

IN VITRO DRUG METABOLISM

ADME deficiency is one of the major factors that cause failures during drug development. To prevent those costly failures from occurring, *in vitro* screening of potential drug candidates in the early drug discovery phase has been employed as a more cost-effective approach to identify compounds that have unfavorable ADME characteristics^{1,2}. Cerep provides a wide variety of valuable metabolism screening assays using assay matrices including recombinant cytochrome P450 enzymes (CYPs), recombinant uridine diphosphate-glucuronosyl transferases (UGTs), liver microsomes, liver S9 fraction, intestinal microsomes, intestinal S9 fraction, and hepatocytes from human and animal species.

A drug, once it enters an organism, can be converted to metabolite(s), which is/are typically more soluble in aqueous solutions and readily excreted than the corresponding parent drug. The chemical conversion is catalyzed by phase I (mainly CYPs) and/or phase II (such as glutathione-S-transferases and UGTs) drug-metabolizing enzymes, which are present at high levels in the liver, intestine, and kidney.

Phase I reaction includes oxidation, dealkylation, and hydroxylation, which introduce or expose polar functional groups, such as hydroxyl, amino, carboxyl, and sulfhydryl groups, in a drug.

Phase II reactions, such as glucuronidation, sulfation, and glutathione conjugation, add bulky hydrophilic substituents, such as glucuronic acid, sulfate, or glutathione, to those polar functional groups to make the test compounds even more water soluble. Also, the formation of a glutathione conjugate often suggests that a reactive metabolite is generated after phase I reaction.

From metabolism perspective, an "ideal" drug should:

- 1) Be relatively stable, i.e. have a small first-pass effect and maintain an effective concentration in blood for a reasonable period of time;
- 2) be metabolized by multiple CYP enzymes and not largely dependent on CYPs that are polymorphically-expressed, such as CYP2C9, CYP2C19, and CYP2D6;
- 3) lead to no pharmacologically-active (unless starting as a prodrug) or chemically-reactive metabolites.

Cerep's rapid *in vitro* metabolism screening assays allow identification of compounds that have these favorable characteristics for further development as drug candidates.

METABOLIC STABILITY

- ▶ FOR EVALUATING THE OXIDATIVE OR CONJUGATION METABOLISM, CHOICE OF MATRICES TO USE:
 - . liver microsomes (with NADPH for oxidation or UDPGA for glucuronide conjugation)
 - . liver S9 (with NADPH for oxidation, glutathione for glutathione conjugation, UDPGA for glucuronide conjugation, or PAPS for sulfation)
 - . hepatocytes
 - . intestinal microsomes (with NADPH for oxidation or UDPGA for glucuronide conjugation)
 - . intestinal S9 (with NADPH for oxidation, glutathione for glutathione conjugation or UDPGA for glucuronide conjugation)
- ▶ FOR EVALUATING HYDROLYTIC METABOLISM, CHOICE OF MATRICES TO USE:
 - . plasma
 - . blood

Many compounds with promising pharmacological characteristics never become drugs because they are rapidly metabolized in the liver and, therefore, have very low oral bioavailability. The metabolic stability assays are designed to measure the stability of a test compound in a variety of the aforementioned assay matrices from human and animal species. Metabolic stability can be conducted at 2 time points or 5 time points (for determination of half-life, $T_{1/2}$ and/or intrinsic clearance, Cl_{int}). The determination of Cl_{int} values can help confirm whether metabolism is the major elimination pathway when it is compared with the total body clearance *in vivo*. Cl_{int} values are also helpful in rank-ordering drug candidates based on their metabolic stabilities, assessing species and gender differences in metabolic clearance, and projecting the metabolic clearance of drug candidates in humans.

A microsomal preparation from the human liver contains all CYP isozymes and other membrane-bound drug metabolizing enzymes, such as flavin monooxygenase (FMO) and UGTs, which are responsible for the metabolism of majority of drugs in humans. Liver microsomes are well characterized and are the most frequently used form of tissue preparations for *in vitro* drug metabolism studies, especially for the phase I oxidations (Fig. 1). By supplementing the cofactor, UDPGA (uridine diphosphoglucuronic acid), liver microsomes can also be used to screen compounds for phase II glucuronide conjugation.

S9 is another choice of matrix for *in vitro* drug metabolism studies. S9 contains both membrane-bound and soluble enzymes, including esterases, glutathione-S-transferases (GST), and all the enzymes present in the microsomes. By supplementing proper co-factors, S9 can be used to study both phase I oxidations and hydrolysis and phase II conjugations of test compounds.

