Review Article

In vitro test methods for metabolite identification: A review

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Abstract

Metabolite identification and profiling are relevant in several stages of drug discovery & development. Studies modelling both the human and experimental animal metabolism of a drug are useful in the design of toxicological studies. An imperative issue in toxicology is the suitability of the information obtained with experimental animals for human risk assessment. However, the data obtained in animal studies can be better extrapolated to the patient by utilizing bridging studies with invitro models of human drug metabolism. There are two basic categories of in vitro methods for the examination of human liver drug metabolism. The first group of invitro methods consists of the cellular models, which include primary hepatocytes, liver slices, and cell lines. The second group is the utilization of preparations of the drug metabolizing enzymes such as supersomes, cytochrome P450, cytosolic fraction, S9 fraction, human liver microsomes and rat liver microsomes. Invitro studies can identify the species specific metabolic routes, and the experimental animal models that best reflects the potential human exposure to the drug and its metabolites. In this review, detailed procedures, advantages, disadvantages and applications of various invitro metabolite identification methods have been discussed.

Keywords: Metabolite identification, drug discovery & development, toxicology, cellular models, metabolizing enzymes

Introduction

The development of a new therapeutic agent always involves a preclinical screening stage which involves the investigation of the main pharmacokinetic, pharmacodynamic, and toxicological properties of the drug. The preclinical investigation is based on both in vitro models and in vivo experiments in various animal species (Koster et al., 1997). In vitro drug metabolism studies using models of human drug metabolism that are performed using in vitro comparative studies, utilizing tissue or enzymes from human and experimental species provides the better extrapolation of the risk assessment information obtained in the toxicology studies to the patient. As a result of the ever increasing regulation of the pharmaceutical industry, the process of developing drug is increasing in cost and time. Studies using human tissue or enzymes in vitro can be utilized in a cost-effective and timely manner to target or guide the clinical studies. For example, in vitro studies can examine potential drug-drug interactions and the effect of environmental agents, ethnic background, gender, and genetics on the metabolism of a drug.

Biotransformation occurs in many tissues, with the liver as the most important organ, but also the kidneys, skin, lungs, and intestine can be involved. The in vitro methods available to study human hepatic drug metabolism can be divided into 2 categories. The first broad categories composed of cellular systems. Included in this group are primary hepatocytes in suspension or monolayer culture, liver slice culture and hepatocyte derived cells lines. The second category is composed of preparations of the drug-metabolizing enzymes. Included in this group are the use of subcellular fractions such as microsomal and cytosolic fractions and the use of isolated enzyme preparations that can be obtained by purification techniques or more easily through heterologous complementary DNA (cDNA) expression system (Steven et al., 1995).

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The in vitro systems can be used to compare the profile of metabolites formed by the different species, which may be useful in explaining species-specific toxic or pharmacologic effects of a drug. The effect of the drug directly on the hepatocyte can be examined with the cellular systems. Finally, the information obtained with the in vitro systems can aid in the rational and justifiable species selection for toxicological testing. The information obtained from in vitro studies can also be used to support the clinical development of a drug candidate. Specifically, the in vitro systems can be used to identify human metabolites of a drug candidate, which then can be compared to those predicted by the studies with the experimental species, thus assuring that the animals were exposed not only to the parent compound but also to the major human metabolites. Furthermore, the human enzyme(s) responsible for the metabolic clearance of the drug candidate can be determined (Wrighton et al., 1992).

Metabolities

Metabolites are intermediate results of metabolic responses catalysed by different enzymes that normally occur inside cells. Some examples of human metabolites include arachidonic acid, inosine-5'-monophosphate, steroid harmones, catecholamines such as norepinephrine or dopamine. A greater part of the drug is disposed off from the body by metabolism through detoxification process. The metabolites adjust the efficacy of drugs in the treatment of disease. The metabolites may have pharmacological action and might be toxic (N-acetyl-p-benzoquinone imine (NAPQI), a metabolite of paracetamol when taken in overdoses). Metabolites may provide lead to new and more complex drugs. Metabolite identification occurs once the compound is selected for drug development. Because of the toxicity of the drug metabolites, drug metabolite identification is much more serious and carefully monitored and the metabolite identification investigations are very significant in the early periods of drug selection.

