Int. J. Pharm. Investigation, 2026; 16(3):885-891.
https://www.jpionline.org

Review Article

In vitro Models for Studying Drug Metabolites and Metabolizing Enzymes: A Comprehensive Review

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ABSTRACT

Background: *In vitro* assays play a crucial part in evaluating the metabolic fate of drug candidates and understanding the activity of drug-metabolizing enzymes. **Materials and Methods:** Model systems including Hepatocytes, S9 fractions, and liver microsomes are frequently employed to investigate xenobiotic metabolism and assess potential toxicity and recombinant enzymes, provide controlled environments to investigate biotransformation pathways, metabolite formation, and enzyme kinetics without the complexity of whole-animal studies. **Results and Discussion:** The assessment of drug metabolites *in vitro* helps predict pharmacokinetic behavior, drug–drug interactions, and potential toxicity, thereby supporting drug discovery and development. Enzyme-specific assays further enable identifying the main isoforms of cytochrome P450 and conjugating enzymes take part in metabolism of drug. **Conclusion:** Overall, *in vitro* assays of drug metabolites and enzymes involved in drug metabolism serve as essential tools in preclinical research, facilitating safer and more effective pharmaceutical development and regulatory evaluation.

Keywords: Assay, Cytochrome P450, Enzyme Kinetics, Hepatocytes, Liver microsomes, Metabolizing Enzymes, S9 fraction.

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Received: 24-12-2025;
Revised: 16-02-2026;
Accepted: 06-04-2026.

INTRODUCTION

The pharmacokinetic and pharmacodynamic characteristics of medicinal medicines are significantly influenced by drug metabolism. To assess a drug's effectiveness, safety, and potential for side effects, it is crucial to comprehend how it gets biotransformed into metabolites. The Cytochrome P450 (CYP) superfamily and phase II conjugating enzymes, including UDP-glucuronosyltransferases, sulfotransferases, and glutathione-S-transferases, are mostly responsible for the majority of drug metabolism. The investigation of these pathways is essential to drug development since variations in enzyme activity might result in interindividual variations in drug responsiveness and drug–drug interactions.

In vitro assays have emerged as indispensable tools for studying drug metabolism and enzyme activity under controlled laboratory conditions. Commonly employed systems include liver microsomes, S9 fractions, primary hepatocytes, and recombinant enzymes, each offering unique advantages for identifying metabolic pathways and quantifying metabolite formation. These assays not only help predict *in vivo* drug disposition but also aid in the early detection of potentially toxic metabolites and inhibitory effects on metabolic enzymes.

Compared with *in vivo* studies, *in vitro* assays are cost-effective, ethically favorable, and highly reproducible, allowing for rapid screening of multiple compounds. Despite certain limitations, such as the inability to fully mimic whole-body physiology, they remain central to preclinical research. By combining metabolite profiling with enzyme-specific assays, researchers can generate valuable data to support drug discovery, regulatory submissions, and the design of safer, more effective therapeutic agents (Anzenbacher & Anzenbacherova, 2012).

Metabolites

Metabolites are intermediate products formed during metabolic reactions, typically catalyzed by enzymes within cells. These

DOI: 10.5530/ijpi.20260116

compounds play a vital role in various biological processes, including drug metabolism. Some common Arachidonic acid, inosine-5'-monophosphate, steroid hormones, and other human metabolites and catecholamines such as norepinephrine and dopamine. A significant portion of administered drugs is eliminated from the body through metabolism, primarily via detoxification pathways. Metabolites can influence the therapeutic efficacy of drugs, and while some possess beneficial pharmacological activity, others may be harmful. Crucially, metabolites can also act as building blocks for the creation of more sophisticated and novel medications. Metabolite identification is a crucial stage in assessing safety and efficacy and starts as soon as a molecule is chosen for therapeutic development. This procedure is carried out very carefully and is constantly watched, particularly in the early phases of drug selection, because of the possible toxicity of some metabolites (Asha & Vidyavathi, 2010).

