Chapter 1

In Vitro Biotransformation in Drug Discovery

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Abstract

In vitro Biotransformation studies play a crucial role in drug discovery program that determine the fate of the new chemical entities (NCE's). Enzyme rich matrices such as microsomes, hepatocytes, liver fractions and S9 fractions transform the new chemical entities to different metabolites. Metabolites could be pharmacologically important or toxic. Newly formed metabolites are identified using liquid chromatography interfaced with mass spectrometry. Identification of the biotransformation sites in the new chemical entity helps the medicinal chemists to optimize its structure and develop the NCE as a pharmaceutical drug. Screening pharmaceutical drugs using *in vitro* biotransformation studies assist in selecting the right new chemical entity for further *in vivo* studies in animal systems and later in human clinical trials.

Keywords: *In vitro* biotransformation, metabolism, drug discovery, chromatography, mass spectrometry

1. Introduction

Organisms such as Enzymes, Bacteria and Fungi play a crucial role in the conversion of organic compounds to different products. This process of transformation is termed as biotransformation. In different fields of science, biotransformation has a significant impact. In the case of drug discovery and development, metabolism of drug to many different compounds is catalyzed by the enzymes in the liver [1, 2]. Similarly microorganisms in the gut are responsible for microbial biotransformation of organic compounds [3]. Most of the pharmaceutical drugs are organic moieties and undergo enzymatic biotransformation or microbial biotransformation. Therefore, *in vitro* biotransformation studies that mimics the actual *in vivo* system gains importance.

In vivo studies on various species are not economical and have restrictions; therefore *In vitro* biotransformation studies are vital in drug discovery programs [4]. Screening pharmaceutically important compounds using *in vitro* matrices such as microsomes, hepatocytes, liver slices and S9 fractions from different species directs the drug discovery team to make an appropriate decision to advance the molecules. Incubation of new chemical entities as well as pharmaceutical compounds with different *in vitro* matrices [5–8] results in metabolites. Metabolites could be pharmacologically important or it could be toxic [9].

Metabolites are identified and characterized using liquid chromatography and mass spectrometry (LC–MS/MS) [10]. *In vitro* biotransformation studies performed on various species guides the researchers to choose the right animal model to validate the developed molecule [11, 12]. Thus the *in vitro* biotransformation studies assist to identify and eliminate the false positives and make it possible to choose the relevant molecule for further *In Vivo* studies and later for clinical trials. Biotransformation of pharmaceutical drugs with different structural moieties results in different metabolite structures. Liquid chromatography assists in separation of the metabolites and drugs according to the polarity of the molecules. Liquid chromatography also provides a vital information to purify the drugs, metabolites and nano materials that serves as delivery systems for various biomedical applications [13, 14]. Mass Spectrometry resort to identify the mass of the drugs, metabolites and thus helpful to assign the biotransformation sites in the metabolites [10, 12].

2. *In vitro* metabolism of drugs by Microsomes

The important site of the body for drug metabolism is liver. Membrane bound drug metabolizing enzymes were present in liver microsomes as subcellular fractions. Hepatic CYP-450 enzymes were the reason for metabolism and clearance of more than 60% of marketed compounds [5, 15]. *In Vito* intrinsic clearance of a compound is determined using microsomes and interspecies differences in drug metabolism using species-specific microsomes. Commercially available microsomes for rat, mouse, dog and cyanomolgus monkey were used to understand the interspecies differences [11, 12].

Microsomal stability assays performed for a drug is used to calculate *in vitro* half-life ($T_{1/2}$) for a particular drug and also intrinsic clearance (CLint). Thus microsomes are ideal model systems to determine the metabolic stability, phase-I metabolism and intrinsic clearance of a compound that can be scaled to *in vivo* situation to predict human clearance [5]. Control compounds such as Verapamil, Diazepam, Diphenylhydramine, Quinidine and Dextromethorphan are used as matrices for human, rat, mouse, monkey and dog respectively to compare the deviation of test compounds. Both the control and test incubations were performed in the absence of NADPH (Nicotinamide Adenine Dinucleotide Phosphate) to identify the chemical stability of compounds or non enzyme mediated pathway and in the presence of NADPH to determine the enzyme mediated metabolism.