Metabolite identification

Identification certainty can change widely because the process of metabolite identification is intricate and depends on the analytical stage and robustness of the techniques applied, and in addition the databases and assets utilized. Identification of drug metabolites is typically conducted using a radiolabelled version of parent drug. Different estimations such as the percentage of the dose found in urine and faeces can be calculated. Radiometric, chromatographic and spectroscopic methods (HPLC/UPLC, LC-MS, LC-MS/MS, GC-MS, capillary electrophoresis for oligonucleotides, TOF, MALDI-TOF, etc.,) are then used to determine the molecular identity of the radioactive compounds present in the biological samples, permitting the derivation of more specific information for each metabolite (Sofia et al., 2007). The various approaches to invitro metabolite identification are discussed below.

Cell lines

Cell lines are used for drug metabolism studies only in enzyme induced state and in cytotoxicity studies of drug and its metabolite. This in vitro model is less popular than other described models due to de-differentiated cellular characteristic and lack of complete expression of all families of metabolic enzymes. The sources of cell lines are primary tumours of liver parenchyma. Currently available cell lines are Hep G2, Hep 3B, SNU-398, SNU-449, SNU-182 and SNU-475 (from hepatocellular carcinoma), BC2 and PLC/PRF/5 (from hepatoma), C3A (hepatoblastoma). These are easy to culture and have stable enzyme concentration relatively and among them Hep G2 cell line with cytochrome P (CYPs) 1A, CYP 3A and uridine diphosphoglucuronosyl transferase (UGT) is most frequently used for biotransformation studies (Esther et al., 2003). The metabolic activity of cell line is generally low compared to freshly isolated human hepatocytes. Metabolic activity of some metabolic enzyme is not detected and this problem of low activity could be partly overcome by the pre-treatment of cell lines by inducers of various metabolic enzymes. But still the induced activity is below the enzymatic activity in freshly isolated human hepatocytes. Liver cell lines require appropriate culture medium whose composition significantly influences the metabolic activity. On the other hand, the absence or low expression of most important phase I and phase II drug metabolizing enzymes limit the application of this in vitro model. Moreover, metabolites are not easily detected and it is difficult to investigate individual CYPs or other enzymes and metabolites in cell lines.

Method: Cells can be isolated from the liver tumor of patients suffering from hepatocarcinoma and hepatitis C infection (Philippe et al., 2002). The samples are minced into small pieces, washed with Hepes buffer (pH 7.7; 140 mM NaCl/2.68 mM KCl/0.2 mM Na_2HPO_4/10 mM Hepes), and digested with 0.025% collagenase D diluted in the same buffer supplemented with 0.075% $CaCl_2$ under gentle stirring at 37°C. The cell suspension is washed twice in Hepes buffer and resuspended in a William's E medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, and 5×10^{-7} M hydrocortisone hemisuccinate. Cell suspension is distributed in several dishes without any coating feeder layer. After several weeks, cell growth is sufficient to fulfil the culture dishes. Cells appear well differentiated, with a hepatocyte like morphology. Dishes

having the most homogenous cell population are passaged by gentle trypsinization. After three passages, all cells are aliquot and frozen in 10% DMSO and kept in liquid nitrogen vapours. After thawing, cells originating from one single dish showing a high proportion of cells with a hepatocyte morphology are further passaged in the culture medium used for their isolation. The selection procedure relies on two main steps: (i) Induction of cell differentiation with DMSO, and (ii) Partial purification of the differentiated cells.

Induction of cell differentiation is performed by treating a dish containing confluent cells with the culture medium supplemented with 2% DMSO and hydrocortisone 5×10^{-5}M for 4 weeks. The differentiated cells, showing a typical aspect of hepatocytes clustered in small colonies, are then selectively isolated from non differentiated cells. The procedure includes a brief trypsinization which allows the hepatocyte like cells to remain aggregated, whereas non differentiated cells are mostly present as single cells. To this end, the cell culture is washed with a phosphate saline buffer and then incubated with a commercial trypsin-EDTA solution at 37°C. When most cells are detached from the substratum, 25% FCS is added to neutralize trypsin activity. Cells are then gently collected and allowed to sediment for 10 minutes at 1×g. The pellet consisting mostly of aggregated cells is resuspended, and the sedimentation procedure is repeated once. Cells selected by this method are plated in new dishes. Cells are passaged every 2 weeks (1/5 dilution) by trypsinization. The phenotypic stability of the cell line is greatly favoured by continuously culturing cells in the presence of a high hydrocortisone concentration (5×10^{-5} M). Therefore, the growth medium is composed of William's E medium supplemented with 10% FCS, 100 units/ml penicillin,100 µg/ml streptomycin, 5µg/ml insulin, and 5×10^{-5}M hydrocortisone hemisuccinate. For the routine differentiation process, a two-step procedure is used. Cells are first maintained for 2 weeks in growth medium. Thus, they are maintained in the differentiation medium (the same culture medium supplemented with 2% DMSO) for two more weeks. The medium is renewed every 2 or 3 days.