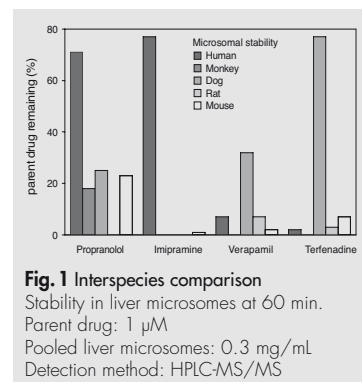


Fig. 1 Interspecies comparison
Stability in liver microsomes at 60 min.
Parent drug: 1 μ M
Pooled liver microsomes: 0.3 mg/mL
Detection method: HPLC-MS/MS

¹ FDA (1997) *Guidance for Industry - Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro*

² FDA (2006) *Drug Interaction Studies - Study Design, Data Analysis, and Implications for Dosing and Labeling*

Hepatocytes not only contain all drug metabolizing enzymes but also are a living system, which closely mimic the *in vivo* situation. Cryopreserved hepatocytes retain sufficient viability and enzyme activities for several hours and are an excellent assay matrix for metabolic stability (Fig. 2).

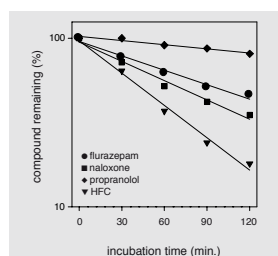


Fig.2 Stability in hepatocytes
Parent drug: 0.5 μ M
Pooled human cryopreserved hepatocytes from 10 donors: 10⁶ viable cells/mL
Detection method: HPLC-MS/MS

The intestine also contains high activities of drug metabolizing enzymes and exerts a first-pass effect. Metabolic stability of test compounds can be screened using intestinal microsomes or S9.

The plasma and blood contain esterases and proteases, which have hydrolytic activity toward compounds that have ester and/or amide bonds. Because of the large volume of the blood, the hydrolytic effect on some compounds can be substantial. The stability of test compounds can be screened using plasma (Fig. 3) or blood (Fig. 4).

The standard conditions for Cerep's stability assays include incubation of test compound (1 μ M for microsomes, S9, or hepatocytes, or 5 μ M for plasma or blood) in a

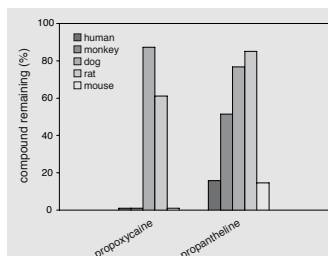


Fig. 3 Interspecies comparison
Stability in plasma at 60 min.
Parent drug: 5 μ M
Detection method: HPLC-MS/MS

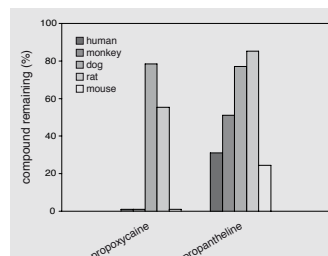


Fig. 4 Interspecies comparison
Stability in blood at 60 min
Parent drug: 5 μ M
Detection method: HPLC-MS/MS

selected assay matrix for up to 60 minutes (liver microsomes or S9, plasma, or blood) or 120 minutes (hepatocytes, intestinal microsomes or S9) in duplicate. The protein concentration is 0.3 mg/mL (microsomes), 1 mg/mL (S9), or 10⁶ cells/mL (hepatocytes). The parent compound is detected by HPLC-MS/MS analysis. The percent compound remaining is calculated by comparing the peak area of the parent compound at each time point to time zero. Half-life is estimated from the slope of the initial linear range of the logarithmic curve of parent compound remaining vs. time, assuming first order kinetics. The apparent intrinsic clearance is further calculated from the half-life value for assays with microsomes, S9, or hepatocytes.

The assay can be customized as per the customer's specifications. Some examples of customization include incubation time, test compound concentration, and the number of replicates, among others.

► CYP REACTION PHENOTYPING

- Metabolic stability with individual CYP isoforms (Step I)
- Metabolic stability with HLM and CYP selective inhibitors (Step II)

If a test compound is substantially metabolized by microsomes, CYPs are likely to be the enzymes that are responsible for the metabolism. Consequently, the next step is to find out which CYP is responsible for the metabolism. The process of identifying which CYP is responsible for the metabolism of a compound is often referred to as CYP reaction phenotyping or CYP phenotyping. A CYP phenotyping study consists of a combination of assays designed in step-wise to determine which of the individual CYPs is involved in the metabolic conversion of a test compound. Compounds are first tested in the metabolic stability assays with each of the 10 human recombinant CYPs (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5). Compounds (1 μ M) are incubated with each CYP in phosphate buffer (pH 7.4) containing the cofactor NADPH. Incubation is for 60 minutes at 37°C. The disappearance of the parent compound is determined by HPLC-MS/MS analysis. If a compound displayed a significant turnover (e.g. 50% remaining or less) with a particular CYP(s), the compound can be further tested in the metabolic stability assay with human liver microsomes (HLM) in the absence and presence of the respective CYP inhibitor(s).

