biological matrices. These studies help researchers structurally and chemically modify the drug to increase its efficacy, reduce its toxicity, and facilitate the synthesis of a molecule with enhanced therapeutic activity [53–56]. The FDA guidance "Safety Testing of Drug Metabolites" states that the metabolic drug profile must be determined by *in vitro* and *in vivo* models at various phases of the drug development. *In vitro* metabolite profiling of a

drug can be performed using liver microsomes, hepatocytes or liver slices collected from humans or animals [57]. The regulatory bodies (ICH, EMEA, and FDA) recommend studying *in-vitro* and *in-vivo* hepatic drug metabolismFDA guidance mentions the importance of metabolism studies via the kidney and the gastrointestinal tract as most orally administered medications interact with the gastrointestinal enzymes [58, 59]. Other regulatory authorities lay minimal emphasis on extra-hepatic metabolism studies.

Using a single high concentration of drug or a series of concentrations produces a high concentration of metabolites that meets the demands of quantification. A concentration of 50 μM or concentrations of 5, 50, and 500 μM can be chosen for novel metabolites. Concentration should be higher than or equal to the K_m value (Michaelis constant) recorded for the CYP substrates to generate metabolites in measurable amounts. A positive control having testosterone or phenacetin should be included for measuring the formation of 6β-hydroxytestosterone acetaminophen metabolites. A negative control without NADPH for each test compound helps determine the sources of metabolites other than oxidative metabolism (e.g., carboxylesterases, nonenzymatic metabolite formation, substrate impurities) [11].

4.4 Metabolic stability

Metabolic stability defines the liability of a drug compound to its metabolism. It is determined by estimating the disappearance of the drug substrate in a relevant *in vitro* system over a particular period. Further, metabolic stability data gives information on the secondary pharmacokinetic parameters such as bioavailability and half-life of the drug. Therefore, optimizing metabolic stability plays a vital role in the drug discovery and development phase [60]. These obtained parameters explain the drug's pharmacological and toxicological profile and shed light on the patient adherence to the drug. **Figure 4** shows the different stages in the metabolic stability study.

Metabolic stability studies are performed at a drug concentration less than K_m value, where enzymatic reactions follow the first-order process. While dealing with an unknown K_m value, 1 μM concentration of a drug is recommended. In general, the metabolic system is incubated with the drug substrate for a specified period at 37°C. The disappearance of the drug substrate is monitored at individual time points using a

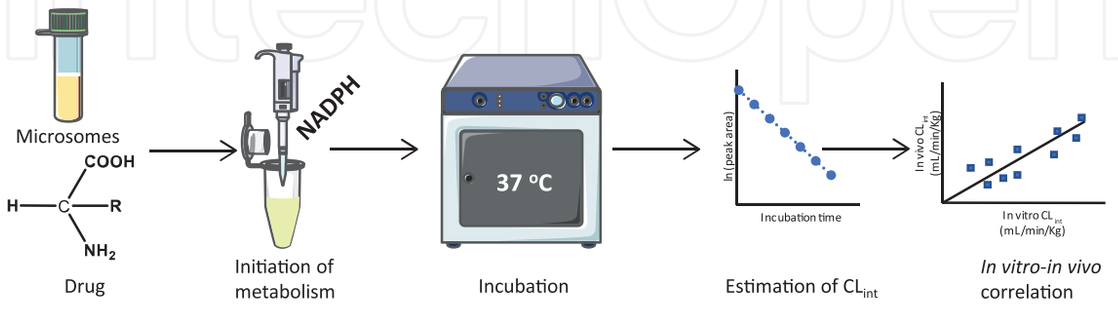

Figure 4.
Metabolic stability of drug substance. This assessment involves the incubation of the drug with microsomes in 100 mM potassium phosphate buffer. The addition of 1 mM NADPH solution followed by incubation at 37°C initiates the metabolic reaction. The disappearance of the drug substrate is monitored at individual time points using the analytical technique. Plotting the natural log of peak area ratio with time yields a straight line, where the slope of the line gives the elimination rate constant that helps in predicting the intrinsic clearance. The conversion scaling factors involves in the correlation between the clearance values obtained from the in vitro *intrinsic clearance data and* in vivo *clearance values.*

suitable analytical technique. Testosterone or DL-propranolol is added as a positive control to ensure the adequate execution of the assay. A negative control without NADPH is included to ascertain drug loss due to thermal degradation. Negative controls could also serve as matrix controls if they lack the drug or responsible enzyme. Plotting natural log of peak area ratio (drug substance peak area/internal standard peak area) with time yields a straight line, where the slope of the line gives the elimination rate constant (k).