Applications

1. Croft et al. (1992) suggested an invitro model for screening antileishmanial drugs in the human leukaemia monocyte cell line, THp-1.

2. Meussen-Elholm et al. (2004) reported inhibition of cell growth in the human breast cancer cell line MCF7 by antiepileptic drugs.

3. Pesic et al. (2006) studied induced resistance in the human non-small cell lung carcinoma (NCI-H460) cell line in vitro by anticancer drugs.

Transgenic cell line

Another approach to obtain a cell line expressing phase I and/or phase II enzymes is the recombinant expression of the human enzyme in a cell line. At present all known human CYPs involved in drug biotransformation have successfully been over-expressed in cells and these cell lines are available for research. Cell lines are transfected at high efficiency using protoplast fusion, centrifugation of lysozyme-treated bacteria bearing the desired vector with parent cells in the presence of polyethylene glycol. Crespi et al. (1993) achieved the first stable expression of human CYPs in a human cell line. These cell lines are used to generate metabolites for structure elucidation, pharmacological characterization and to assess drug-drug interactions.

Advantages are the ease of culturing, high expression of CYP and UGT isoenzymes, possibility to study single enzyme reactions and the influence of one isoenzyme or a combination of number of isoenzymes.

Disadvantage is that only one or a few of isoenzymes are expressed, therefore the complete in vivo situation cannot be reflected. Moreover, transgenic cell lines are more expensive than other enzyme-based technology.

Applications

1. Gene overexpression

2. Promoter characterization and cell lineage markers

3. Gene knockdown by RNA interference

4. Complementation and mutation mapping

5. Double or multiple transgenes

6. Site-specific recombinase and conditional transgenic lines

7. Inducible transgene expression (Liu C, 2013).

Liver slices

The incubation of liver slices in nutrient-rich media is another tool to study metabolism in vitro. Owing to impaired diffusion of nutrients and oxygen in the liver slices, the duration of activity of CYP is short. There are no commercially available human liver slices yet. There is possibility for studying the induction of CYP isoforms by new drugs.

Its disadvantages include inadequate penetration of culture medium into the slices and short viability time period of 5 days.

Method

Albino rats of the Wistar strain (maintained on a diet of vitamin-enriched rat-bread or on MRC diet 41 B) may be killed by decapitation. Rats of either sex, usually weighing about 250g, are starved overnight before use. Tissue slices are prepared and incubated essentially as described by

Hultin et al. (1962) except that Krebs ringer phosphate medium may sometimes be used instead of the bicarbonate medium. At the end of the incubation period the vessels are plunged into ice-cold water and the medium is removed from the slices and discarded. The slices are washed twice with ice-cold medium containing a large excess of non- radioactive dimethyl nitrosamine and are disintegrated in water, again containing excess of the unlabelled compound, in a homogenizer of the type described by Potter et al. (1936). An equal volume of 10 % w/v trichloroacetic acid is then added to the tissue suspension with stirring and the precipitate is centrifuged down. In some experiments the supernatant acid-soluble fraction is retained for isolation of formaldehyde.

Applications

1. Montesano et al. (1970) reported the metabolism of dimethyl nitrosamine by human liver slices in vitro.

2. James et al. (1997) proposed liver slices as a reliable in vitro model for studying the metabolism of a wide variety of structurally diverse chemicals.

3. Viktoriia et al. (2017) reported the maintenance of drug metabolism and transport functions in human precision-cut liver slices during prolonged incubation for 5 days.