Identification of Metabolites

The certainty of metabolite identification can vary significantly due to the complexity of the process, which is influenced by the stage of analysis, the resilience of the methods used, and the quality of databases as well as resources used. Typically, drug metabolite identification involves the use of a radiolabelled version of the parent compound, allowing researchers to trace and quantify the metabolites formed. Measurements like the proportion of the administered dose recovered in urine and feces provide valuable insights into drug disposition. In order to identify these radioactive substances' chemical identities in biological samples, a combination of radiometric, chromatographic, and spectroscopic techniques is applied. These include methods like Capillary electrophoresis (particularly for oligonucleotides), Time-of-Flight (TOF), GC-MS, LC-MS, LC-MS/MS, HPLC/UPLC, and MALDI-TOF. These advanced analytical tools enable the generation of detailed and specific information about each metabolite. Various *in vitro* approaches to metabolite identification further enhance the understanding of drug metabolism and are explored in subsequent discussions (Bale *et al.*, 2016).

In vitro Assay System

Liver Microsomes

Source: Endoplasmic reticulum fraction of liver cells.

Usually prepared by differential centrifugation of liver homogenates derived from fresh human liver tissue, liver slices, liver cell lines, or primary hepatocytes, Human Liver Microsomes (HLM) are vesicular fragments formed from the endoplasmic reticulum of hepatocytes. Important drug-metabolizing enzymes such as cytochrome P450s, carboxyl esterases, epoxide hydrolases, Flavin-Containing Monooxygenases (FMOs), and UDP-Glucuronosyltransferases (UGTs) are abundant in this subcellular fraction.

Due to their enzymatic composition, HLMs are widely used as an *in vitro* model for drug metabolism profiling and drug interaction studies. Researchers often investigate the role of specific isoenzymes by applying selective inhibitors during microsomal assays. Since enzyme activity can vary between individuals, HLMs are also valuable for studying interindividual variability in drug metabolism. To minimize this variability in general assessments, pooled microsomes from a broad donor bank are commonly used. Additionally, microsomes derived from other human organs such as the intestines, kidneys, and lungs are employed to evaluate extrahepatic metabolism. Gender-specific microsomes are available as well, enabling the study of sex-based differences in drug biotransformation. In the drug discovery process, HLMs serve multiple purposes, including metabolite identification, assessment of interspecies metabolic differences, prediction of *in vivo* drug clearance, reaction phenotyping, and elucidation of metabolic pathways (Bayliss, 1999; Berry *et al.*, 1992; Cross *et al.*, 1995).

Enzymes present: Flavin-containing Monooxygenases (FMO), Cytochrome P450 (CYP450), and some phase II enzymes (UGTs).

Metabolism studied: Phase I (oxidation, reduction, hydrolysis) and some glucuronidation (Phase II).

Advantages: Cheap, easy to prepare, high metabolic activity.

Limitations: No complete cell system → cannot study transporters or full conjugation pathways.

Hepatocytes (Primary or Cryopreserved)

Source: Intact liver cells (human or animal).

Enzymes present: All major Phase I & Phase II enzymes, transporters.

Metabolism studied: Both Phase I and Phase II reactions (oxidation, conjugation, etc.).

Advantages: Closest to *in vivo* liver metabolism, can predict clearance.

Limitations: Expensive, limited lifespan, inter-donor variability (Eichelbaum & Burk, 2001; Gomez-Lechon & Castell, 2007).

S9 Fraction

Source: Post-mitochondrial supernatant (contains both microsomes + cytosol).

The S9 fraction, obtained from organ tissue homogenates, is widely employed in biological assays to investigate the metabolism of drugs and other xenobiotics. Although liver S9 fractions have been in use since the 1970s, they are less commonly applied than microsomes. Unlike microsomes, S9 fractions include both microsomal and cytosolic components, providing a broad range of metabolic enzymes such as Cytochrome P450s (CYPs), Flavin-containing Monooxygenases (FMOs), carboxylesterases,

epoxide hydrolases, UDP-Glucuronosyltransferases (UGTs), Sulfotransferases (SULTs), methyltransferases, acetyltransferases, and Glutathione S-Transferases (GSTs). This extensive enzymatic repertoire makes S9 fractions a valuable *in vitro* system for assessing drug metabolism, toxicity, and mutagenicity. To sustain CYP activity, an external NADPH-regenerating system is usually added to fulfill their energy requirements. Representing the post-mitochondrial supernatant of liver homogenates, S9 fractions capture both phase I and phase II reactions, offering a more integrated view of metabolism. Notably, they can generate metabolites not formed by microsomal or cytosolic fractions alone. However, their overall enzyme activity is lower than that of isolated subcellular fractions, which may result in certain metabolites remaining undetected (Figure 1) (Gajula *et al.*, 2022; Richardson *et al.*, 2016).