Microsomes were prepared, stored and used conveniently in comparison to whole cell models. Microsomes are generally used to screen hundreds of compounds in drug discovery and development and rank order the compounds. Compounds are rank ordered as high,

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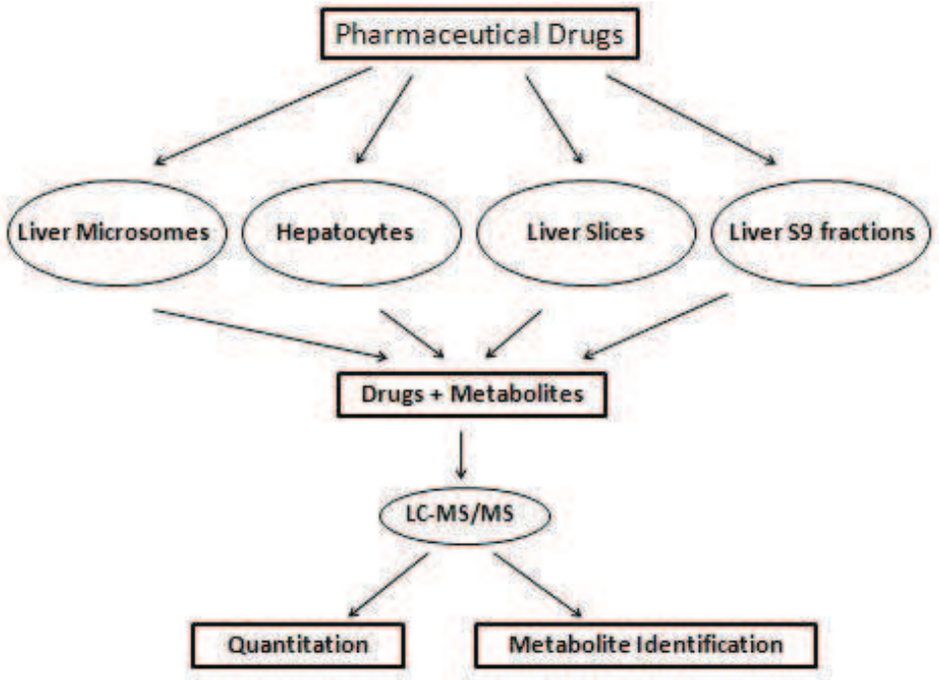

Figure 1. *In vitro* biotransformation scheme to screen pharmaceutical drugs.

medium and low clearance compounds. For example intrinsic clearance value of >47 μL/min/mg protein for a particular drug in human liver microsomes is considered to be high and intrinsic clearance value of <8.6 μL/min/mg protein is considered as low clearance. Intrinsic clearance value between 47 μL/min/mg protein and 8.6 μL/min/mg protein is considered to be moderate intrinsic clearance. Similarly for monkey, dog, rat and mouse high, medium and low clearance values were assigned based on the extensive studies done for many compounds [15, 16].

Predominantly microsomal stability studies are performed to assure the Phase-I metabolism in the presence of NADPH as cofactor (**Figure 1**). Besides this microsomes can also be utilized to understand the Phase-II metabolic pathway of a compound. Incubating the test compound in the presence of microsomes, NADPH and UDPGA (Uridine Diphosphate Glucoronic Acid) would reveal the possible glucoronide pathway. Similarly incubation of a parent drug with NADPH and Glutathione helps to identify the conjugation site for glutathione and its pathway.

3. *In vitro* metabolism of drugs by hepatocytes

Intact cell of hepatocytes consists of both Phase-I and Phase-II drug metabolizing enzymes. Hence, hepatocytes based studies to identify the intrinsic clearance of compound mimics *In Vivo* system to a greater extent than microsomes [16]. While, microsomes are rich in Phase-I enzymes, hepatocytes consists of both Phase-I and Phase-II enzymes. Quantity of Phase-I enzymes were abundant in microsomes and helps to detect the Phase-I metabolites with accuracy, but lacks Phase-II enzymes. Hepatocytes derived from various species such as rat, mouse, dog, monkey and human were imperative to understand the impact of species

differences in metabolism. Hepatocytes studies help to identify the species akin to that of humans and choose the model system for further studies of any drug [17]. Thus Human hepatocytes serve as a gold standard to understand the metabolism and toxicity of drugs.