Recombinant human CYP and UGT enzymes (supersomes/baculosomes)

Insect cells lack endogenous cytochrome P450 (CYP) and UGT activity. Therefore, microsomes, which consist of vesicles of the hepatocyte endoplasmic reticulum, of human CYP or UGT transfected insect cells can be a valuable tool in human biotransformation studies. Since the expression is baculovirus mediated, microsomes of these cells are sometimes referred to as baculosomes, but more often as supersomes. The availability of specifically expressed human CYPs and UGTs in supersomes allows the investigation of the contribution of a single metabolic enzyme to the biotransformation pathway of the compound under investigation. At present all common human CYPs, co-expressed with NADPH-cytochrome P450 reductase and optionally cytochrome B5, and UGTs are offered in supersomes. A control experiment, incubation with non-transfected supersomes, must always be conducted. A NADPH-regenerating system (which consists of β-NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase) or NADPH is required to supply the energy demand of the CYPs. For the UGT activity, uridine diphosphoglucuronic acid (UDPGA) has to be added as cofactor. The specific CYP and UGT activity can be measured with various model substrates like midazolam Cl'-hydroxylation for CYP3A4 and estradiol 3-glucuronidation for UGT1A (Asha and Vidyavathi, 2010). They are valuable supplement to human liver microsomes and therefore it is likely that their application will increase in the future.

Advantage is that enzyme activity of one single CYP and UGT isoform is expressed and in this way the evaluation of individual metabolic enzyme and its commitment to the metabolic pathway could be performed. Additionally, this in vitro system could be utilized for the evaluation of drug-drug interactions. Moreover, due to availability of supersomes with different CYP and UGT genotypes, the influence of different polymorphisms on drug biotransformation pattern could be estimated (Yao et al., 2001).

Disadvantage is that, in UGT supersomes, the UGT active site is shielded behind a hydrophobic barrier resulting in latency of glucuronidations. However, this disadvantage can be overcome by using a pore forming agent, e.g. alamethicin.

Applications

1. Anderson et al. (2002) explained the sex differences in drug metabolism using cytochrome P-450 and uridine diphosphate glucuronosyltransferase.

2. Kim et al. (2008) identified the human cytochrome P450 enzymes involved in the metabolism of IN-1130, a novel activin receptor-like kinase-5 (ALK5) inhibitor.

3. Sylvie et al. (2014) identified the metabolic pathways and enzyme systems involved in the in vitro human hepatic metabolism of dronedarone, a potent new oral antiarrhythmic drug using supersomes.

Cytochrome P450 inhibition

The cytochrome P450s (CYPs) constitute a superfamily of isoforms that play an important role in the oxidative metabolism of drugs. Each CYP isoform possesses a characteristic broad spectrum of catalytic activities of substrates. Whenever 2 or more drugs are administered concurrently, the possibility of drug interactions exists. The ability of a single CYP to metabolise multiple substrates is responsible for a large number of documented drug interactions associated with CYP inhibition. In addition, drug interactions can also occur as a result of the induction of several human CYPs following long term treatment.

Mechanism: The mechanism of CYP inhibition can be divvied into 3 categories: (a) reversible inhibition; (b) quasi-irreversible inhibition; and (c) irreversible inhibition. In mechanistic terms, reversible interactions arise as a result of completion at the CYP active site and probably involve on the first step of the CYP catalytic cycle. On the other hand, drugs that act during and subsequent to the oxygen transfer step are generally irreversible or quasi-irreversible inhibitors. Irreversible and quasi-irreversible inhibition require at least one cycle of the CYP catalytic process.

Because human liver samples and recombinant human CYPs are now readily available, in vitro systems have been used as screening tools to predict the potential for in vivo drug interactions. Although it is easy to determine in vitro metabolic drug interactions, the proper interpretation and extrapolation of in vitro interaction data to in vivo situations require a good understating of the pharmacokinetic principles. From the view point of drug therapy, to avoid potential drug-drug interactions, it is desirable to develop a new drug candidate that is not a potent CYP inhibitor or inducer and the metabolism of which is not readily inhibited by other drugs. In reality, drug interaction by mutual inhibition between drugs is almost inevitable, because CYP-medicated metabolism represents a major route of elimination of many drugs, which can compete for the same CYP enzyme.

