In Vitro Drug Metabolism Using Liver Microsomes

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Knowledge of the metabolic stability of newly discovered drug candidates eliminated by metabolism is essential for predicting the pharmacokinetic (PK) parameters that underpin dosing and dosage frequency. Further, characterization of the enzyme(s) responsible for metabolism (reaction phenotyping) allows prediction, at least at the qualitative level, of factors (including metabolic drug-drug interactions) likely to alter the clearance of both new chemical entities (NCEs) and established drugs. Microsomes are typically used as the enzyme source for the measurement of metabolic stability and for reaction phenotyping because they express the major drug-metabolizing enzymes cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT), along with others that contribute to drug metabolism. Described in this unit are methods for microsome isolation, as well as for the determination of metabolic stability and metabolite formation (including kinetics). © 2016 by John Wiley & Sons, Inc.

Keywords: in vitro drug metabolism • liver microsomes • metabolic stability • microsome isolation

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INTRODUCTION

Hepatic metabolism is the primary elimination mechanism for the majority of drugs, as well as for other xenobiotics and endogenous compounds. As such, metabolism is a determinant of drug oral bioavailability, clearance, and elimination half-life, and therefore of dose and dosing frequency. Consequently, the metabolic stability of new chemical entities (NCEs) is typically characterized during compound discovery/lead optimization. Hepatic microsomes, derived largely from the smooth endoplasmic reticulum, are commonly used as the enzyme source for the measurement of metabolic stability, as they contain the main drug-metabolizing enzymes (DMEs) cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT), together with other DMEs, including flavin-containing monooxygenases (FMO) and esterases (hCE1, hCE2) (Penner et al., 2012). Metabolic stability may be determined from the measurement of substrate depletion during incubation with hepatic microsomes or, when the metabolites are known, from the measurement of metabolite formation kinetics. Additionally, hepatic microsomes are

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employed for reaction phenotyping, which entails the identification of the particular CYP or UGT enzyme(s) involved in metabolite formation. Described in this unit is a method for determining metabolic stability (Basic Protocol). Detailed in Alternate Protocols are: (1) a higher-throughput procedure, (2) methods to incorporate UGT catalysis in addition to NADPH-dependent metabolism, and (3) a procedure for measuring metabolite formation instead of substrate depletion. Additionally, the preparation of human liver microsomes (HLM; Support Protocol 1), the measurement of microsomal P450 content (Support Protocol 2), the yield of microsomal protein per gram of liver (MPPGL; Support Protocol 3), and an example of the measurement of metabolite formation kinetics for a compound metabolized by UGT enzymes (Support Protocol 4) are described.

NOTE: All protocols using live animals or animal tissues must first be reviewed and approved by the appropriate institutional committee(s) [e.g., Institutional Animal Care and Use Committee (IACUC) or Animal Ethics and Welfare Committee (AEWC)] and must conform to governmental regulations regarding the care and use of laboratory animals.

NOTE: Collection and use of human tissues must adhere to all institutional and governmental guidelines and regulations and be reviewed and approved by the relevant institutional Human Research Ethics Committee (HREC). Additionally, appropriate Occupational Health and Safety procedures (e.g., disposal of biological waste) and Handling of Biological Specimen Guidelines (e.g., use of protective clothing, gloves, etc.) should be followed when handling human tissues.

BASIC PROTOCOL

MEASUREMENT OF COMPOUND METABOLIC STABILITY USING LIVER MICROSOMES

Described in this protocol is a screening assay for comparing the metabolic stabilities of a group of compounds. Only one compound concentration (traditionally 1 μM) thought to be well below the apparent K_m is examined to provide a better approximation of the initial slope of the velocity versus drug concentration curve that defines intrinsic clearance (Jia and Liu, 2007). Two time points (0 and 30 min) are examined for each agent for rank ordering of stability. Additional time points (e.g., 5, 10, 20, 30, 45 min) are usually needed to define precisely the log-linear portion of the curve and for calculating intrinsic clearance (CL_{int}). A positive control, such as testosterone or DL-propranolol, should be included to ensure that the assay is performing satisfactorily. A negative control (without NADPH) for each test compound is generated to determine sources of compound loss other than oxidative metabolism (e.g., carboxylesterases, thermal instability of the compound, etc.). Additional negative controls that may be included are matrix controls in the absence of enzyme or test compound (for baseline scans for LC/MS/MS).

Materials

Test compound(s)
0.1 M potassium phosphate buffer, pH 7.4 (*APPENDIX 2A*), pre-warmed to 37°C
10 mM NADPH freshly prepared in 0.1 M potassium phosphate buffer, pH 7.4, *or* NADPH-generating system (see recipe).
Positive control (e.g., 10 mM DL-propranolol, see recipe)
Human or animal microsomes [see Support Protocol 1 or purchase commercially from Corning Life Sciences (*http://www.corning.com*), Bioreclamation/IVT (*http://www.bioreclamationivt.com*), or XenoTech (*http://www.xenotech.com*)]
Acetonitrile

1.5-ml microcentrifuge polypropylene tubes
37°C water bath
Tabletop centrifuge

Additional reagents and equipment for analytical method, e.g., LC/MS/MS (Korfmacher, 2013)

1. Dissolve or dilute the test compound(s) to a suitable stock concentration (usually ≥100× the final concentration) in a volume ≥50 µl.

   If the test compounds are not soluble in potassium phosphate buffer, then acetonitrile, methanol, or DMSO can be used. However, the final organic solvent concentration in the assay should always be ≤1% (acetonitrile, methanol) or ≤0.2% (DMSO). Making a 1000× stock in DMSO, followed by 10-fold dilution with 1:1 acetonitrile:water to prepare a working stock, takes advantage of the strong solvent properties of DMSO and increases volume to facilitate pipetting while at the same time maintaining a low volume percentage of organic solvent.

2. Prepare five 1.5-ml microcentrifuge tubes for each test compound concentration and each positive control. The five 1.5-ml microcentrifuge tubes include duplicate tubes for both 0- and 30-min time points and one tube for the negative control. To each tube add:

   432 µl 0.1 M potassium phosphate buffer, pH 7.4, pre-warmed to 37°C
   50 µl 10 mM NADPH (see annotation below; replace with 0.1 M potassium phosphate buffer for negative control)
   5 µl 100× test compound or positive control.

   Incubate 5 min at 37°C.

   Volumes and the number of replicates can be scaled proportionally. 50 µl of an NADPH-generating system can be used in place of 50 µl NADPH.

   The choice between using either NADPH or an NADPH generating system using $NADP^+$ is based on user preference, taking into consideration that $NADP^+$ solutions (a stock component of the generating system) are stable for several freeze-thaw cycles and with storage (6 months) at −20°C, and that NADPH is more expensive than $NADP^+$ and degrades, turning yellow after a few days at −20°C, necessitating disposal and preparation of a fresh solution. In addition, generating systems rely on a functional enzyme (e.g., glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase) to generate NADPH from $NADP^+$. As a generating system "regenerates" NADPH, a lower initial concentration of $NADP^+$ (e.g., 0.1 mM) can be used without concern about depletion.

3. During the incubation in step 2, thaw microsomes and dilute them to 20 mg protein/ml in 0.1 M potassium phosphate buffer.

   Microsomes should be placed on ice immediately after thawing. The unused portion is returned to storage at −70°C. Under these conditions, the microsomes should retain activity over several freeze/thaw cycles. Nevertheless, use of individual portions sufficient for one experiment eliminates a potential source of experimental variability.

4. Initiate the reaction by adding a 13-µl aliquot of microsomes (final concentration 0.5 mg/ml). Mix the sample well by briefly vortexing and then incubate at 37°C in a water bath. Add 250 µl ice-cold acetonitrile to the 0-min tubes immediately after addition of microsomes to stop the reaction. Mix well by vortexing.

   Some investigators prefer: (1) to conduct incubations in a shaking water bath (~150 rpm), although stationary incubations perform equally well; and (2) to initiate the reaction by the addition of NADPH. For this case add buffer, then microsomes, then test compound. Warm for 5 min then add NADPH or the NADPH generating system.