Hepatocytes are cryopreserved and stored for longer duration of time. Cell viability and activity are better with cryopreserved hepatocytes and best alternatives for fresh cells. Presence of both phase-I and phase-II enzymes result in better assessment for clearance of drugs [18, 19]. Based on intrinsic clearance values compounds can be rank ordered as low, medium and high using hepatocytes for different species. Intrinsic clearance for humans range from <3.5 to >19.0 μL/min/10^6 cells, in the case of monkey <5.2 and >28.3 μL/min/10^6 cells; for dog <1.9 and >10.5 μL/min/10^6 cells; for rat <5.1 and >27.5 μL/min/10^6 cells and mouse <3.3 and 17.8 μL/min/10^6 cells.

4. Microsomes versus hepatocytes

Compounds that are metabolized by phase-II enzymes are best studied by hepataocytes, compounds whose primary metabolic pathway is through phase-I enzymes is best understood by microsomes (**Figure 2**). Microsomes are rich in CYP450 enzymes and therefore metabolic turnover is very high compared to that of hepatocytes. Compounds with the property of poor permeability through cell membranes are more stable in hepatocytes than microsomes. Identification of phase-I metabolites using microsomes are confirmatory while phase-II metabolite identification can be authenticated using hepatocytes. Quantities of enzymes are less in hepatocytes compared to microsomes resulting in less quantity of metabolites for hepatocytes. Hence, in order to confirm few of the phase-II metabolites such as glucuronidation and glutathione conjugation, pharmaceutical drugs are incubated with microsomes along with cofactors such as UDPGA or Glutathione. Incubation of microsomes with drugs and cofactors result in more quantity of metabolites and can be quantified accurately.

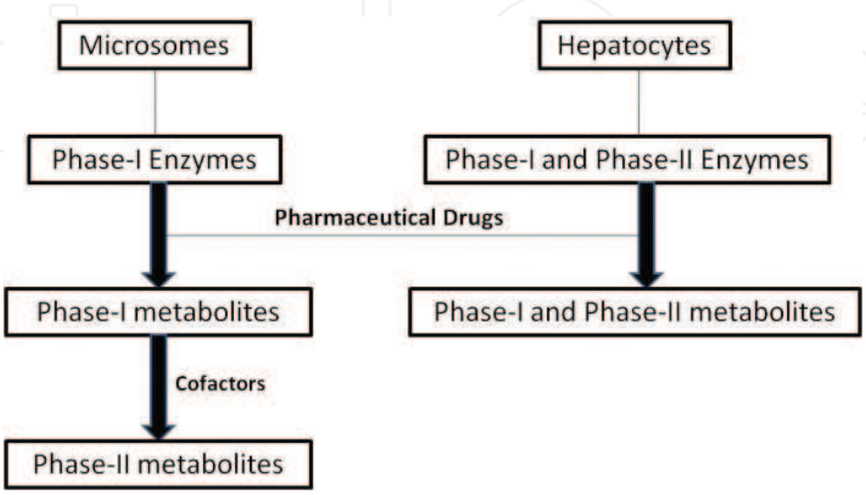

Figure 2. Comparison of outcome of microsomes and hepatocytes upon incubation with pharmaceutical drugs.

Thus both microsomes and hepatocytes are very much essential as a matrix to study the xenobiotics of pharmaceutical drugs. Prediction of clearance using hepatocytes is better and results are similar to that of *in vivo* systems [20, 21].