The clinical significance of a metabolic drug interaction depends on the magnitude of the change in the concentration of active species (parent drug/ or active metabolites) at the site of pharmacological action and the therapeutic index of the drug. The smaller the difference between toxic and effective concentration, the greater the likelihood that a drug interaction will have serious clinical consequences. Thus, careful evaluation of potential drug interactions of a new drug candidate during the early stage of drug development is essential (Lin and Lu, 1998).

Method: CYP isoform specific substrates are incubated with human liver microsomes at a single concentration or at a range of test compound concentrations (typically 0.1-50 µM). At the end of the incubation, the amount of parent remaining relative to each substrate is monitored by LC-MS/MS at each of the test compound concentrations. For IC_{50} value determinations the substrate is incubated at concentrations below its K_M. For K_i determinations, both the substrate and inhibitor concentrations are varied to cover ranges above and below the drug's K_M and inhibitor K_i. Each compound is analysed by reversed phase HPLC and the MS detection is performed. The amount of parent compound is determined on the basis of the peak area ratio.

Applications

1. Baldwin et al. (1999) performed the invitro metabolism of rosiglitazone, a potential oral antidiabetic agent for the treatment of type 2 diabetes-mellitus using human cytochrome P450 enzyme(s).

2. Ashton et al. (1999) identified that the human CYP450 enzymes involved in the invitro metabolism of artemisinin.

3. Kanayama et al. (2007) studied drug-drug interactions in the metabolism of imidafenacin. They explained the role of human cytochrome P450 enzymes, UDP-glucuronic acid tranferases and potential of imidafenacin to inhibit human cytochrome P450 enzymes.

Cytosolic fraction

Cytosolic fraction is an in vitro model that has not been utilized very often. Cytosol is produced by differential centrifugation of whole liver homogenate. Solvent enzymes of phase II, such as N-acetyltransferases (NAT), Glutathione S-transferase (GST), sulfotransferases (SULT), carboxylesterase, soluble epoxide hydrolase, diamine oxidase, xanthine oxidase and alcohol dehydrogenase are expressed in cytosolic fraction, however just initial three are expressed at higher concentration. This in vitro model requires cofactors like acetyl coA, dithiothreitol and acetyl coA-regenerating system for NAT, three-prime-phosphoadenosine 5-prime-phosphosulfate (PAPS) for SULT, glutathione for GST activity.

Advantage is the presence of aforementioned enzymes at higher concentrations in cytosolic fraction compared to human liver S9 fraction. The biotransformation by NAT, GST or SULT can be examined independently or in combination depending upon the cofactors added (Robert et al., 2012).

Disadvantage is the absence of UGT and therefore glucuronidations cannot be contemplated by this model.

Method: Cells (up to 5 x 10^6) are collected by centrifugation for 5 minutes at 4°C and washed once with ice cold phosphate buffer saline (PBS). The supernatant is removed and discarded. Cell pellet is resuspended with 500 µl of ice cold, IX cytosol extraction buffer (containing dithiothreitol (DTT) / protease inhibitors) by pipetting up and down. Suspension is transferred into a pre-chilled micro centrifuge tube, incubated for 10 minutes, 25 µl of cell lysis reagents are added and vortexed for 10 seconds at the highest setting. It is centrifuged for 10 minutes at 4°C and supernatant is then transferred carefully to a clean, chilled micro centrifuge tube. Cytoplasmic fraction can be stored at -80°C for future use.

Applications

1. Arilla et al. (1984) reported the characterization of somatostatin binding sites in cytosolic fraction of rat intestinal mucosa.

2. Miao et al. (2005) reported the characterization of a novel metabolite intermediate of ziprasidone in hepatic cytosolic fractions of rat, dog, and human by ESI-MS/MS, hydrogen/deuterium exchange, and chemical derivatization.

3. Cytosolic fractions are used to study few phase-II metabolism reactions such as sulfonation, glutathione conjugation and acetylation.