5. At the predetermined time points, stop the reaction by adding 250 µl ice-cold acetonitrile. Mix well by vortexing.

6. Centrifuge the tubes for 5 min at 10,000 × *g*, 4°C. Pipet the supernatant fraction into labeled HPLC tubes (or appropriate containers for the chosen analytical technique). Store the fractions at −20°C until analysis.

   The sample-containing pellet should always be stored at −20°C until the analysis is performed. If the compound is not extracted completely by acetonitrile, another solvent system (such as methanol or 4% perchloric acid) may be needed to separate the compound from the pelleted protein. The extent of extraction can be determined by comparing the expected quantity in the 0-min sample to the standards. However, this assumes that the compound does not partition into or become significantly bound to the reaction vessel. Acetonitrile generally provides a more compacted pellet than methanol. Adding a larger volume of organic solvent (e.g., 500 µl) to terminate the reaction may be more effective for precipitating microsomes, lipids, and salts, thereby providing a cleaner sample and reducing LC/MS/MS maintenance. Subsequent dilution of the sample supernatant fraction with water (e.g., to 20% aqueous v/v) may be necessary to ensure effective retention of compound on a typical reversed-phase HPLC column.

7. Prepare a standard curve for each test compound by diluting the 1 mM stock solution to 1, 0.1, and 0.01 µM in 0.1 M potassium phosphate buffer. Develop an analytical method for each compound using an appropriate technique such as HPLC or LC/MS. Determine the concentration of the parent compound for each sample.

   Quantification using a standard curve is not absolutely necessary. Rather, the extent of depletion at 30 min can be calculated by comparison of peak area to that of the 0-min samples. However, low compound recovery may not be detected, confounding the results. Maintaining the matrix of analytical standards as close as possible to samples (e.g., inclusion of NADPH) may help avoid matrix effects such as ion suppression that can confound quantification by mass spectrometry (Hall et al., 2012).

8. Calculate the extent of metabolism (as substrate depletion) and % recovery of the test compounds and positive controls using the following equations:

$$\text{rate of depletion (pmol/ min /mg)} = \frac{(\Delta C \times 1000)}{(B \times T)}$$

   where ΔC = [concentration (or peak area) at 0 min] – [concentration (or peak area) at 30 min (nmol/ml or µM)]; B is the microsome protein concentration (mg/ml); T is the incubation time (min); and 1000 is the conversion factor from nmol to pmol.

   Alternatively, express the extent of metabolism as a percentage of the parent compound depletion using the following equation:

$$\%\ \text{parent compound depletion} = 100 - \left[\frac{\text{concentration (or peak area) at 30 min}}{\text{concentration (or peak area) at 0 min}} \times 100\right]$$

   The concentration (or peak area) of the control without NADPH at 30 min should be >90% of the compound concentration at 0 min. A lower percentage indicates confounding factors such as compound instability or non-NADPH-dependent enzyme degradation (neither CYP- or FMO-catalyzed metabolism). For low-clearance compounds, metabolic loss of compound may not be detected with this assay even though metabolism may be a major clearance pathway for the agent. Conduct incubations at 0.1 µM substrate to check for saturation of enzyme (e.g., that might occur at 1 µM substrate) or conduct Alternate Protocol 3 for metabolically stable compounds.

9. Calculate the percent recovery for the extracted compound as follows:

$$\%\ \text{recovery} = \frac{\text{concentration (or peak area) at 0 min } (\mu\text{M})}{\text{concentration (or peak area) determined for } 1\mu\text{M standard}} \times 100$$

The recovery should be >90%. If the recovery is low, a different extraction solvent may be needed. Alternatively, in high-throughput laboratory environments, recovery may not be determined at this stage if the compound remains a viable drug candidate, but evaluated later in the drug-development process.

10. Calculate the intrinsic clearance as follows. Plot the log of the test compound peak area versus incubation time and determine the slope [elimination rate constant (k) is 1/slope is for the initial, linear portion of the curve, or calculate it directly using the equation $c_t = c_0 e^{-kt}$ where $t = 30$ for a 30-min incubation. Calculate the half-life (min) as $0.693/k$. Calculate V (µl/mg) as the volume of the incubation (in µl) divided by the protein amount in the incubation (mg). Calculate the intrinsic clearance (CL_{int}, µl/min.mg) as $V \times 0.693$/half-life.

The clearance "intrinsic" to the microsomal test systems may be scaled up to hepatic clearance (see Chiba et al., 2009; Klopf and Worboys, 2010; Obach, 2012, for a more detailed discussion of scaling).

HIGHER-THROUGHPUT SCREENING FOR METABOLIC STABILITY USING LIVER MICROSOMES

ALTERNATE PROTOCOL 1

Utilizing specialized equipment, this protocol is a higher-throughput version of the Basic Protocol. It can be employed to measure the extent of metabolism of up to 32 compounds at a time in a 96-well plate.

Additional Materials *(also see Basic Protocol)*

96-well plates (polypropylene; Axygen Scientific, cat. no. P-DW-20-C)
Multichannel pipettor (Rainin Instrument, LLC)
Reagent reservoirs for multichannel pipetting (e.g., 50-ml polystyrene; Corning Costar, cat. no. 4870)
Centrifuge with a plate holder–capable rotor
96-well autosampler for LC/MS/MS

1. Dissolve or dilute the test compounds to a stock concentration of 0.1 mM (100× the final concentration to be tested).

Refer to guidance on compound stock solution preparation in Basic Protocol, step 1.

2. Add the following to the designated wells (see plate layout in Fig. 7.8.1) of a 96-well plate in the order indicated:

197.5 µl 0.1 M potassium phosphate buffer, pH 7.4, pre-warmed to 37°C
25 µl 10 mM NADPH (replace with buffer for negative control)
2.5 µl 100× test compound

Incubate 5 min at 37°C.

Use of a multichannel pipettor for buffer and NADPH additions decreases preparation time.

3. To a separate 96-well plate, add 50 µl ice-cold acetonitrile to rows 1 through 9, and maintain at 4°C.

An internal standard is usually added to the acetonitrile (e.g., 0.4 µM labetalol) to control for suppression or fluctuation in the LC/MS response.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Compound 1 1μM replicate A	Compound 1 1μM replicate B	Compound 1 1μM no NADPH	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
B	Compound 2 1μM replicate A	Compound 2 1μM replicate B	Compound 2 1μM no NADPH	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
C	Compound 3 1μM replicate A	Compound 3 1μM replicate B	Compound 3 1μM no NADPH	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
D	Compound 4 1μM replicate A	Compound 4 1μM replicate A	Compound 4 1μM no NADPH	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
E	Compound 5 1μM replicate A	Compound 5 1μM replicate B	Compound 5 1μM no NADPH	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
F	Compound 6 1μM replicate A	Compound 6 1μM replicate B	Compound 6 1μM no NADPH	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
G	Compound 7 1μM replicate A	Compound 7 1μM replicate B	Compound 7 1μM no NADPH	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty
H	Compound 8 1μM replicate A	Compound 8 1μM replicate B	Compound 8 1μM no NADPH	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

Figure 7.8.1 Sample plate (incubation plate) layout shown for 8 compounds; up to 32 compounds can be assayed per plate.

4. During the incubation in step 2, thaw microsomes and dilute to 5 mg protein/ml in potassium phosphate buffer.

   Store microsomes on ice and use within 2 hr of thawing (Pearce et al., 1996).

5. Initiate the reaction by adding 25 μl of microsome suspension (final concentration 0.5 mg/ml) to all wells in the 96-well plate from step 2 with a multichannel pipettor, and gently mix by pipetting up and down two to three times. Immediately remove 100 μl of each sample for the 0-min time point and add to the acetonitrile plate in the wells indicated in Figure 7.8.2. Store acetonitrile-quenched samples at 4°C. Return the sample plate to the incubator at 37°C.

   Some prefer to initiate the reaction by the addition of NADPH. In this case, add buffer, then microsomes, and then the test compound. Warm for 5 min and then add NADPH or the NADPH generating system.