Enzymes present: Phase I enzymes (CYPs) + Phase II enzymes (e.g., sulfotransferases, N-acetyltransferases).

Metabolism studied: Wide range of pathways (oxidation + conjugation).

Advantages: Covers more enzymes than microsomes.

Limitations: Lower activity than hepatocytes.

Recombinant Enzymes (cDNA-expressed)

Source: Human CYP450 isoenzymes expressed in cell lines (e.g., *E. coli*, yeast).

Enzymes present: Individual CYP isoforms (e.g., CYP3A4, CYP2D6, CYP2C9).

Metabolism studied: Isoenzyme-specific metabolism.

Advantages: Identify which specific enzyme metabolizes a drug.

Limitations: Artificial system, lacks enzyme–enzyme interactions (Lin & Lu, 1998; Marques *et al.*, 2023).

Precision-Cut Liver Slices (PCLS)

Source: Thin slices of fresh liver tissue.

Incubating liver slices in nutrient-rich media serves as a valuable *in vitro* approach for studying drug metabolism. This method preserves the structural integrity of liver tissue, allowing for the examination of metabolic processes in a more physiologically relevant context. However, the limited diffusion of nutrients and oxygen within the slices restricts the duration of Cytochrome P450 (CYP) enzyme activity, making it a short-lived system. Currently, commercially available human liver slices are not accessible, which limits broader application. Despite these challenges, liver slice models offer potential for investigating the induction of specific CYP isoforms by new drug candidates, providing insights into enzyme regulation and metabolic response (Knights *et al.*, 2016; Martiny & Miteva, 2013).

Enzymes present: All metabolic enzymes + intact tissue architecture.

Metabolism studied: Phase I & II reactions, plus transport.

Advantages: Preserves natural cell–cell interactions.

Limitations: Short viability (few hours), technically demanding (Mondal *et al.*, 2008; Naritomi & Terashita, 2003).

Enzyme Inhibition Studies

Enzyme inhibition studies are a critical tool for assessing the influence of known Cytochrome P450 (CYP) inhibitors on drug metabolism, using either pooled human liver microsomes (HLM) or individual CYP isoforms. The use of selective chemical inhibitors enables precise characterization of metabolic pathways. For reliable outcomes, careful optimization of both drug and inhibitor concentrations during incubation is essential, since excessive inhibitor levels may cause non-selective effects. For instance, quinidine and ketoconazole are selective inhibitors of CYP2D6 and CYP3A4, respectively, when used at concentrations below 1 μM, but at higher levels they may also inhibit other isoforms. CYP inhibition is generally divided into two categories: reversible inhibition, which includes competitive and non-competitive mechanisms, and irreversible inhibition, also referred to as mechanism-based or suicide inhibition. In irreversible inhibition, the enzyme converts the drug into a reactive intermediate that permanently binds to the active site, leading to sustained inactivation. Such inhibition experiments can be conducted either before or after studies with cDNA-expressed recombinant CYP enzymes. They provide complementary evidence to recombinant enzyme assays and are particularly useful in identifying the specific CYP isoforms responsible for drug metabolism (Figure 2) (Olavi *et al.*, 1974).

In vitro Assay Procedure For Drug Metabolites

In vitro assays provide indispensable approaches for drug metabolism studies, as they provide controlled systems to investigate the biotransformation of new chemical entities. The primary objective is to identify and characterize metabolites, determine metabolic stability, and understand enzyme pathways before advancing to *in vivo* studies. Among the widely used systems, Human Liver Microsomes (HLM) and hepatocytes offer reliable models of hepatic metabolism. The following procedure outlines the use of HLM for drug metabolite profiling, with extensions for hepatocyte use to capture Phase II pathways (Pinheiro *et al.*, 2017).

MATERIALS AND METHODS

The assay setup for evaluating drug metabolism typically involves pooled Human Liver Microsomes (HLM) at a final protein concentration of 0.5–1.0 mg/mL, suspended in a potassium phosphate buffer (100 mM, pH 7.4) supplemented

with magnesium chloride (3 mM). To support cytochrome P450 enzyme activity, a NADPH-regenerating system—comprising $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase—is included.