S9 fractions

The S9 fraction is the product of an organ tissue homogenate used in biological assays that measure the metabolism of

drugs and other xenobiotics. Liver S9 fractions have been used since the 1970s, but not as widely as microsomes. The liver S9 fraction contains both microsomal and cytosolic fractions and consequently expresses a wide range of metabolic enzymes – CYP, flavin-containing monooxygenase (FMO), carboxylesterases, epoxide hydrolases, glucuronosyltransferase (UGT), SULT, methyl transferases, acetyltransferases, glutathione S transferase (GST) and others. This in vitro model could be utilized for metabolic, toxicity and mutagenicity studies. Exogenous cofactor, NADPH-regenerating system is supplied to meet the energy demand of the CYP enzymes. These are useful in the study of xenobiotic metabolism and drug interactions which represent the post-mitochondrial supernatant fraction from homogenized liver. They offer a more complete representation of the metabolic profile because they contain both phase I (cytochrome P450 isoforms) and phase II (transferases) activities. In some cases, metabolites which are not produced by either the cytosolic fraction or the microsomal fraction alone are formed with S9 fractions. As S9 fractions contain lower enzyme activity when compared to cytosol or microsomes, it may leave unnoticed metabolites.

Advantage over microsomes and cytosolic fraction is a more complete representation of the metabolic profile due to the presence of phase I and phase II enzymes.

Disadvantage is overall lower enzyme activity in the S9 fraction compared to microsomes and cytosol, which may leave some metabolites unnoticed.

Method: Richardson et al. (2016) used both human (gender pooled, 10 individuals) and rat (Sprague dawley; male; pooled) liver S9 fractions. Several phase I and phase II enzymes retain their activity, when stored at -70°C for up to 10 years and up to 10 freeze/thaw cycles. The incubation conditions are developed by using commercial compounds like 7-ethoxycoumarin (7-EC), diclofenac, 4-nitrocatecal, and phenolphthalein with known metabolic profiles (both phase I and II metabolisms). The rat and human S9 protein concentration and the cofactor concentrations are optimized to match the results of hepatocyte stability, quantitively.

The phase I and phase II metabolites; 7-hydroxycoumarin (7-HC), 7-HC sulphate, 7-HC glucuronide, 4-hydroxydiclofenac (4-HD), 4-HD glucuronide, diclofenac acyl glucuronide, 4-nitrocatechol sulphate and phenylalanine glucuronide are monitored, the identity of each of the metabolite is assessed by comparison of their retention time and mass spectra with those of authentic standards. Each new lot of S9 is passed through this optimization process before being used for high throughput compound screening in the discovery drug metabolism and pharmacokinetics (DMPK) flowchart. A cocktail of four activation cofactors may be used in order to stimulate phase I (NADPH) and phase II (UDPGA, PAPS, Glutathione (GSH)) metabolism. NADPH, UDPGA, GSH at concentrations of 1, 0.5, and 2.5 mM, respectively with 0.05 mg/mL of PAPS are commonly used.

Tris buffer (200 mM solution) containing 2 mM magnesium chloride (included $MgCl_2$ as a source for Mg^{+2} ions to stimulate CYP activity) in deionised water, adjusted with 1 M NaOH to pH 7.4 can be used. Stock reference solutions (7-EC as the positive control) and the test compounds are prepared at 5 mM concentrations in DMSO, and then diluted to 0.3 mM with ACN prior to use. NADPH, UDPGA, and GSH solutions at 40, 20 and 2 mM respectively and PAPS at 2 mg/ml are prepared in tris buffer prior to mixing together in a 1:1:1:1 ratio for use. S9's should be pre-incubated with test compound for 5 minutes at 37°C in tris buffer, pH 7.4 and then the reaction is initiated by adding the cofactor mixture. At two time points, zero and sixty minutes, aliquots of the sample mixture are removed and quenched by addition of two volumes of ice cold 50:50 ACN: MeOH. The plate of quenched sample is centrifuged at 4000g for 10 minutes to sediment the precipitated protein before injection onto LC-MS/MS for analysis of parent compound remaining. Percent of the parent compound remaining is calculated by comparing peak areas.

Applications

1. Suzuki et al. (1983) studied metabolism of N, N - dibutylnitrosamine by rat liver S9 fraction and oxidation by chemical model system.

2. Mori et al. (2001) used N-Benzyl imidazole for preparation of S9 fraction with multi-induction of metabolizing enzymes in short term genotoxicity assays.