6. At 30 min, quench the reaction by adding 100 μl of the reaction mixture from the sample plate to the acetonitrile plate in the indicated wells. Mix by pipetting up and down two to three times.

7. Prepare a standard curve for each compound by diluting the 1 mM stock solution (prepared in organic solvent) to 1, 0.1, and 0.01 μM in potassium phosphate buffer and adding 100 μl of each dilution to the acetonitrile plate in the indicated wells.

8. Centrifuge the acetonitrile plate for 10 min at 3500 × *g*, 4°C. Pipet 100 μl of the supernatant fraction into an analysis plate, taking care not to disturb the pellet. Store at −20°C until analyzed.

9. Develop an analytical method for each compound using a conventional technique such as HPLC or LC/MS (Korfmacher, 2013). Determine the concentration of the parent compound for each sample.

	1	2	3	4	0-min 5	0-min 6	30-min 7	30-min 8	30-min 9	10	11	12
A	Compound 1 blank	Compound 1 1μM standard	Compound 1 0.1μM standard	Compound 1 0.01μM standard	Compound 1 1μM replicate A	Compound 1 1μM replicate B	Compound 1 1μM replicate A	Compound 1 1μM replicate B	Compound 1 1μM no NADPH	Empty	Empty	Empty
B	Compound 2 blank	Compound 2 1μM standard	Compound 2 0.1μM standard	Compound 2 0.01μM standard	Compound 2 1μM replicate A	Compound 2 1μM replicate B	Compound 2 1μM replicate A	Compound 2 1μM replicate B	Compound 2 1μM no NADPH	Empty	Empty	Empty
C	Compound 3 blank	Compound 3 1μM standard	Compound 3 0.1μM standard	Compound 3 0.01μM standard	Compound 3 1μM replicate A	Compound 3 1μM replicate B	Compound 3 1μM replicate A	Compound 3 1μM replicate B	Compound 3 1μM no NADPH	Empty	Empty	Empty
D	Compound 4 blank	Compound 4 1μM standard	Compound 4 0.1μM standard	Compound 4 0.01μM standard	Compound 4 1μM replicate A	Compound 4 1μM replicate B	Compound 4 1μM replicate A	Compound 4 1μM replicate B	Compound 4 1μM no NADPH	Empty	Empty	Empty
E	Compound 5 blank	Compound 5 1μM standard	Compound 5 0.1μM standard	Compound 5 0.01μM standard	Compound 5 1μM replicate A	Compound 5 1μM replicate B	Compound 5 1μM replicate A	Compound 5 1μM replicate B	Compound 5 1μM no NADPH	Empty	Empty	Empty
F	Compound 6 blank	Compound 6 1μM standard	Compound 6 0.1μM standard	Compound 6 0.01μM standard	Compound 6 1μM replicate A	Compound 6 1μM replicate B	Compound 6 1μM replicate A	Compound 6 1μM replicate B	Compound 6 1μM no NADPH	Empty	Empty	Empty
G	Compound 7 blank	Compound 7 1μM standard	Compound 7 0.1μM standard	Compound 7 0.01μM standard	Compound 7 1μM replicate A	Compound 7 1μM replicate B	Compound 7 1μM replicate A	Compound 7 1μM replicate B	Compound 7 1μM no NADPH	Empty	Empty	Empty
H	Compound 8 blank	Compound 8 1μM standard	Compound 8 0.1μM standard	Compound 8 0.01μM standard	Compound 8 1μM replicate A	Compound 8 1μM replicate B	Compound 8 1μM replicate A	Compound 8 1μM replicate B	Compound 8 1μM no NADPH	Empty	Empty	Empty

Figure 7.8.2 Acetonitrile plate layout (quenching plate with 50 μl acetonitrile/well) shown for 8 compounds.

A single LC analytical method can often be used for compounds with similar chemical structures.

10. Calculate the extent of metabolism (substrate depletion) and % recovery of the test compounds as described in the Basic Protocol, steps 8 and 9.

HIGHER-THROUGHPUT SCREENING FOR METABOLIC STABILITY USING LIVER MICROSOMES SUPPLEMENTED WITH NADPH AND UDP-GlcUA

ALTERNATE PROTOCOL 2

This procedure is similar to Alternate Protocol 1 except that an additional cofactor, UDP-glucuronic acid (UDP-GlcUA), is added to activate the UDP-glucuronosyltransferases (UGT) present in the liver microsomes. Therefore, this protocol is used to also identify metabolic instability attributable to UGT enzymes.

Additional Materials *(also see Basic Protocol 1)*

500 mM magnesium chloride
25 μg/ml alamethicin (see recipe)
UDP-GlcUA (see recipe)

96-well plates (polypropylene; Axygen Scientific, cat. no. P-DW-20-C)
Multichannel pipettor (Rainin Instrument LLC)
Reagent reservoirs (e.g., 50 ml polystyrene, Corning Costar catalog no. 4870)
Centrifuge with a plate holder–capable rotor
96-well auto sampler for LC/MS/MS

1. Dissolve or dilute the test compounds to a stock concentration of 0.1 mM (100× the final concentration for study).

   Refer to guidance on compound stock solution preparation in the Basic Protocol, step 1.

2. Add the following to the designated wells (see plate layout in Fig. 7.8.1) of a 96-well plate in the order indicated:

   168 µl 0.1 M potassium phosphate buffer, pH 7.4, pre-warmed to 37°C
   25 µl of 5 mg/ml microsomes (final concentration 0.5 mg/ml)
   4 µl of 500 mM magnesium chloride
   2.5 µl 100× test compound
   0.625 µl 25 µg/ml alamethicin.

   Incubate 5 min at 37°C.

   A 5× solution of alamethicin, magnesium chloride solution, and 0.1 M potassium phosphate can be prepared beforehand (use within 30 min) to facilitate addition of these reagents in a single pipetting step. Scale to the appropriate volumes. Store microsomes on ice if not used immediately. The UDP-GlcUA and alamethicin/$MgCl_2$ premixes are commercially available (Corning cat. no. 451300 and cat. no. 451320, respectively)

3. To a separate 96-well plate, add 50 µl of ice-cold acetonitrile to rows 1 through 9 and maintain at 4°C.

   An internal standard is usualy added to the acetonitrile (e.g., 0.4 µM labetalol) to control for suppression or fluctuation in the LC/MS response.

4. During the incubation in step 2, combine (1:1 v/v) 20 mM UDP-GlcUA and 10 mM NADPH cofactor solutions. Scale volumes as needed.

5. Initiate the reaction by adding 50 µl of the UDP-GlcUA/NADPH solution (replace with buffer for negative control) to all wells in the 96-well plate from step 2 with a multichannel pipettor and gently mix by pipetting up and down two to three times. Immediately remove 100 µl of each sample for the 0-min time point and add to the acetonitrile plate in the wells indicated on Figure 7.8.2. Store acetonitrile-quenched samples at 4°C. Return the sample plate to the incubator at 37°C.

   Although not absolutely required for UGT activity, it is generally accepted practice to preincubate microsomes with alamethicin, which forms pores in microsomal membranes, promoting access of substrate and cofactor to UGT enzymes. Initiation of the reaction by addition of microsomes is not recommended when evaluating UGT activity (Boase and Miners, 2002; Gill et al., 2012; Caldwell and Yan, 2014).

6. At 30 min, quench the reaction by adding 100 µl of the reaction mixture from the sample plate to the acetonitrile plate in the indicated wells. Mix by pipetting up and down two to three times.

7. Prepare a standard curve for each compound by diluting the 1 mM stock solution to 1, 0.1, and 0.01 µM in potassium phosphate buffer and adding 100 µl of each dilution to the acetonitrile plate in the indicated wells.

8. Centrifuge the acetonitrile plate for 10 min at 3500 × *g*, 4°C. Pipet 100 µl of the supernatant fraction into an analysis plate, taking care not to disturb the pellet. Store at −20°C until analyzed.