The test compound is usually added at a concentration ranging from 1 to 10 μM, alongside a positive control substrate such as midazolam and an internal standard for analytical consistency. Organic solvents like acetonitrile or methanol are used to quench the reactions.

For Phase II metabolic studies, additional cofactors such as UDP-Glucuronic Acid (UDPGA) for glucuronidation, 3'-Phosphoadenosine-5'-Phosphosulfate (PAPS) for sulfation, and Glutathione (GSH) for conjugation may be incorporated. To simulate whole-cell metabolism more accurately, cryopreserved hepatocytes can also be introduced into the assay system (Potter & Elvehjem, 1936).

Preparation

Microsomes are thawed slowly on ice, mixed gently, and pre-equilibrated to 37°C. A master incubation mixture is prepared with buffer, $MgCl_2$, microsomes, and optional cofactors. Test compound solutions are diluted to ensure organic solvent concentration remains below 1% to avoid enzyme inhibition. Separately, the NADPH-regenerating solution is freshly prepared to initiate Phase I metabolism (Riley & Grime, 2004).

Incubation

For each time point, pre-warm incubation tubes containing the master mix at 37°C for 5 min. Reactions are initiated by adding the NADPH system to reach a final NADPH concentration of 1 mM. Typical final conditions are 0.5 mg/mL microsomal protein and 1 μM substrate concentration. Reactions are performed in triplicate, alongside controls lacking NADPH, heat-inactivated microsomes, and matrix blanks. Incubations run for 5-60 min, with aliquots withdrawn at intervals (0, 5, 15, 30, 45, 60 min) to capture time-dependent metabolite formation (Sowjanya, 2019).

Quenching and sample processing

To prepare samples for LC–MS analysis, each aliquot is quenched by adding two to three volumes of ice-cold acetonitrile containing an internal standard. The mixture is then vortexed thoroughly and placed on ice for 10 min to stabilize the reaction. Following this, the samples are centrifuged at 14,000 g for 10 min at 4°C to separate the supernatant, which contains the analytes of interest. This supernatant is subsequently collected for LC–MS analysis. For enhanced sensitivity and cleaner metabolite profiles, solid-phase extraction may be optionally employed to concentrate and purify the metabolites. After extraction, the samples can be