3. Jawecki (2008) studied in vitro biotransformation of amitriptyline and imipramine with rat hepatic S9 fraction.

Human liver microsomes (HLM)

HLM are vesicles of hepatocytes endoplasmic reticulum obtained by differential centrifugation of liver preparations (homogenates) from fresh human liver, liver slices, liver cell lines and primary hepatocytes. This subcellular fraction is a rich source of following enzymes: cytochrome P450s, flavin-monooxygenase (FMO), carboxyl esterases, epoxide hydrolase and UGTs. Therefore, HLM are most frequently utilized in vitro model in drug metabolic profiling and drug interaction studies. Moreover, the influence of specific isoenzymes is studied using liver microsomes in the presence of specific inhibitors. There are some interindividual variations in the activity of human liver microsomes; therefore, they can be utilized also to

study interindividual variability. In case of general estimation of drug metabolism, pooled microsomes from a large bank of individual liver tissues can be used to overcome the influence of interindividual variability. Microsomes from other human organs (intestines, kidney, lung) are also available and are utilized to evaluate extrahepatic metabolism. Additionally, gender-specific microsomes are available for the estimation of gender-based discrepancies in drug biotransformation. In drug discovery process HLM are used for metabolite identification, evaluation of interspecies differences in drug metabolism, prediction of in vivo clearance reaction phenotyping and metabolic pathway identification. NADPH or NRS is required in the incubation for the estimation of CYP activity. In order to evaluate the UGT activity UDPGA and alamethicin (pore-forming reagent) are required.

Advantages of HLM are ease of use, low costs, best characterized in vitro model for estimation of drug biotransformation, easy and long term storage, appropriate for studying of inter-individual and population-based variation, provide qualitative estimation of in vitro drug metabolism, convenient tool for high throughput screening of compounds, appropriate for lead compound optimization studies and drug interaction studies.

Disadvantage is HLM are not appropriate for quantitative estimation of drug biotransformation because of absence of enzymes like NAT, GST and SULT and cofactors needed. This limits the expected metabolic completion and formation of some in vivo metabolites. Another disadvantage is a very difficult assessment of the fraction of drug bound to plasma proteins versus to microsomes which is an important factor in the estimation of in vivo biotransformation.

Applications

1. Von Moltke et al. (1996) studied the biotransformation of triazolam by human liver microsomes and also reported the effects of metabolic inhibitors and clinical confirmation of predicted interaction with ketoconazole.

2. Von Moltke et al. (1996) used human liver microsomes to study midazolam hydroxylation and the inhibition by fluoxetine, norfluoxetine by azole antifungal agents.

3. Fisher et al. (2000) performed the in vitro glucuronidation study on the pore-forming peptide alamethicin using human liver microsomes.

Method: Tissue sample is thawed on ice and the weighed samples are finely homogenized on ice using a glass homogenizer in 0.5M Tris-HCl (pH 7.0) buffer containing 1.12 % w/v KCl and 1.12 % v/v EDTA (10 ml per gm liver). After mixing, 0.5 ml of the homogenate is retained for POR activity analysis while the remaining sample is centrifuged at 9000xg for 20 min at 4°C. The supernatant is collected and centrifuged at 100,000xg at 4°C with an ultracentrifuge. The resulting microsomal pellet is re-suspended in 0.15M Tris-HCl (pH 7.6) buffer and centrifuged for an additional hour at 100,000xg at 4°C. The final microsome pellet is suspended in 0.25M sucrose (2 ml/gm original sample). Both the homogenate and microsomal suspension are frozen in liquid nitrogen and stored at 80°C until analysis. Microsomal protein concentrations are determined (Zhang et al., 2015).

Rat liver microsomes (RLM)

Rat liver microsomes contain un-degraded membrane-bound polysomes and can function very well in an in vitro translation system. It uses endogenous ribonuclease inhibitor in all steps, avoiding pelleting rough microsomes in all steps and sacrificing good recovery (Sabatini, 2014).

Method: Sun et al. (2004) prepared liver microsomes of wistar rats using ultracentrifuge method. The invitro metabolism of verapamil is studied with rat liver microsomal incubation at a concentration of 1.0 µl and 5.0 µl. The metabolites are separated and assayed by liquid chromatography-ion trap mass spectra.

An alternate procedure as described by Horak et al. (2010) involves harvesting of livers from wistar and brown rats and bobwhite quail. Following euthanasia by CO_2 a longitudinal incision is made in the abdomen, the liver is removed and weighed. The tissue is rinsed thoroughly and either perfused with ice cold 0.9% NaCl or immediately frozen liquid nitrogen. The perfusion is continued until the liver appeared blanched; the perfused tissue is then snap-frozen in liquid nitrogen. The frozen samples are stored at -80°C until further processing.