5. *In vitro* metabolism of drugs by liver slices

Metabolism plays a key role in detoxification of xenobiotics. Metabolic enzymes as well as transporter proteins that transfer the metabolites and parent drugs through the cells are essential for *in vitro* metabolism to mimic the *in vivo* metabolic systems [7]. Liver slices from human and various species are precious and valuable to conduct drug metabolism studies and acute toxicity studies [22, 23]. Metabolic enzyme activity of liver slices declines rapidly and restricts its prolonged use for drug metabolism studies. In spite of this inherent problem of liver slices pharmacological and toxicological studies have been performed and liver slices were proved to be one of the efficient model systems [23]. Precision cut liver slices consists of all liver cell types that are present in natural cells with cell to matrix and cell to cell interactions, thus representing actual liver functions [24]. Thus precision cut liver slices helps to recognize and identify the mechanisms for exposure in humans. Most of the precision cut liver slices with active enzyme components and cells are viable for not more than 2 days [25, 26]. Therefore modifications of culture conditions such as medium composition were attempted in recent years [27] to extend the viability of precision cut liver slices to 5 days with promising results.

6. *In vitro* metabolism of drugs by liver S9 fractions

Liver S9 fractions are rich in both microsomal and cytosolic fractions. Optimum metabolic information about a compound is obtained from liver S9 fractions than microsomes. Liver S9 fractions consist of both microsomal and cytosolic fractions and corresponding enzymes, whereas microsomes have only microsomal proteins. Isolation of liver S9 fractions is uncomplicated and obtained during the initial stages of microsomal preparations [28, 29]. Major components of cytosolic S9 fractions are cytochrome P450's, Uridine 5′-diphospho-glucuronosyltransferase, aldehyde oxidase, xanthine oxidase, sulfotransferases, methyl transferases, N-acetyl transferases, glutathione transferases and represents the *in vivo* system to a greater extend. Microsomes consists of CYP 450's and Uridine 5′-diphospho-glucuronosyltransferase and lack other enzymes that are present in S9 fractions [30]. Similar to microsomes liver S9 fractions need cofactors such as β-Nicotinamide adenine dinucleotide phosphate-regenerating system (for oxidation), Uridine 5′-diphospho-α-D-glucuronic acid (glucuronidation), 3′-phosphoadenosine-5′-phosphosulphate (Sulfate conjugation) and glutathione (glutathione conjugation) for phase-I and phase-II assays. Hepatocytes do not require any of these cofactors that are used during microsomal or liver S9 fractions based assays [31]. Human hepatocytes are considered to be golden standard and 7-ethoxy coumarin has been used as an appropriate substrate to understand the phase-I and phase-II metabolism [31] in different matrices. Recently liver S9 fractions were shown as a matrix comparable to that

of hepatocytes to screen compounds for metabolic stability assays [32]. Researchers have used 7-ethoxy coumarin and performed a comparative study on three different matrices i.e. microsomes, hepatocytes and liver S9 fractions. Phase-I metabolite 7-hyroxy coumarin was observed in all the three matrices, whereas glucuronide and sulfate conjugates were observed for hepatocyte and liver S9 fractions [33]. Therefore activities of liver S9 fractions are more akin to hepatocytes than that of microsomes.

7. Purified CYP enzymes for reaction phenotyping

Compounds that exhibit medium or high clearance in microsomes and hepatocytes assays are subjected to reaction phenotyping studies.

Expressed enzymes or purified CYP enzymes are used to identify the enzyme responsible for the metabolism of a particular compound and is termed as reaction phenotyping [34]. CYP enzymes CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 are primarily responsible for phase-I metabolism of most of the drugs. If phase-II metabolite glucuronide conjugate is expected, enzymes UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7 and UGT2B15 need to screened. Thus the above mentioned purified enzymes need to be incubated with a particular drug to identify the percentage contribution of each enzyme for the metabolism of a drug. During the reaction phenotyping assay positive controls are tested for each purified enzyme. Ethoxycoumarin is a positive control for CYP1A2; Amodiaquine for CYP2C8; Diclofenac for CYP2C9; Diazepam for CYP2C19, Dextromethorphan for CYP2D6; Testosterone for CYP3A4 and Efavirenz for CYP2B6. There are instances where a particular drug can result in different metabolites. Formation of each metabolite can be from same enzyme or due to different enzymes or due to the contribution of mixture of enzymes. Hence reaction phenotyping is essential to identify and confirm the enzyme responsible for the formation of particular metabolite [35]. Knowledge from reaction phenotyping helps to suppress or enhance the formation of a particular metabolite using inhibitors or enhancers. In case a particular metabolite is expected to be toxic in expected quantity, then the formation of that metabolite can be suppressed using inhibitors. Reaction phenotyping studies will also help the medicinal chemist to design a synthetic strategy to avoid the formation of a toxic metabolite.