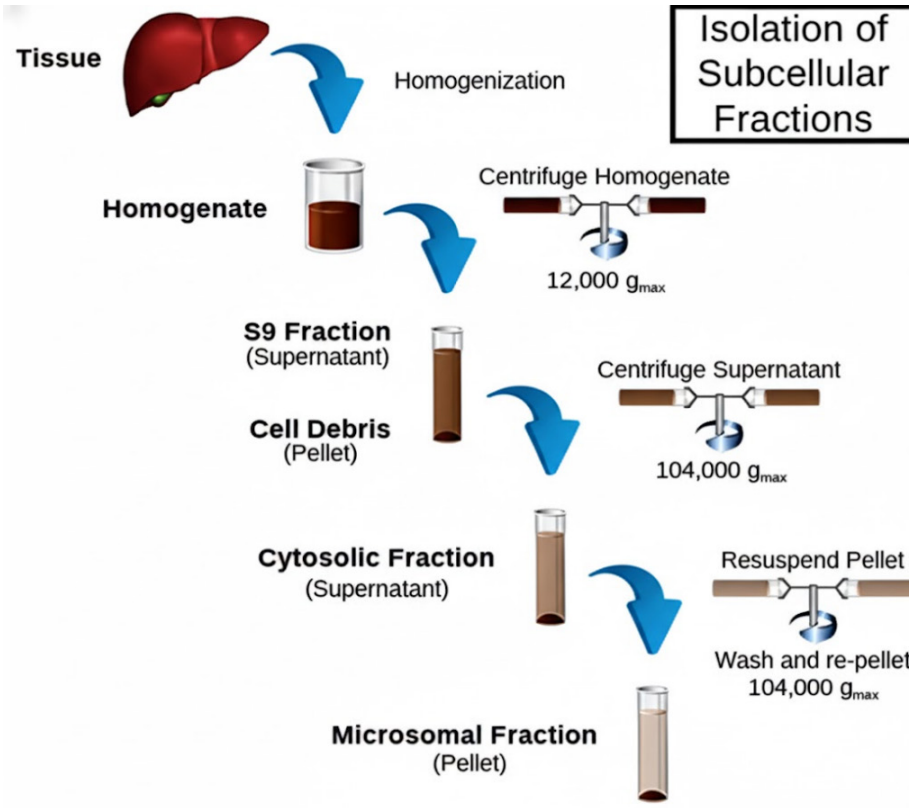

Figure 1: This diagram outlines the sequential differential centrifugation method for the isolation of subcellular fractions from tissue (e.g., liver). The process begins with homogenization, followed by a series of centrifugation steps at increasing *g*max forces (12,000 *g*max and 104,000 *g*max). This technique separates the homogenate into distinct fractions, including the S9 fraction, cell debris, cytosolic fraction, and the microsomal fraction.

dried using nitrogen evaporation and reconstituted in the initial mobile phase prior to analysis (Sabatini, 2014; Wojcikowski & Daniel, 2009).

LC–MS analysis

Samples are analyzed using UHPLC coupled with high-resolution mass spectrometry. Separation is achieved on a C18 column with a gradient from aqueous 0.1% formic acid to acetonitrile. Data acquisition is performed in both positive and negative ion modes, using full-scan and MS/MS fragmentation to identify metabolites. Characteristic mass shifts such as +16 Da (hydroxylation), +176 Da (glucuronidation), or +305 Da (glutathione adduct) guide metabolite detection.

RESULTS AND DISCUSSION

Data analysis and interpretation

Extracted ion chromatograms are compared against controls to eliminate artifacts. Relative metabolite abundances are quantified using internal standards, and parent drug depletion is used to estimate intrinsic clearance. Enzyme involvement can be probed using chemical inhibitors or recombinant CYP/UGT enzymes (Yao *et al.*, 2001).

Metabolic stability

Metabolic stability describes the tendency of a drug compound to undergo metabolic transformation. It is typically evaluated by monitoring the rate of substrate depletion within an appropriate *in vitro* system over a defined time period. The resulting data offer important insights into pharmacokinetic properties such as bioavailability and half-life, which determine how long a drug remains active in the body and how effectively it is absorbed. Consequently, improving metabolic stability represents a key objective in the drug discovery and development process. The parameters obtained not only help characterize the drug's pharmacological and toxicological profile but also influence patient adherence by affecting dosing frequency and therapeutic consistency (Figure 3) (Zhang, *et al.*, 2015).

Advantages of *in vitro* Assay of Drug Metabolites

Ethical and safer than in vivo – No need for human/animal administration in early stages, reducing ethical concerns and risks.

Controlled environment – Conditions (pH, enzyme concentration, cofactors) can be precisely controlled and manipulated.

Identification of metabolites – Helps in detecting primary and secondary metabolites before moving to *in vivo* studies.

High-throughput screening – Multiple drug candidates can be tested rapidly for metabolic stability.

Cost- and time-effective – Much cheaper and quicker than animal or clinical studies (Uehara *et al.*, 2022; Zanger & Schwab, 2013).