Application

1. Hamilton et al. (1958) performed ultra-centrifugal studies on ribonucleoprotein from rat liver microsomes.

2. Feller et al. (1971) have performed enzymatic reduction of niridazole by rat liver microsomes.

3. Mondal et al. (2008) reported optimization of rat liver microsomal stability assay using HPLC for atenolol, propranolol HCl, verapamil HCl, imipramine HCl, midazolam HCl, andrographolide and 14-deoxyandrographolide.

4. Guodong et al. (2008) reported the structural characterization of in vitro rat liver microsomal metabolites of antihistamine desloratadine using LTQ-orbitrap hybrid mass spectrometer in combination with online hydrogen/deuterium exchange HR-LC/MS.

Table 1. Detail of *in vitro* methods used for metabolite identification

In vitro methods	Applications	References
Cell lines	Screening of antileishmanial drugs in the human leukaemia monocyte cell line, THp-1	Croft et al. (1992)
	Inhibition of cell growth in the human breast cancer cell line MCF7 by antiepileptic drugs	Meussen-Elholm et al. (2004)
	Study of induced resistance in the human non-small cell lung carcinoma (NCI-H460) cell line by anticancer drugs.	Pesic et al. (2006)
Transgenic cell line	Gene overexpression,	Liu (2013)
	Promoter characterization and cell lineage markers,	
	Gene knockdown by RNA interference, Complementation and mutation mapping, Double or multiple transgenes,	
	Site-specific recombinase and conditional transgenic lines,	
	Inducible transgene expression	
Liver slices	Invitro metabolism of dimethyl nitrosamine	Montesano et al. (1970)
	In vitro model for studying the metabolism of a variety of structurally diverse chemicals	James et al. (1997)
	Study of maintenance of drug metabolism and transport functions during incubation	Viktoriia et al. (2017)
Recombinant human	Study of sex differences in drug metabolism	Anderson et al. (2002)
CYP and UGT enzymes	Metabolism of IN-1130, a novel activin receptor-like kinase-5 (ALK5) inhibitor	Kim et al. (2008)
(supersomes/ baculosomes)	Identification of the metabolic pathways and enzyme systems involved in the in vitro human hepatic metabolism of dronedarone (antiarrhythmic drug)	Sylvie et al. (2014)
Cytochrome P450 inhibition	Invitro metabolism of rosiglitazone (an oral antidiabetic agent)	Baldwin et al. (1999)
	Invitro metabolism of artemisinin	Ashton et al. (1999)
	Study of drug-drug interactions in the metabolism of imidafenacin	Kanayama et al. (2007)
Cytosolic fraction	Characterization of somatostatin binding sites of rat intestinal mucosa	Arilla et al. (1984)
	Characterization of a novel metabolite intermediate of ziprasidone in hepatic cytosolic fractions of rat, dog, and human by ESI-MS/MS	Miao et al. (2005)
S9 fractions	metabolism of N, N - dibutylnitrosamine	Suzuki et al. (1983)
	Genotoxicity assays	Mori et al. (2001)
	In vitro biotransformation of amitriptyline and imipramine with rat hepatic S9 fraction	Jawecki (2008)
Human liver microsomes	Biotransformation of triazolam	Von et al. (1996)
(HLM)	Study of midazolam hydroxylation	Von et al. (1996)
	In vitro glucuronidation study on alamethicin	Fisher et al. (2000)
Rat liver microsomes (RLM)	Ultra-centrifugal studies on ribonucleoprotein	Hamilton et al. (1958)
	Study of enzymatic reduction of niridazole	Feller et al. (1971)
	Microsomal stability HPLC assay for atenolol, propranolol HCl, verapamil HCl, imipramine HCl, midazolam HCl, andrographolide and 14-deoxyandrographolide	Mondal et al. (2008)

Conclusion

Although, at present, in vitro models are unable to replace in vivo screening completely, they offer promising features. They can reduce the number of animals needed and offer a less complex way to elucidate the human biotransformation pathway of a new drug. It is likely that the different in vitro techniques used to study human biotransformation will become increasingly important in the early development stage of a new drug before starting in vivo experiments, so that the most promising drugs are selected and in vivo testing can be performed as efficiently as possible.

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Conflict of Interest

The authors declare no conflict of interest.

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