Thus the information derived from reaction phenotyping studies can be utilized to (1) predict drug–drug interactions with the coadministered drugs and (2) metabolism of a drug by an isoform can lead to increase or decrease in the concentration of drugs, metabolites in plasma and therefore difficult to determine the therapeutic range of a compound (3) helps medicinal chemist to modify the structure of drug or pharmacologist to use an inhibitor or enhancer to address the safety of the drug (4) Generally compounds which exhibit high or medium clearance in the microsomal or hepatocyte stability assays are subjected to reaction phenotyping assays.

Food and Drug Administration also highly recommends *in vitro* studies to understand the systemic clearance and also to determine the responsible CYP450 or UDPGA enzyme for the metabolism of new investigational drug.

8. CYP450 time dependent inhibition assay

Drug-drug interactions is caused by the inhibition of CYP450 enzymes. Inhibition is of three types (1) irreversible (2) Quasi reversible and (3) reversible. Irreversible CYP450 inhibition reactions are of major concern compared to that of reversible CYP450 inhibition, because synthesis of inactivated enzyme is essential to restore the activity of deactivated enzymes. Hence it is mandatory to understand the mechanism of new chemical entities at an early stage of the drug discovery and development [36]. Two types of time dependent inhibition are generally referred as mechanism based inhibition (MBI) and time dependent inhibition (TDI). Mechanism based inhibition is due to the inhibition of enzyme by chemically derived metabolite. Mechanism based inhibition is classified as a subset of time dependent inhibition. Time dependent inhibition arises when there is an increased inhibition during the incubation of test compound with metabolizing system prior to the addition of the substrate. Available CYP isoforms such as CYP3A4, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP2D6 are generally tested for CYP inhibition. The substrate used for CYP3A4 inhibition is midazolam and control compound is mifepristone; substrate for CYP1A2 is ethoxyresorufin and positive control compound is furafylline; substrate for CYP2B6 is bupropion and positive control compound is ticlopidine; substrate for CYP2C8 is paclitaxel and control compound is gemfibrozil; substrate for CYP2C9 is diclofenac and control compounds is tienilic acid; substrate for CYP2C19 is S-mephenytoin and control compound is ticlopidine and substrate for CYP2D6 is dextromethorphan and control compound is paraxetine. These control compounds are inhibitors which are selected by screening few time dependent inhibitors. For example probe substrate of CYP3A4 midazolam can be subjected to inhibition studies using time dependent inhibitors like clarithromycin, verapamil, troglitazone, mfepristone and mibefradil. The inhibitor that exhibits maximum inhibition is chosen as the inhibitor for a CYP isoform. During the CYP inhibition experiments quantity of substrate, inhibitor and formation of metabolites is analyzed using liquid chromatography interfaced with mass spectrometry [37].

9. LC-MS/MS and *In vitro* biotransformation studies

Accurate bioanalytical methods are essential in drug discovery to assess the concentrations of drugs and metabolites. Pharmacokinetic investigations, toxicokinetic analysis and several *in vitro* studies such as metabolite identification, metabolic stability, caco-2 permeability studies, drug–drug interactions and protein binding studies are extensively studied using liquid chromatography hyphenated with mass spectrometry. Therefore, high throughput screening to develop methods is challenging and time dependent.