The following equation determines half-life ($t_{1/2}$)

$$t_{\frac{1}{2}} = \frac{0.693}{k}$$

Metabolic stability studies derive various parameters that include half-life, intrinsic clearance, and total hepatic clearance. These parameters can be calculated using "well-stirred" and "parallel tube" approaches. In the "well-stirred" approach, the liver is characterized by a single compartment where the intracellular free concentration of drugs in hepatocytes is in equilibrium with the free concentration of drug in blood eliminating from the liver. Whereas in a "parallel tube" approach, the liver comprises numerous parallel tubes, in which the enzymes are evenly distributed. In every tube, the intracellular free concentration of drug in hepatocytes is in equilibrium with the free concentration of the drug in blood [61].

The whole liver CL_{int} is determined by using *in vitro* half-life:

$$\text{Whole liver } CL_{int} = \frac{0.693 \times \text{liver weight}}{invitro t_{\frac{1}{2}} \times \text{amount of the liver in incubation} \times \text{fraction unbound to microsomal protein}}$$

According to the "well-stirred" model, hepatic clearance (CL_H) and hepatic extraction ratio (E_H) are given by: [62].

$$CL_H = \frac{Q_H \times CL_{int}}{Q_H + CL_{int}}$$

$$E_H = \frac{CL_H}{Q_H}$$

where Q_H is the hepatic blood flow.

4.5 Enzyme kinetics in drug metabolism using HLM

It is essential to determine the enzymes involved in the metabolism process and their respective kinetic parameters throughout the drug discovery process. Enzyme kinetics involves studying reaction rates affected by different experimental variables such as enzyme concentration, substrate concentration, enzyme activators, enzyme inhibitors, temperature, pH, and ionic strength [63, 64]. Chakraborty et al. demonstrated the effect of pH, temperature, pressure and dwell time on enzyme inhibition kinetics in pineapple puree and concluded that the temperature had the highest impact on enzyme inactivation [65]. Further, they elucidate the role of polymorphism in determining drug clearance and aid in predicting drug–drug interactions associated with metabolites [66].

The CYP enzyme family metabolizes numerous xenobiotics, thus making it an integral part of drug–drug interactions [67]. Inhibition studies predict most P450

oxidations and drug–drug interactions, owing to their competitive Michaelis–Menten kinetics. Models with a single binding site are explained by competitive, noncompetitive, and uncompetitive inhibition, or activation of the enzyme, whereas some CYP3A4 oxidations tend to demonstrate unusual kinetics [67, 68]. Michaelis–Menten kinetics determines the enzyme kinetic constants such as K_m and V_{max}. The reaction velocity (V), i.e., the formation rates of metabolites with a fixed amount of HLM, is given by: [69].

$$V = \frac{V_{max} \cdot C}{K_m + C}$$

where C depicts the initial drug concentration, V_{max} gives the maximum reaction velocity of the enzyme, and K_m represents the Michaelis–Menten constant.

Intrinsic clearance (CL_{int}) is defined as the ratio of the rate of product formation to the substrate concentration and can be ascertained by using the K_m and V_{max} values [70].

$$CL_{int} = \frac{\upsilon}{[S]} = \frac{V_{max}}{K_m + [S]}$$

where [S] is the substrate concentration, V_{max} gives the maximum reaction velocity of the enzyme,

and K_m represents the Michaelis–Menten constant.

When the concentration of the substrate is considerably lower than the K_m value, then intrinsic clearance is augmented to total clearance [70]. Then, the above equation is simplified to:

$$CL_{int} \approx \frac{V_{max}}{K_m}$$

In cases where more than one CYP is involved in a drug metabolism reaction, a biphasic relationship is observed between V_{max} (maximal reaction velocity) and [S] (substrate concentration). It can be explained by using a two-enzyme model [8]:

$$V = \frac{V_{max\,1} \cdot [S]}{K_{m1} + [S]} + \frac{V_{max\,2} \cdot [S]}{K_{m2} + [S]}$$

Where K_{m1} and K_{m2} are high-affinity and low-affinity component constants, V_{max1} and V_{max2} are the maximal velocities of the enzymes for high and low-affinity components, respectively.