9. Develop an analytical method for each compound using a conventional technique such as HPLC or LC/MS (Korfmacher, 2013). Determine the concentration of the parent compound for each sample.

A single LC analytical method can often be used for compounds with similar chemical structures.

10. Calculate the extent of metabolism (substrate depletion) and % recovery of the test compounds as in the Basic Protocol, steps 8 and 9.

MEASURMENT OF METABOLITE FORMATION IN INCUBATIONS USING LIVER MICROSOMES

ALTERNATE PROTOCOL 3

Described below is a method for quantifying metabolite formation from a test compound using liver microsomes. Unlike the previous protocols, where the substrate concentration is typically much lower than the apparent K_m, a single high concentration or a range of concentrations is employed with this procedure to achieve higher concentrations of metabolites for accurate quantification. This protocol is recommended when: (1) determination of intrinsic clearance is required but substrate depletion is undetectable (e.g., <15% loss); (2) there is a need to investigate metabolites and their pharmacological and toxicological properties; (3) detailed analysis of reaction kinetics is required or; (4) a combination of the above. With no prior knowledge of metabolites or their rate of formation, a single concentration (e.g., 50 μM) or several concentrations (range-finding experiment, e.g., 5, 50, and 500 μM) are used. Concentrations are chosen to be near or above the historical range of K_m values reported for CYP substrates to help ensure formation of detectable quantities of metabolite. A positive control should be included to confirm that the assay is performing satisfactorily. Formation of 6β-hydroxytestosterone from testosterone, or acetaminophen (paracetamol) from phenacetin, are common positive control reactions. A negative control without NADPH for each test compound is useful for determining the sources of metabolites other than oxidative metabolism (e.g., carboxylesterases, nonenzymatic metabolite formation, substrate impurities, etc.). Additional negative controls that may be considered include matrix controls without the enzyme or without the test compound (for LC/MS baseline scans).

Materials

Test compound(s)
0.1 M potassium phosphate buffer, pH 7.4 (*APPENDIX 2A*), pre-warmed to 37°C
10 mM NADPH freshly prepared in 0.1 M potassium phosphate buffer, pH 7.4, or an NADPH-generating system (see recipe)
Positive control (e.g., 20.8 mM testosterone and 3 mM 6β-hydroxytestosterone, see recipes)
Human or animal microsomes [see Support Protocol 1 or purchase commercially from Corning Life Sciences (*http://www.corning.com*), Bioreclamation/IVT (*http://www.bioreclamationivt.com*), or XenoTech (*http://www.xenotech.com*)]
Acetonitrile

1.5-ml microcentrifuge polypropylene tubes
37°C water bath
Tabletop centrifuge

Additional reagents and equipment for analytical method such as LC/MS (Korfmacher, 2013)

1. Dissolve or dilute the test compound(s) to a suitable stock concentration (usually 100× or greater than final test concentration) in a volume of ≥50 μl.

Refer to guidance on compound stock solution preparation in Basic Protocol, step 1.

2. Prepare seven 1.5-ml microcentrifuge tubes for each test compound concentration and each positive control. This includes duplicate tubes for 0-, 10- and 60-min time points and one tube for the negative control. To each tube, add:

   432 μl 0.1 M potassium phosphate buffer, pH 7.4, pre-warmed to 37°C
   50 μl 10 mM NADPH (replace with 0.1 M potassium phosphate buffer for negative control)
   5 μl 100× test compound or positive control.

   Incubate for 5 min at 37°C.

   50 μl of an NADPH-generating system can be used instead of 50 μl NADPH.

3. During the incubation in step 2, thaw microsomes and dilute to 20 mg protein/ml in 0.1 M potassium phosphate buffer, pH 7.4, if necessary.

   Refer to guidance on use of microsomes (see Basic Protocol, step 3).

4. Initiate the reaction by adding 13 μl of microsome suspension (final concentration 0.5 mg/ml). Mix the sample well by briefly vortexing and then incubate at 37°C in a water bath. Add 250 μl ice-cold acetonitrile to the 0-min tubes immediately after addition of microsomes. Mix well by vortexing.

   Refer to notes Basic Protocol, step 4.

5. Stop the reaction at the predetermined time points by adding 250 μl ice-cold acetonitrile. Mix well by vortexing.

6. Centrifuge the tubes for 5 min at 10,000 × *g*, 4°C. Pipet the supernatant fraction into labeled HPLC tubes (or appropriate containers for the analytical procedure). Store at −20°C until analysis.

   The sample containing pellet should always be stored at −20°C until analysis. Refer to notes in the Basic Protocol, step 6.

7. Develop an analytical method for each analyte [e.g., substrate and/or the metabolite(s) for each compound] using a common technique such as HPLC/UV or LC/MS (Korfmacher, 2013). Prepare a standard curve for each analyte by diluting analyte stock and/or working solution. A standard curve of at least 10 concentrations is recommended for metabolite analysis. The concentration range should span 3 to 4 orders of magnitude, with the lowest concentration at or near the limit of detection. Standards should be prepared in a matrix identical to or approximating the composition of the incubation matrix. Determine the concentration of the analyte for each sample.

   Because ionization efficiencies, and therefore mass spectrometer response, may be vastly different than for the substrate, authentic standard metabolites are normally needed for reliable quantitative analysis. Even when authentic metabolite standards are available, maintaining the matrix of the standards as close as possible to that of the samples (e.g., inclusion of NADPH) may help avoid matrix effects, such as ion suppression, that can confound mass spectrometric quantification. Ensure that the matrix for preparation of metabolite standards is catalytically inactive by omitting microsomes or adding quench solution prior to cofactors. Differences in ionization efficiency may be encountered even for the same analyte. This may be caused by slight differences in matrix or normal fluctuation of mass spectrometer response over the course of the analytical run. In this case, inclusion in the reaction quench solution of a stable-labeled isotope metabolite internal standard is recommended. If using ultraviolet light absorbance as a means of detection, the metabolite may be detected and quantified without an authentic standard, with the assumption that metabolites possess similar absorbance properties (i.e., extinction coefficients) as the substrate.

8. Calculate the extent of metabolism and positive controls using the following equations:

$$\text{rate of metabolite formation (pmol/ min /mg)} = \frac{(C \times 1000)}{(B \times T)}$$

where C = concentration determined at each incubation time point, using the standard curve (nmol/ml or μM); B is the microsomal protein concentration (mg/ml); T is the incubation time (min); and 1000 is the conversion from nmol to pmol.

The summed molar concentrations of metabolites should equal the concentration of substrate compound lost over the course of the incubation. A discrepancy of >25% suggests unsuitable analyte recovery or the presence of a significant amount of undetected metabolites that warrant further investigation. The concentration of metabolites in the control without NADPH at each time point should be negligible. Investigate the source and subtract the amount of any quantifiable metabolite to obtain accurate reaction velocities. If a range-finding experiment is conducted, reaction velocities should increase with increasing substrate concentration until enzyme saturation occurs. If the compound is a substrate and reaction velocities do not increase, or increase modestly with substrate concentration, enzyme saturation is probable and the K_m is lower than the concentration range being tested. In this case, conduct experiments over a lower substrate concentration range. If reaction velocities are linear or approximately linear with substrate concentration, the K_m is greater than the concentration selected. In this case, conduct a follow-up experiment over a higher concentration range. After determining an estimate of K_m, a definitive experiment may be conducted with 10 to 15 concentrations of substrate, preferably with an equal number of concentrations bracketing the K_m. A higher number of concentrations better defines the curve and provides greater confidence regarding whether the compound exhibits Michaelis-Menten or atypical kinetics. The apparent K_m and V_{max} can be calculated for each metabolite using a variety of methods. Curve fitting by nonlinear regression (e.g., Prism software from Graphpad; XLfit software from IDBS) is most commonly used. The K_m/V_{max} or intrinsic clearance may be determined for each metabolite. Use the 10- versus 60-min time-point data to select appropriate incubation time(s) in subsequent experiments to ensure measurement of initial rate velocities. Summing the intrinsic clearance for each metabolite yields total intrinsic clearance for the compound.