In the Step II assays, compounds (1 μ M) are incubated with pooled HLM in phosphate buffer (pH 7.4) containing the cofactor NADPH in the presence or absence of a CYP selective inhibitor. Nine selective CYP inhibitors are used in the Step II CYP phenotyping assays: furafylline (for 1A), tranlycypromine (for 2A6), ticlopidine (for 2B6), quercetin (for 2C8), sulfaphenazole (for 2C9), oxybutynin (for 2C19), quinidine (for 2D6), clomethiazole (for 2E1), ketoconazole (for 3A). Incubation is again for 60 minutes at 37°C. The disappearance of the parent compound is determined by HPLC-MS/MS analysis. A result where higher % remaining in the presence of a CYP selective inhibitor than in the absence confirms the involvement of the respective CYP in the metabolism of the test compound. Results from a CYP phenotyping study will also indicate whether the metabolism of a test compound is catalyzed by a single or multiple CYPs including polymorphically-expressed CYPs.

► UGT REACTION PHENOTYPING

- Metabolic stability with human recombinant UGT isozymes

UGTs catalyze the conjugation reaction that adds glucuronic acid to a polar functional group in a compound. Often, the polar functional group is generated after a Phase I reaction. However, if a compound already has polar functional groups; it may directly undergo glucuronide conjugation. Compounds can be screened for direct glucuronide conjugation in the metabolic stability assays using liver microsomes or S9 in the presence of UDPGA. If a compound is a UGT substrate, a UGT phenotyping study can be performed to identify which of the UGTs is responsible for the conjugation. Compounds (1 μ M) are incubated with each of the 10 human recombinant UGT isozymes, (UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, and 2B15), in Tris buffer (pH 7.5) containing the cofactor UDPGA. The incubation is allowed for 60 minutes at 37°C. The disappearance of the parent compound is determined by HPLC-MS/MS analysis (Fig. 5). Unlike the CYP phenotyping study, UGT Phenotyping is typically conducted using recombinant enzymes only since isozyme specific inhibitors are not available.

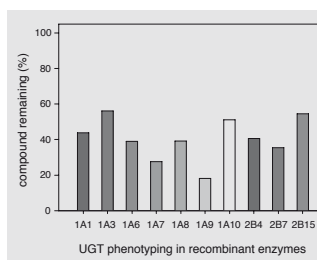


Fig. 5 UGT Phenotyping
Metabolic stability of 7-hydroxy-4-(trifluoromethyl) coumarin (HFC) with each individual human recombinant UGTs at 60 min.
Parent drug (1 μ M)
Detection method: HPLC-MS/MS

■ QUALITY CONTROL

For each of Cerep's metabolic stability assays, one or more reference compounds are concurrently incubated in the same assay with the test compounds. The assay is rendered valid if the results of the reference compounds fall within the specifications as defined in the corresponding Standard Operating Procedure.

The reference compounds used in each type of assays are listed as follows:

| Assay matrix | Reference compounds |
|-----------------------------------|---|
| microsomes or S9 | imipramine, propranolol, terfenadine, and verapamil |
| hepatocytes | propranolol, terfenadine, flurazepam, and 7-hydroxy-4-trifluoromethylcoumarin |
| plasma or blood | propranolol, propoxycaïne |
| CYP1A2 | propranolol, ethoxyresorufin |
| CYP2A6 | propranolol, coumarin |
| CYP2B6 | propranolol, benzphetamine |
| CYP2C8 | propranolol, paclitaxel |
| CYP2C9 | propranolol, diclofenac |
| CYP2C19 | propranolol, omeprazole |
| CYP2D6 | terfenadine, dextromethorphan |
| CYP2E1 | propranolol, chlorzoxazone |
| CYP3A4 or CYP3A5 | propranolol, midazolam |
| HLM with furafylline (CYP1A) | ethoxyresorufin |
| HLM with tranylcypromine (CYP2A6) | coumarin |
| HLM with ticlopidine (CYP2B6) | bupropion |
| HLM with quercetin (CYP2C8) | paclitaxel |
| HLM with sulfaphenazole (CYP2C9) | diclofenac |
| HLM with nootkatone (CYP2C19) | omeprazole |
| HLM with quinidine (CYP2D6) | dextromethorphan |
| HLM with clomethiazole (CYP2E1) | chlorzoxazone |
| HLM with ketoconazole (CYP3A) | midazolam |
| UGTs | 7-hydroxy-4-trifluoromethylcoumarin |



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