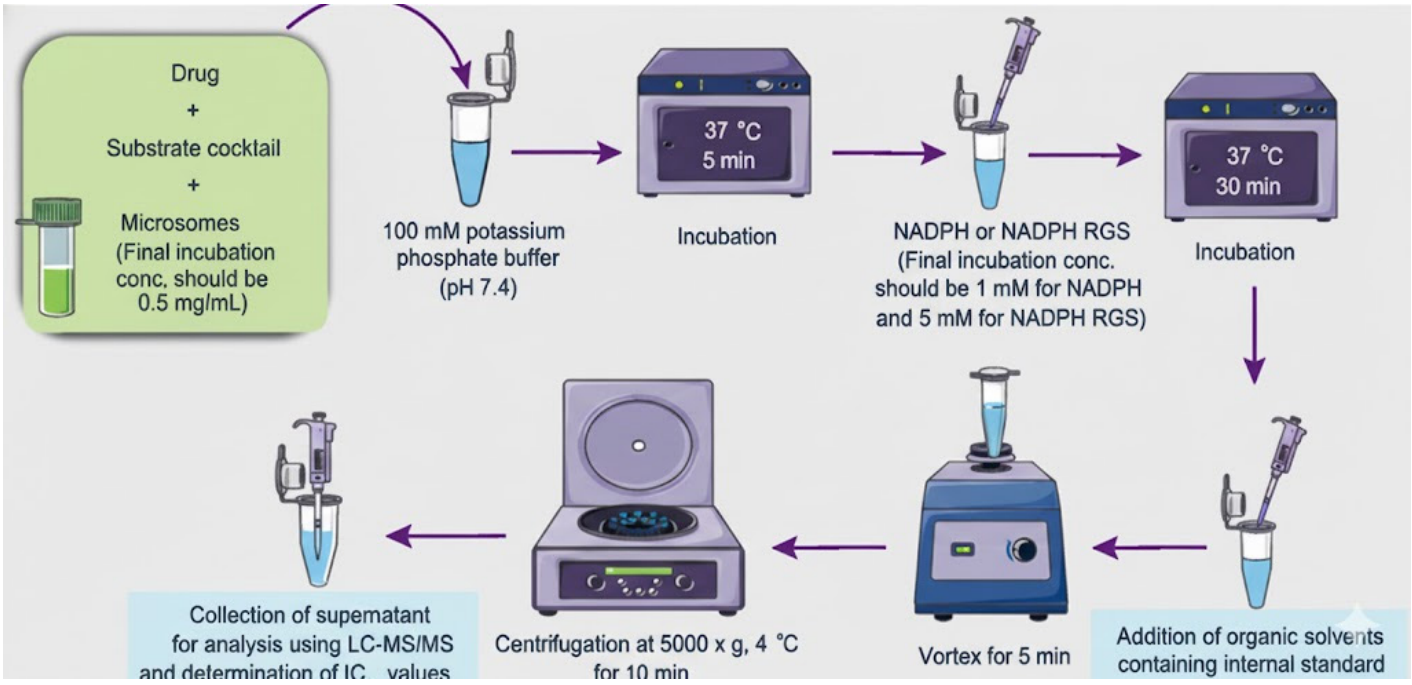

Figure 2: This figure illustrates the experimental workflow for Cytochrome P450 (CYP) inhibition studies using liver microsomes. The process involves sequential steps of incubation with the drug, substrates, and cofactors (NADPH/NADPH RGS) at 37∘C, Sample preparation includes protein precipitation via organic solvents, vortexing, and centrifugation, followed by the collection of the supernatant for LC-MS/MS analysis to determine IC_{50} values.

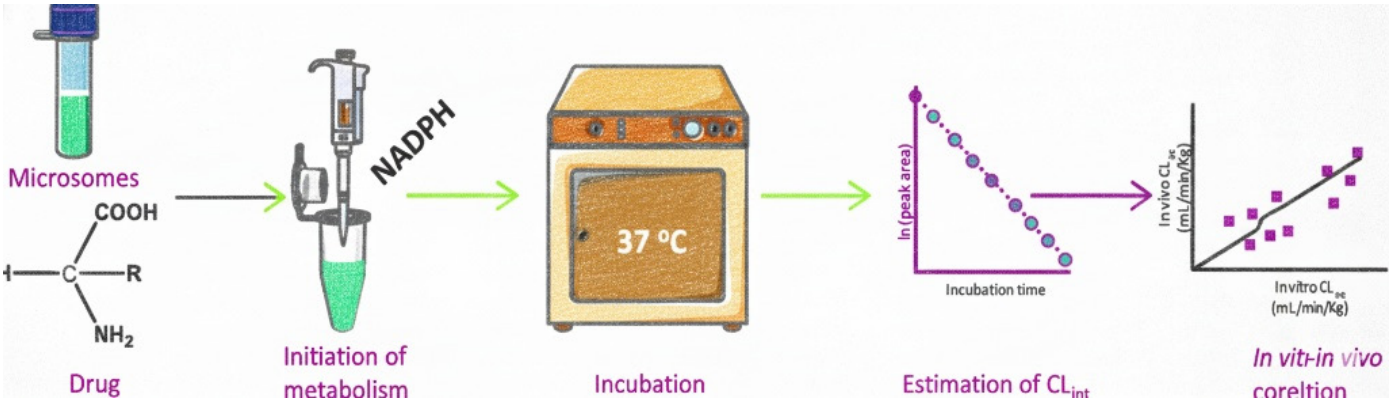

Figure 3: This diagram illustrates the process of estimating the intrinsic Clearance (CL_{int}) of a drug using liver microsomes and NADPH. The procedure involves incubation, kinetic analysis (ln (peak area) vs. Incubation time), and finally establishing an *in vitro-in vivo* correlation (IVIVC) for systemic clearance (CL_{sys}). This methodology predicts *in vivo* drug metabolism based on *in vitro* data.