9. If desired, calculate the percent recovery for the substrate as follows:

$$\%\ \text{recovery} = \frac{\text{concentration at } 0 \text{ min } (\mu\text{M})}{\text{concentration determined for X } \mu\text{M substrate standard}} \times 100$$

where X is the concentration of substrate desired.

Recovery of metabolites is usually greater than or equal to that of the parent unless the metabolite is covalently or tightly bound to microsomal protein/lipid matrix. This is because metabolites are usually more polar than the parent compound. Measuring the recovery of the parent substrate may serve as a worse-case surrogate for recovery of metabolites.

PREPARATION OF MICROSOMES FROM HUMAN LIVER

SUPPORT PROTOCOL 1

Microsomes can be prepared from human or laboratory animal liver tissue that is either fresh or snap frozen in liquid nitrogen, and both tissues and microsomes can be stored up to 5 years at −70°C (Yamazaki et al., 1997). In our experience, CYP and UGT activities in human liver microsomes (HLM) are stable for at least 10 years when stored at −70°C (unpub. observ.). This protocol for the preparation of HLM is a slightly modified version of one first reported by Bowalgaha et al. (2005). Additionally, HLM can be

characterized for the measurement of P450 content and microsomal yield (see Support Protocol 2). Microsomes from a variety of human tissues and laboratory animal tissues and species can be purchased from commercial suppliers such as Corning Life Sciences (*http://www.corning.com*), Bioreclamation/IVT (*http://www.bioreclamationivt.com*), or XenoTech (*http://www.xenotech.com*).

Materials

Human liver tissue (1 gm of tissue per 5 ml of microsome preparation buffer)
Microsome preparation buffer (see recipe)
Microsome storage buffer (see recipe)

Open-top polycarbonate centrifuge tubes (50 ml)
Surgical scissors
Tissue homogenizer (e.g., Ultra Turrax T25, *http://www.ika.com*)
Potter-Elvehjem tissue grinder (30 ml) with Teflon pestle (motor driven)
Refrigerated centrifuge
Syringe (50 ml) with blunt-end aspiration needle
Ultracentrifuge tubes (~25 ml; e.g., Beckman-Coulter)
Ultracentrifuge, refrigerated to 4°C before use
Ultracentrifuge rotors, maintained at 4°C for at least 16 hr before use.
Plastic round-ended probe
Potter-Elvehjem tissue grinder (10 ml) with Teflon pestle (hand held)
Glass storage vials (suitable for −70°C)

Additional reagents and equipment for determining protein concentration (*APPENDIX 3A*; Olson, 2016)

Prepare tissue

When preparing HLM it is essential to precool the equipment and to maintain, using ice buckets, the liver tissue, solutions, centrifuge tubes, and grinders/homogenizers at 4°C. A full-face shield and protective clothing, including gloves, must be worn when working with human tissue and high-speed homogenizing equipment.

1. Weigh frozen liver tissue into clean (labeled) open-top 50-ml polycarbonate centrifuge tube(s) containing 5 ml of ice-cold microsome preparation buffer per g of liver tissue. Allow tissue to thaw on ice.
2. Finely mince the liver tissue with surgical scissors.
3. Insert the tip of the mechanical tissue homogenizer (e.g., Ultra Turrax T25 set at 20,500 rpm) into the tissue solution twice, each time for 30 sec, with a 30-sec cooling period between bursts.

   If using less than 1 g of human liver tissue, increase the volume of the microsome preparation buffer to ensure that the tip of the tissue homogenizer probe is covered. This will enable homogenization without frothing.

   The time and number of bursts required for mechanical disruption of tissue will vary (e.g., 2 to 4 30-sec bursts) for certain tissues such as human kidney and dog and monkey liver, which are more fibrous than rat and mouse liver.

4. Transfer the ground liver solution into an ice-cold 30-ml Potter-Elvehjem grinder. Homogenize using eight full strokes using the matched motorized Teflon pestle.

   A minimum of two full strokes is necessary to ensure sufficient homogenization. Increasing the number of strokes increases the yield of microsomes, but prolonged shear force and possible heating during this step can cause contamination of the homogenate by other proteins and possible degradation of microsomal enzymes.

Isolate microsomes

5. Pour the homogenate into clean (labeled), open-top 50-ml polycarbonate centrifuge tubes. Balance pairs of tubes using ice-cold microsome preparation buffer.

6. Centrifuge the homogenate for 10 min at 700 × g, 4ºC, then increase to 10,000 × *g* for a further 10 min.

   Starting at the lower centrifugal force ensures that fibrous tissue sediments first, followed by organelles such as mitochondria. This approach reduces entrapment and improves microsomal protein yield.

7. Carefully remove the supernatant fraction using a syringe with a blunt-end needle and transfer to ice-cold ultracentrifuge tubes (~25 ml). Discard tissue pellets. Balance pairs of tubes using ice-cold microsome preparation buffer.

   Try to avoid transferring the light-colored fluffy fat layer that 'floats' towards the top of the supernatant fraction layer. The supernatant fraction is commonly referred to as the S9 homogenate fraction.

8. Ultracentrifuge the supernatant fraction for 60 min (once maximum speed has been attained ~15 min) at 105,000 × *g*, 4°C.

   For example, a Beckman L8-70M ultracentrifuge equipped with a Ti 50.2 rotor is run at 34,000 rpm. Ultracentrifugation sediments the membrane fraction, leaving non-membranous proteins in the supernatant fraction.

9. Decant the supernatant (cytosolic) fraction.

10. Add 3 ml of microsome preparation buffer per g of liver tissue (as determined in step 1) to the ultracentrifuge tube(s).

11. Using a plastic round-ended probe, gently lift the pellet away from the wall of the ultracentrifuge tube(s). Pour the pellet and buffer into a cold 10-ml Potter-Elvehjem grinder.

12. Add an additional 2 ml of microsome preparation buffer to the ultracentrifuge tube(s) and wash away any remaining protein. Combine with the liquid in the Potter-Elvehjem grinder.

13. Resuspend the pellet by gentle hand homogenization (three to four full strokes).

14. Pour the homogenate back into the ultracentrifuge tube(s). Balance pairs of tubes using ice-cold microsome preparation buffer.

15. Ultracentrifuge the homogenate 60 min at 105,000 × g, 4°C, as for step 8.

16. Decant the supernatant fraction and add 1 ml of microsome storage buffer per g of liver tissue. Repeat step 11 and step 13.

17. Set aside a small portion to determine the protein concentration (*APPENDIX 3A*; Olson, 2016).

18. Aliquot the remainder of microsomal homogenate into labeled glass vials cooled on dry ice to snap freeze before storing at −70°C.

    While microsomes are stable for up to 10 freeze-thaw cycles (Pearce et al., 1996), freezing in portions sufficient for a single experiment eliminates multiple freeze/thaw cycles as a source of variability.

    For proteomic analyses, omit glycerol from the microsome storage buffer, which may affect the stability of CYP enzymes.

Be aware that the glucuronidation of acidic agents in the presence of glycerol in the storage buffer may also result in formation of compound-glycerol esters, which are not metabolites but artifacts due to the presence of glycerol (Obach, 2009).

If using fresh isolated rat liver, perfuse the tissue with the microsome preparation buffer before mincing with the surgical scissors. Overnight fasting of animals is advisable to reduce the glycogen content. Glycogen forms a brownish-orange, transparent layer at the bottom of the centrifuge tube, and microsomal protein adheres loosely to glycogen. Microsomes can be gently dislodged from the glycogen using a glass rod while the glycogen remains bound to the centrifuge tube.

SUPPORT PROTOCOL 2

DETERMINATION OF CYTOCHROME P450 (P450) CONTENT OF MICROSOMES

All P450 enzymes contain a heme iron (Fe^{2+}) that gives a characteristic absorption spectrum at 450 nm when complexed with CO. The quantity of P450 is determined by measuring the absorbance difference between 450 and 490 nm and using an extinction coefficient (ϵ) of 91 mM^{-1} cm^{-1} (Omura and Sato, 1964). A yield of 0.2 to 0.5 nmol P450/mg microsomal protein from human livers can be expected.