Atypical kinetics are elucidated through a particular enzyme by binding more than one drug molecule concomitantly or through other active site interactions [71, 72]. Hence, it is essential to analyze kinetics as an *in vivo* or *in vitro* effect to prevent an erroneous prediction of intrinsic clearance and ultimately impact the *in vivo* clearance [72]. Eadie-Hofstee plots are used to resolve multiple-enzyme kinetics when Michaelis–Menten plots fail to be beneficial [73]. It is essential to model the non-additive interactions to understand multidrug cocktails usage [74]. Further, it is necessary to consider the non-specific binding in enzyme kinetics. The chances of these events are high, resulting in the *in vitro* clearance values being closer to the measured values. However, if the non-specific binding is absent, the obtained values of intrinsic clearance will be lower than the real-time *in vivo* clearance [75].

Electrochemical methods determine enzyme kinetics where electron transfers are involved. The catalytic activity of the cytochrome P450 enzyme is electro analyzed as the catalytic cycle requires electron transfer. Electroanalysis paves the way for multicomponent studies entailing many drugs to describe interactions under the mutual influence or drug interference, which in turn is manifested by an alteration in the kinetic constants of enzymatic catalysis [76]. Novel microfluidic tools and detection methods have made the high throughput measurement of enzyme kinetics possible using droplet-based optofluidic systems [77]. A nanochannel-array enzyme reactor has been developed to comprehend the basics of enzymatic reactions restricted to nano-spaces and also gives an outreach to design productive enzyme reactors [78].

Many *in vitro* systems like HLM, human hepatocytes, recombinantly expressed CYP enzymes, S9 fractions and human liver slices are used to determine the intrinsic clearance of the drug for speculating the *in vivo* clearance by estimating the kinetic parameters, K_m and V_{max} values. HLM is a well-established in vitro system for studying drug metabolism through CYP450 kinetics due to its low cost, ease of use, and commercial availability [79]. CYP activity levels differ in different microsomal preparations. This variation between different donor's preparations can be used to understand the effects of age, sex, and genotype on CYP-regulated kinetics [22].

4.6 Glutathione conjugation assay

The tripeptide, L-γ-glutamyl-L-cysteinyl-glycine, known as Glutathione (GSH), is a low molecular mass, thiol-reducing compound, synthesized from L-glutamate, L-cysteine, and glycine amino acids [80]. The cysteine sulfhydryl group (-SH) is responsible for reduction and conjugation reactions for eliminating reactive electrophiles and enhancing a lipophilic compound's solubility [81, 82]. GSH is usually found at concentrations between 1 and 10 mM and involves scavenging reactive oxygen species and detoxifying foreign compounds [83, 84]. The glutathione conjugation is followed by a series of metabolic and transport phases that eventually leads to the mercapturic acid formation (S-conjugates of N-acetylcysteine) [85, 86]. The formed mercapturic acid is more polar and, thus, easily excreted in urine [87]. Glutathione S-transferases (GSTs) include drug-metabolizing enzymes responsible for catalyzing glutathione conjugation with many foreign compounds [88]. They belong to the superfamily of Phase II enzymes and exist as dimeric proteins [89, 90].

The human GST is present in the cytosol, is expressed in the liver, and is subdivided into classes α, π, and μ. In a human liver, 80–90% of the GST is present in the form of GST-α [91]. The expression of these GST-α enzymes may lead to resistance toward anticancer drugs, and thus, GSTs can also be used as markers for malignant tumors [92, 93]. The main functions of GSTs are redox signaling, antioxidation, and detoxification of many cancer drugs [94–96]. The detoxification phenomenon is not always valid, and in some instances, GSH S-conjugates were observed to be toxic [97]. Christoph Englert et al. reported that nanocarriers' coupling to glutathione aided in effectively crossing the blood–brain barrier [98].

4.7 Glucuronidation and its kinetics

Glucuronidation reaction results in the conjugation of glucuronic acid obtained from uridine diphosphate-glucuronic acid (UDPGA) to compounds that contain hydroxyl, carboxyl, thiol, amino, and acidic functional groups by UDP-glucuronosyltransferase enzymes (UGTs) [99, 100]. UGTs are abundant in the