CONCLUSION

In vitro assays of drug metabolites are powerful tools in drug discovery and development. They provide valuable information about the metabolic stability, pathways, and potential interactions of potential drug compounds tested under controlled conditions. By using systems such as liver microsomes, S9 fractions, or recombinant enzymes, researchers can predict *in vivo* metabolism, identify major metabolites, and assess safety concerns at an early stage. These assays are cost-effective, time-saving, and ethically preferable to extensive animal testing, while also reducing risks in human trials. However, *in vitro* test investigations should be correlated with *in vivo* studies for a complete understanding of drug metabolism.

ACKNOWLEDGEMENT

The authors are thankful to the management and Principal Dr. M. Ganga Raju of Gokaraju Rangaraju College of Pharmacy for providing facilities for this review work.

ABBREVIATIONS

CYP: Cytochrome P450; **UDP:** Uridine diphosphate; **TOF:** Time of Flight; **GC-MS:** Gas chromatography - mass spectrometry; **LC-MS:** Liquid chromatography-mass spectrometry; **LC-MS/MS:** Liquid chromatography tandem mass spectrometry; **HPLC:** High performance liquid chromatography; **UPLC:** Ultra-performance liquid chromatography; **MALDI-TOF:** Matrix assisted laser desorption ionization – time of flight; **cDNA:** Complimentary deoxyribonucleic acid; **HLM:** Human liver microsomes; **FMOs:** Flavin containing monooxygenases; **UGT:** UDP-glucuronosyltransferases; **SULTs:** Sulfotransferases; **GSTs:** Glutathione S-transferases; **NADPH:** Nicotinamide adenine dinucleotide phosphate; **PCLS:** Precision-Cut Liver Slices; **UDPGA:** UDP-glucuronic acid; **PAPS:** 3'-phosphoadenosine-5'-phosphosulfate; **GSH:** Glutathione; **UHPLC:** Ultra-High Performance Liquid Chromatography; **$NADP^+$:** Nicotinamide adenine dinucleotide phosphate (oxidized form).

CONFLICT OF INTEREST

We have no conflicts of interest related to this work. We affirm that the research, authorship and publication of this article have not been influenced by any personal or financial relationships. Kindly provide the competing interest if any or declare none.

ETHICAL STATEMENT

This article is a review and does not involve any original experiments or data collection with human participants, animals, or biological specimens. All information has been obtained from previously published studies. Therefore, ethical approval and informed consent were not required for this manuscript.

FINANCIAL SUPPORT AND SPONSORSHIP

The authors declare that there was no financial support, funding, or sponsorship received for the conduct, preparation, or publication of this review article.

AUTHOR CONTRIBUTIONS

Durga Pani Kumar Anumolu: Conceptualization, supervision, expert review of scientific content, and critical revision of the manuscript.

Bugga Veena: Lead author; major contributor to the study design, extensive literature review, data interpretation, drafting of the full manuscript, preparation of figures/tables, and final editing for intellectual content.

Syed Sara Afreen: Literature search, compilation of enzyme-related studies, and assistance in writing the methodology sections.

Jahnavi Bandla: Data organization, comparative analysis of *in vitro* assay techniques, and proofreading.

Chenchu Lakshmi K.N.V.: Technical evaluation of metabolizing enzyme studies and refinement of the discussion section.

Veera Shakar Pulusu: Final manuscript review, reference validation, and quality assurance.

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Cite this article: Anumolu DPK, Veena B, Afreen SS, Bandla J, Lakshmi KNVC, Pulusu VS. *In vitro* Models for Studying Drug Metabolites and Metabolizing Enzymes: A Comprehensive Review. Int. J. Pharm. Investigation. 2026;16(3):885-91.