Materials

Crystalline sodium dithionite ($Na_2S_2O_4$)
Carbon monoxide (CO; e.g., BOC, Praxair) in a tank with a two-stage regulator; attach flexible tubing with the appropriate end outlet (e.g., blunt end needle, fine Pasteur pipet) to the outlet of the regulator.
Microsome storage buffer (see recipe)

Semi-micro quartz spectrophotometric cuvettes (1.4 ml) with 10-mm light path
UV/vis scanning spectrophotometer, preferably a dual-beam instrument (e.g., Agilent, Eppendorf, JASCO)

Additional reagents and equipment for preparation of human liver microsomes (HLM; see Support Protocol 1)

1. Using microsome storage buffer, prepare 2 ml of HLM at a concentration of 1 mg/ml protein (see Support Protocol 1). Mix either by repeated inversion of the tube or by very gentle vortexing.

   Approximately 4 ml of microsome solution is needed for standard cuvettes.

2. Dispense 1 ml of the microsomal preparation into matched sample and reference cuvettes, wipe the outside of the cuvettes to remove any condensation, and place them into the spectrophotometer. Record a baseline spectrum between 400 and 500 nm.

3. Add a few crystals of sodium dithionite (<5 mg, which approximates the amount that can be held on the tip of a tiny spatula) to both cuvettes. Cap or cover with Parafilm and mix gently by inversion to dissolve.

   Dithionite acts as a reducing agent to ensure that all heme iron is in a reduced state. Because reduced P450 is unstable, it is important to minimize the time between dithionite addition and CO treatment.

4. Remove the sample cuvette and, in a controlled environment (e.g., fume hood), bubble CO via a fine blunt-end needle adaptor into the sample cuvette only for ~30 sec at a rate of 1 bubble/sec.

CAUTION: *Remember that CO is toxic and can be fatal at high exposures. Follow all relevant Occupational Health and Safety procedures when using CO and ensure the cylinder is turned off when not in use.*

An excessively high flow rate of CO will cause frothing and protein denaturation.

5. Replace the sample cuvette in the spectrophotometer and re-scan and record the spectrum from 400 to 500 nm.

   The spectrum should exhibit an absorption maximum at 450 nm. A prominent peak at 420 nm indicates the presence of hemoglobin or inactivated CYP.

6. Calculate P450 content in the diluted sample as follows:

$$\text{P450 concentration (m M)} = \text{A} / \varepsilon$$

   where $A = A_{450} - A_{490}$, the absorbance difference between 450 and 490 nm and $\varepsilon = 91\ \text{mM}^{-1}\ \text{cm}^{-1}$, the extinction coefficient (450 to 490 nm) for CYP.

7. Express results as nmol P450/mg microsomal protein.

DETERMINATION OF THE AMOUNT OF MICROSOMAL PROTEIN PER GRAM OF LIVER (MPPGL)

SUPPORT PROTOCOL 3

In vitro kinetic data using HLM as the enzyme source may be used to calculate the in vivo hepatic clearance of a compound using in vitro–in vivo extrapolation (IV-IVE; Miners et al., 2010; Beaumont et al., 2014). The IV-IVE calculation requires knowledge of the amount of microsomal protein per g of liver (MPPGL), which is corrected for the loss of microsomal protein during preparation of microsomes and the size of the liver. The MPPGL can be estimated by determining P450 content on both the liver homogenate (S9 fraction) and the microsomal fraction. Due to the presence of hemoglobin in the S9 fraction (Support Protocol 1, step 7), the method of Matsubara et al. (1976) is commonly employed, with the usual extinction coefficient of 91 $\text{mM}^{-1}\text{cm}^{-1}$ being replaced by 104 $\text{mM}^{-1}\text{cm}^{-1}$ to correct for the presence of oxidized hemoprotein.

The recovery of microsomal protein, and hence fraction of microsomal protein loss during preparation, is determined as follows (Wilson et al., 2003):

Recovery factor = P450 microsomes (nmol per tissue sample)/P450 homogenate (nmol per tissue sample).

Values of MPPGL are corrected for the loss of microsomal protein during preparation using the following equation:

MPPGL = nmol P450 in homogenate per g liver/nmol P450 in microsomes per mg microsomal protein.

MEASUREMENT OF THE KINETICS OF DRUG GLUCURONIDATION BY HUMAN LIVER MICROSOMES

SUPPORT PROTOCOL 4

By way of example, this protocol describes the incubation conditions for the measurement of zidovudine (3′-azido-3′deoxythymidine; AZT) glucuronidation formation by HLM. Negative controls without substrate, cofactor (UDP-glucuronic acid, UDP-GlcUA), and HLM should be included in each set of experiments. Incubations at each substrate concentration should be performed at least in duplicate.

Materials

Human liver microsomes (HLM; Support Protocol 1)
0.1 M potassium phosphate buffer, pH 7.4 (*APPENDIX 2A*),
Alamethicin (from *Trichoderma viride*; A.G. Scientific; *http://www.agscientific.com*)
40 mM magnesium chloride ($MgCl_2$)
3′-azido-3′ deoxythymidine (AZT; Sigma-Aldrich)
50 mM UDP-GlcUA (trisodium salt; Sigma-Aldrich)
11.6 M perchloric acid
AZT-β-D-glucuronide (Sigma-Aldrich) for standards
Tabletop centrifuge

Additional reagents and equipment for the analytical method (typically LC/MS or HPLC; Uchaipichat et al., 2006; Rowland et al., 2007)

1. Dilute HLM to 5 mg/ml in 0.1 M potassium phosphate buffer (pH 7.4) and pre-incubate with alamethicin (50 µg/ml) on ice for 30 min prior to use.

   Treatment with the pore-forming peptide alamethicin is necessary to activate microsomes (i.e., remove the latency) for use in glucuronidation assays (Boase and Miners, 2002).

2. To each microcentrifuge tube on ice, add:

   118 µl of distilled water
   20 µl of 1 M phosphate buffer, pH 7.4 (final concentration of 0.1 M)
   20 µl of activated HLM (final concentration of 0.5 mg/ml microsomal protein)
   20 µl of 40 mM magnesium chloride (final concentration of 4 mM)
   2 µl of AZT dissolved in water (10× desired concentration in incubations).

   Eight to ten AZT concentrations that span the K_m (typically 0.2 to 3 times K_m) for AZT glucuronidation by HLM should be employed for kinetic studies. There should be a sufficient number of data points below the K_m for accurate determination of kinetic constants. Because the known K_m for AZT glucuronidation by HLM is approximately 1 mM, the recommended substrate concentrations for this assay are; 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 2.5, and 3 mM. If the K_m of a substrate is unknown, a range-finding experiment (e.g., 1, 10, 100, 1000 µM) should be conducted prior to the definitive experiment. Bovine serum albumin (BSA, 1% to 2% w/v) is added to incubations of substrates for UGT 1A9, 2B7, and 2B10 (see Commentary). AZT is soluble in water. For substrates with limited aqueous solubility, the preferred solvent is DMSO, with the final concentration of solvent present in incubations being ≤1% (v/v) (Uchaipichat et al., 2004).

   After a 5-min pre-incubation at 37°C, initiate the reaction by the addition of 20 µl of UDP-GlcUA (final concentration 5 mM). Vortex and continue the incubation for 60 min at 37°C.

   For the incubation conditions described here, product (AZT-β-D-glucuronide) formation is linear with respect to both microsomal protein concentration and incubation time, and substrate depletion is 10%.

3. Terminate the reaction after 60 min by the addition of 3 µl of 11.6 M perchloric acid. Vortex, centrifuge for 10 min at 10,000 × *g*, 4ºC, and decant the supernatant fraction for the quantification of AZT-β-D-glucuronide formation by HPLC (Uchaipichat et al., 2006; Rowland et al., 2007) or LC/MS.

   For incubations containing BSA, the volume of perchloric acid used for reaction termination should be increased to 6 µl.