liver and intestine [101]. These membrane-bound enzymes of the endoplasmic reticulum account for the metabolism of more than 35% of drugs [102, 103]. Human UGT enzymes are categorized into four families, namely: UGT1, UGT2, UGT3, and UGT8. These enzymes are further classified into UGT 1A, 2A, and 2B isoforms depending upon the structure of the gene and the analogy of sequence. The isoforms expressed in the liver of UGT1A: UGT1A1, UGT1A3, UGT1A4, UGT1A5, UGT1A6, and UGT1A9 [104, 105] and UGT2B isoforms: UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17 and UGT2B28 [104]. The lumen of the endoplasmic reticulum (ER) serves as an active site for UGTs, and its membrane allows substrates, cofactors, and products to diffuse [106]. The latent action of the UGTs in microsomal incubations can be removed by distorting the barrier. Alamethicin disrupts the barrier by forming pores in the membrane and permits entry to the enzyme, causing no impact on the membrane's structure or its intrinsic catalytic activity [107]. Glucuronidation is a detoxification reaction as it enhances the compound's polarity and facilitates the excretion of compounds through urine and bile [103, 108]. It is necessary to comprehend the involvement of UGTs in the drug's metabolism as it aids in averting drug–drug interactions and adverse drug reactions [109].

In the first stage, the microsomes are activated in 0.1 M potassium phosphate buffer (pH 7.4) pre-incubated with 50 μg/mL concentration of alamethicin on ice for 30 min. The drug is incubated for 5 min at 37°C with 0.1 M potassium phosphate buffer (pH 7.4), 4 mM $MgCl_2$and the activated HLM with the final concentration of 0.5 mg/mL. The metabolic reaction is initiated by adding 5 mM UDPGA and incubating this mixture at 37°C for predetermined time points. A well-known UGT substrate is included as a positive control, and a mixture without UDPGA is used as a negative control to assess the formation of metabolites other than glucuronidation metabolism [110, 111]. The addition of ice-cold extraction solvents (Acetonitrile or methanol) terminates the reaction. The samples are centrifuged, and the supernatant is collected for further analysis using a suitable analytical technique like HPLC [112–114] or LC–MS/MS [115–117].

Kinetic analyses were performed with HLM and commercially available UGTs. The elucidation of the kinetics of glucuronidation has a significant influence on the credibility of the predicted *in vitro* clearance value [118]. Michaelis–Menten kinetic model is used for the determination of the kinetics of glucuronidation via kinetic constants, K_m (Michaelis constant, the concentration of substrate when the reaction rate is 50% of V_{max}) and V_{max} (the maximum rate of reaction when the substrate saturates all the active sites of the enzymes). Kinetics of glucuronidation for several drugs like NSAIDs [119], olanzapine [120], serotonin [121], and ursolic acid [122] were determined using this model. The substrate inhibition equation used is:

$$\nu = \frac{V_{max}\,[S]}{K_s + [S] + {}^{[S]^2}/_{K_{si}}}$$

where v is the reaction rate, [S] is the substrate concentration, V_{max} is the maximum velocity, K_s is the substrate affinity constant, and K_{si} is the substrate inhibition constant [123].

The hill equation is used to determine the sigmoidal kinetics:

$$\nu = \frac{V_{max}\,S^n}{S_{50}^n + S^n}$$

where S_{50}^{n} is the substrate concentration leading to 50% of $V_{max,}$ and n is the Hill coefficient [124].

Eadie–Hofstee plots and Lineweaver-Burk plots determine the model to be selected for the kinetic analyses using nonlinear regression analysis for fitting the experimental data [125, 126]. A straight line in the plot signifies the Michaelis–Menten model's usage. In contrast, if a hook in the upper panel is obtained, it represents the usage of the substrate inhibition model [110, 127].

5. Conclusion

After the drug's oral administration, the drug undergoes various processes like absorption, distribution, metabolism, and excretion. Metabolism of most of the drugs is carried out by CYP and UGT enzymes, which are abundant in the liver. *In vivo* and *in vitro* studies are often used for drug metabolism studies. *In vivo* studies involve screening serum, urine and feces, whereas *in vitro* drug metabolism studies are carried out using HLM, human hepatocytes, and recombinantly expressed cytochrome P450 enzymes. HLM are widely used as it offers several advantages like high throughput screening, ease in storage, economic, and simplicity and convenience in their usage. Metabolism studies play a significant role in identifying, characterizing, and quantifying potential metabolites of a particular drug, thereby elucidating the drug metabolism pathway. Metabolism may convert a drug into a toxic metabolite or render an inactive drug into its active form. Metabolic stability helps predict the metabolic clearance of the drug and thereby helps in dosage adjustment and the frequency of administration of a drug. Therefore, conducting drug metabolism studies using HLM at the drug discovery stage helps screen the potential leads with optimized pharmacological properties.

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Conflict of interest

The authors declare no conflict of interest.

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