Chromatography and Mass Spectrometry plays a major role in determining the concentrations of drugs and metabolites at the levels of attomole or femtomole [38]. The science of separation achieved through chromatography is very important to separate the drugs and metabolites based on the polarity of the molecules. Advent of high performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC) resulted in analyzing samples

in shorter period of chromatography run time (1–5 min). Sensitive mass spectrometers such as triple quadrupole mass spectrometers, quadrupole ion trap mass spectrometers, orbi trap mass spectrometers, time-of-flight mass spectrometers have accelerated the detection of drugs and metabolites at very low quantity such as attomole levels and to identify the structure of drugs and metabolites. Thus chromatography hyphenated with mass spectrometers were supportive to address the LADMET (liberation, absorption, distribution, metabolism, excretion and toxicity) related issues in drug discovery programs [39]. Quantitation by chromatography and mass spectrometry is important to assess the liberation, absorption, distribution, excretion and toxicity of a drug molecule and its metabolites.

10. Quantitation of drugs and metabolites

Efficient sample preparation methods are essential for effective quantitative analysis of drugs and metabolites. Recovery of drugs and metabolites in high amount is an important factor in this process. Several methods such as liquid–liquid extraction, solid–liquid extraction, use of sep-pak cartridges, elution of drugs and metabolites through small columns packed with silica or C-18 were used to purify the samples. Purification of samples removes the matrices and other unwanted impurities resulting in ideal samples for bioanalysis by chromatography and mass spectrometry. Precipitation methods using centrifuges, precipitation using solvents were also employed to purify the samples. Depending on the nature of the drugs and metabolites one or more of the above process has to be practiced for samples with high purity [40]. Highly pure samples result in reproducible quantification results. Along with purification of samples, choosing an appropriate internal standard is also essential for quantification of drugs and metabolites. The chosen internal standard should have structure similar to that of parent drug and should not interfere with parent drug during analysis. For ex internal standards should have different retention time and molecular weight compared to the drugs and metabolites. Thus chosen internal standards can be distinguished from parent drugs by LC–MS/MS during the analysis. Different retention time in chromatography and different multiple reaction monitoring (MRM) transitions in mass spectrometry for internal standards with respect to that of parent drugs is essential for accurate quantitation of drugs and metabolites. Thus the separation power of chromatography and sensitivity of mass spectrometers are an added advantage for a pharmacokineticist and toxicokineticist to determine the fate of new chemical entities (NCE's) [41].

11. Identification of metabolites

Biotransformation of pharmaceutical drugs results in metabolites. Structural identification of metabolites by liquid chromatography and mass spectrometry confirm the metabolic softspots and hotspots [42, 9]. Metabolic softspots involve usual biotransformation pathways such as oxidation, reduction, hydrogenation, dehydrogenation, hydroxylation, dehydroxylation, loss of a functional group, oxidative dehalogenation, epoxidation, decarboxylation, hydration etc. [4].

These are the major phase-I biotransformation along with few other unusual metabolic pathways. Apart from phase-I metabolites, conjugation reactions leads to phase-II metabolites such as glucuronidation, sulfation, glutathione formation, methylation, glycine conjugation, and taurine conjugation [4]. Identification of the structure of phase-I and phase-II metabolites and its quantity is essential to understand the elimination of drugs, metabolites and residence time of drugs, metabolites in human system. LC–MS/MS plays a crucial role in rapid identification of the metabolic soft spots and hot spots and directs synthetic chemists to make appropriate modifications in the drug moiety [43, 44].

12. Conclusions

In vitro biotransformation of pharmaceutical drugs utilizing matrices viz. microsomes, hepatocytes, liver slices or liver S9 fractions results in various metabolites. Each matrix has its advantages and shortcomings. Therefore, all the matrices are essential to identify, confirm and quantify the metabolites. During the preclinical pharmaceutical candidate optimization, screening the pharmaceutical drugs using various matrices helps bioanalytical and biotransformation scientists to identify the metabolic spots of the molecules with the aid of chromatography and mass spectrometry. Identification of metabolic spots at the early stage is beneficial for medicinal chemists to design and alter the structure of pharmaceutical drug for better potency and accelerate the drug discovery and development process.

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Conflict of interest

None declared.

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