4. Prepare a standard curve using five AZT-β-D-glucuronide concentrations ranging from 0.1 to 5 µM. Calculate the concentration of AZT-β-D-glucuronide present in the incubation samples using the standard curve.

Convert the concentrations of AZT-β-D-glucuronide measured in the incubation samples to a rate (pmol/min/mg). Kinetic constants (K_m and V_{max}) are obtained by fitting the equations for empirical kinetic models (Michaelis-Menten, substrate inhibition, etc.) to the experimental data (i.e., rate of product formation at each substrate concentration; see Miners et al., 2010).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and assays. For common stock solutions, see APPENDIX 2A.

Alamethicin, 10 mg/ml

Dissolve alamethicin (A.G. Scientific, *http://www.agscientific.com*) in methanol to a concentration of 10 mg/ml. Store up to 1 year at −20°C.

6β-Hydroxytestosterone

Weigh out approximately 2 mg of 6β-hydroxytestosterone (Sigma-Aldrich, formula weight 304.4). Dissolve the 6β-hydroxytestosterone in 2.19 ml methanol to yield a final concentration of 0.913 mg/ml (3 mM). Store up to 1 year at −20°C. Dilute the stock solution to prepare a 100 μM working solution. The routine dilution procedure is to place 133 μl of the stock solution into 3.87 ml deionized water.

Microsome preparation buffer

Add 2.88 g potassium chloride and 2.5 ml of 1 M potassium phosphate buffer, pH 7.4 (*APPENDIX 2A*) to 100 ml water. Mix well to dissolve the KCl then dilute to 250 ml with water. This buffer must be used within 1 to 2 days. Store at 4°C.

Composition: 10 mM potassium phosphate buffer, pH 7.4, with 1.15% (w/v) potassium chloride.

Microsome storage buffer

Weigh out 20 g of glycerol and mix with 50 ml water. Add 10 ml of 1 M potassium phosphate buffer, pH 7.4 (*APPENDIX 2A*), and dilute to 100 ml with water

This buffer must be used within 7 days. Store at 4°C.

Composition: 0.1 M potassium phosphate buffer, pH 7.4, with 20% (w/v) glycerol.

NADPH generating system (10×)

30 mM glucose-6-phosphate (pH 7.4)
4 U/ml glucose-6-phosphate dehydrogenase
10 mM $NADP^+$
30 mM $MgCl_2$
Prepare immediately before use

DL-Propranolol, 10 mM

Prepare a 2.96 mg/ml solution of DL-propranolol (e.g., Sigma-Aldrich) in DMSO (10 mM). Store up to 6 months at −20°C. Dilute 10 μl with 90 μl of DMSO to prepare a 1 mM working stock.

Testosterone, 20.8 mM

Prepare a 6 mg/ml solution of testosterone (formula weight 288.4) in acetonitrile. Store up to 1 year at −20°C.

UDP-glucuronic acid (UDP-GlcUA), 20 mM

Prepare a 13 mg/ml solution of UDP-GlcUA (Sigma, cat. no. U-6751; formula weight of the trisodium salt = 646.2) in distilled water. Store up to 1 year at −20°C.

COMMENTARY

Background Information

Metabolic biotransformation alters the rate of elimination of a compound from the body and can have a significant effect on the efficacy and safety of the agent (see Gibson and Skett, 2001; Baillie, 2008; Chiba et al., 2009; Nassar, 2009). Although the liver is the major site of drug metabolism, other organs, including intestines, kidneys, and lungs, contribute to metabolic clearance as well. Enzymatic drug metabolism processes are broadly divided into two reaction categories: functionalization (or phase I) and conjugation (or phase II) (Josephy et al., 2005). Functionalization reactions, which include oxidation, reduction, and hydrolysis, create or expose functional groups such as OH, SH, NH_2, and COOH. Conjugation reactions typically involve the covalent linkage ('conjugation') of a polar endogenous compound (e.g., glucuronic acid, sulfate, glutathione) to a nucleophilic 'acceptor' functional group (e.g., –OH, $–CO_2H$, $–NH_2$, –SH) on the substrate. The products of functionalization reactions are often substrates for conjugation enzymes. In some instances, glucuronides may also be substrates for cytochrome P450 (CYP) enzymes, in particular CYP2C8.

The CYP enzymes are a family of heme-containing mixed function oxygenases that catalyze hydroxylation, epoxidation, and N-/O- dealkylation reactions. These membrane-bound enzymes are located primarily on the endoplasmic reticulum in hepatic cells, and can be isolated in subcellular microsomal (literally "small bodies") or S9 (supernatant fraction) by centrifugation (see Support Protocol 1). The major human forms involved in hepatic drug metabolism are CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5. Approximately 60% of marketed drugs are substrates for CYP enzymes. The expression and activity of CYP enzymes are influenced by many factors including diet, drug use, age, genetics, and gender, and can vary greatly between individuals (Zanger and Schwab, 2013). The CYP oxygenation reactions require atmospheric oxygen, NADPH, and NADPH-cytochrome P450 reductase to catalyze the reaction:

$$RH + NADPH + H^+ + O_2 \rightarrow NADP^+ + H_2O + ROH$$

The P450s in microsomes and the S9 supernatant fractions require the addition of the cofactor NADPH for enzymatic activity. The NADPH can be added directly or as an NADPH-generating system:

$$\text{glucose-6-phosphate} + NADP^+ \xrightarrow[Mg^{2+}]{G6PDH} NADPH + \text{6-phosphogluconolactone} + H^+$$

The UDP-glucuronosyltransferases (UGT) are a family of enzymes that also reside in the endoplasmic reticulum, with UGT-catalyzed glucuronidation being responsible for the elimination of about 20% of marketed drugs. The major human forms involved in hepatic drug glucuronidation are UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7, and UGT2B15 (Miners et al., 2010). Their expression, tissue distribution, and activity are influenced by many of the same factors affecting CYP enzymes (Miners and Mackenzie, 1991; Zanger and Schwab, 2013). Catalysis by major drug metabolizing UGTs requires UDP-GlcUA as a cofactor:

$$R - X + UDP - GlcUA \rightarrow R - XGlcUA + UDP$$

Because metabolism influences drug efficacy and safety, the metabolic properties of drug candidates are often determined early in the drug discovery process (Di et al., 2003; Klopf and Worboys, 2010). Microsomes, and less often, S9 supernatant fractions, are commonly used for in vitro screens for metabolic stability (see Basic Protocol and Alternate Protocols 1 and 2).

The advantages of microsomes for metabolic stability assays are (Wrighton et al., 1995) as follows.

1. Convenient storage and stability at −70°C for several years provides a readily available source of metabolic enzymes (Pearce et al., 1996).

2. Because microsomes are a subcellular fraction, plasma-membrane penetration and uptake of the test agent are not limiting factors in the assay. Hepatocytes, engineered cell lines, or in vivo models can be used for cellular uptake studies. Described in *UNIT 7.7* (Silverstein et al., 2004) are uptake and efflux assay procedures.

3. Microsomes contain the major drug metabolizing enzymes, including CYP and UGT.

4. High compound concentrations may be tested, as cytotoxicity is not a factor in the assay.

5. Relative to cell-based assays, microsomes are easy to use and are more cost-effective.

The disadvantages of microsomes for metabolic stability assays are as follows.

1. They are not as physiologically relevant as hepatocytes or in vivo models.

2. They lack soluble conjugation (e.g., sulfotransferases) and other enzyme activities.

3. They require supplementation with cofactors.

Microsomes are also often used to screen for potential drug-drug interactions, such as inhibition of CYP and UGT (Perloff et al., 2009; Miners et al., 2010) as described in *UNIT 3.9* (Delaporte and Rodrigues, 2002). Other uses for microsomes in drug discovery and development include metabolite identification, comparison of metabolism by different species (Chauret et al., 1997), prediction of in vivo clearance (Miners et al., 2010; Obach, 2012), and reaction phenotyping (Zhang et al., 2007; Nassar, 2009; Miners et al., 1994, 2010; Zientek and Youdim, 2014). While the most common use of liver microsomes is to evaluate CYP metabolism, concurrent (Caldwell and Yan, 2014) or separate (Miners et al., 2006, 2010; Gill et al., 2012) analysis of UGT metabolism has become increasingly popular.

Numerous factors are known to affect the activities of human liver microsomal CYP and UGT enzymes. The typically lipophilic substrates of CYP and UGT frequently require solubilization with an organic solvent to remain in solution in the aqueous incubation medium. However, organic solvents variably affect CYP (Busby et al., 1999) and UGT enzymes (Uchaipichat et al., 2004). For this reason, the final concentration of the solvent in microsomal incubations is typically limited to $<1\%$ (v/v).

Lipophilic bases often bind extensively to the microsomal membrane (McLure et al., 2002). Microsomal nonspecific binding reduces the concentration of unbound drug in the incubation medium, resulting in an overestimation of K_m and K_i. As a result, in vitro intrinsic clearance (CL_{int}) and the potential for drug-drug interactions are underestimated. For compounds that bind extensively to microsomes, the fraction bound to incubation constituents should be determined using techniques such as equilibrium dialysis or ultracentrifugation, with the results used to correct calculations of kinetic constants.

It has become recognized in recent years that long-chain unsaturated fatty acids released from the microsomal membrane during incubation are potent inhibitors of several drug-metabolizing enzymes including CYP 1A2, 2C8, and 2C9, and UGT 1A9, 2B4, 2B7, and 2B10 (Rowland et al., 2007; 2008a, 2008b; Wattanachai et al., 2012). Consequently, microsomal K_m and K_i values for substrates/inhibitors of these enzymes are overestimated, and CL_{int} values under-estimated. As indicated in Support Protocol 4, inclusion of BSA (1% to 2%, w/v) to microsomal incubation systems sequesters the inhibitory fatty acids, thereby enhancing the accuracy of the measurement of the kinetic constants. To accomplish this, BSA binding of the test compound must be measured (e.g., by equilibrium dialysis) and accounted for when calculating the kinetic parameters.

As noted (Support Protocol 4), acyl glucuronides may be unstable in microsomal incubations at pH 7.4, undergoing hydrolysis, transacylation, or both. It has been demonstrated (Miners et al., 1997) that degradation of acyl glucuronides may be diminished when incubations are performed under weakly acidic conditions (pH 6.8).

The development of an analytical method for the detection of each test compound is required, with HPLC and LC/MS being the most commonly employed techniques. While it is essential to determine appropriate precursor and fragment ions using a tandem mass spectrometer operated in multiple reaction monitoring mode, "generic" chromatography methods are often used instead (Drexler et al., 2007; Xu et al., 2010; Korfmacher, 2013; Wen and Zhu, 2015).

Critical Parameters and Troubleshooting

Shown on Table 7.8.1 are problems commonly encountered when executing the protocols described in this unit, along with possible causes and suggested remedies.

Anticipated Results

Microsomal isolation usually yields ~80 to 160 mg microsomal protein per 4 g of liver tissue for many animal species. A CYP content of 0.3 to 1.0 nmol CYP/mg liver microsomal protein can be expected from untreated animals.

Table 7.8.1 Troubleshooting Guide for Determination of Compound Metabolism Using Liver Microsomes

Problem	Possible cause	Solution
Low compound recovery	Poor extraction in solvent	Re-extract pellet in a different solvent, such as acetonitrile
	Binding to incubation tube	Repeat using glass tubes. Use alternate protocol adding microsomes before the test compound and initiating with NADPH.
Low metabolic turnover in positive controls	Low activity from microsomes	Repeat with freshly prepared/purchased microsomes. Ensure neutral pH of buffer solution.
	Low NADPH levels	Make fresh NADPH solutions. NADPH will oxidize over time; do not use if a distinct yellow color. Use NADPH-generating system.
	Essential reagent not added	Ensure compound, microsomes and NADPH were added to the incubation
Turnover in 0-min samples	Reaction not immediately terminated with acetonitrile	Stop reaction with ice-cold acetonitrile immediately after addition of microsomes; acetonitrile may be added before microsome addition, but take care to use fresh pipet tips for further microsome pipetting to prevent enzyme degradation
	Substrate contaminant may have identical structure as metabolite	Mathematically correct for contaminant level or obtain higher quality substrate
Low CYP content/activity from prepared microsomes	Degraded enzymes	Always keep tissue at 4°C or below during preparation
Variable results	Varying microsome preparations	Use microsomes pooled from multiple animals or donors
	Analytical samples lack homogeneity	Ensure stored samples prone to phase separation are homogeneous prior to analysis

Metabolic stability can vary greatly between compounds and species. Shown in Table 7.8.2 is an example of the rate of depletion of testosterone using the indicated analytical method.

Representative metabolic stabilities for substrates with human liver microsomes as the enzyme source are displayed on Table 7.8.4. These substrates are selectively metabolized by the CYP enzymes indicated. Other examples of compound microsomal stability can be found in Chauret et al. (1997); Di et al. (2004), and Drexler et al. (2007).

Time Considerations

Microsome preparation and characterization can be accomplished in 5 to 8 hr. Incubations for the determination of metabolic stability of ten compounds can be performed in a day using the Basic Protocol. Using the plate-based protocol (see Alternate Protocol 1), 32 compounds per plate can

Table 7.8.2 Depletion of Testosterone (50 μM) using Liver Microsomes[a]

Species	Depletion rate (pmol/min/mg protein)	Mean
Rat	2272	2518
	2764	
Dog	593	696
	799	
Human	1174	1011
	849	

[a]For analytical conditions see Table 7.8.3. Rate of depletion is based on the amount of substrate depleted and converted to metabolites.

Table 7.8.3 Solvent Gradient Used in Testosterone Analysis Referred to in Table 7.8.2[a]

Run time	Solvent A (%)	Solvent B (%)
0	95	5
1.6	10	90
3.7	10	90
3.9	95	5
7.0	95	5

[a]Aliquots of the supernatant fractions were subjected to LC/MS analysis, with single ion monitoring, with the analysis conditions as follows: Phenomenex AQUA C18 (50 × 2–mm; 125 Å) column; ambient temperature; mobile phase flow rate of 0.4 ml/min; positive ion; MRM: 289.2 to 109.2; injection volume of 20 μl; solvent A: water containing 0.1% formic acid; solvent B: acetonitrile containing 0.1% formic acid.

be prepared for analysis in 8 hr. The time required for method development and analysis by HPLC or LC/MS varies by the structures and degree of variation in the compounds. Auto-tuning software modules are available from instrument manufacturers to reduce LC/MS method development time. Many incubation protocols of higher throughput are described in the literature (Di et al., 2003; Di et al., 2004; Drexler et al., 2007).

Conflict of Interest

David M. Stresser and Charles L. Crespi are employed by Corning Incorporated, a manufacturer of multiwell plates, subcellular fractions, recombinant drug metabolizing enzymes, and other reagents for these assays. Corning Incorporated is also a service provider for in vitro drug metabolism assays using liver microsomes and other sources of active enzymes.

Kathleen M. Knights and John O. Miners declare no conflicts of interest.

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Table 7.8.4 Activities of Cytochrome P450 Enzyme-Selective Substrates with Human Liver Microsomes

Substrate	CYP Enzyme	Substrate concentration	Incubation time (min)	Enzyme activity (pmol/min/mg)
Phenacetin	1A2	25 μM	20	2910
Tolbutamide	2C9	100 μM	30	34
S-Mephenytoin	2C19	100 μM	30	430
Dextromethorphan	2D6	5 μM	20	210
Chlorzoxazone	2E1	30 μM	20	3060
Testosterone	3A4	50 μM	30	1250

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Internet Resources

http://medicine.iupui.edu/flockhart/
Provides drug-drug interaction tables.

http://www.cypalleles.ki.se/
Provides nomenclature and alleles for CYPs.

http://www.flinders.edu.au/medicine/sites/clinical-pharmacology/udp-glucuronosyltransferases.cfm
Provides information on UGT sequences.