Human and Animal Liver Microsome Thawing and Incubation Protocol

Background

The liver is the major organ for metabolism of endogenous substrates as well as exogenous drugs. There are several *in vitro* tools available to help researchers study the metabolic fate of drug candidates, including isolated fresh or cryopreserved hepatocytes, liver slices, and sub-cellular fractions such as liver microsomes and S9 fractions. These sub-cellular fractions are prepared from the liver via a series of homogenization and ultracentrifugation steps. .

An initial lower speed centrifugation of liver homogenate at 10,000g produces the S9 fraction also known as the supernatant of this centrifugation. The S9 fraction contains all phase I and phase II enzymes. A further centrifugation of the S9 fraction at 100,000g yields the endoplasmic reticulum-derived microsomes. Microsomes are an enriched source of cytochrome P450 (CYP) and flavin monooxygenases (FMO) enzymes. Additionally, some phase II enzymes (e.g. certain uridine glucuronide transferases (UGT) isoforms and epoxide hydrolase (EH) enzymes) are present in microsomes. . Microsomes can be used to investigate UGT activity; however, microsomal membranes restrict access of UGT substrates and/or cofactors. Optimal UGT activity can be achieved by the addition of MgCl2 and a pore-forming antibiotic (i.e. alamethicin). These components allow for the efficient transfer of a glucuronide product and the co-factor, uridine 5′-diphospho-alpha-D-glucuronic acid (UDPGA) within the microsomal matrix.

Individual or pooled donor microsomes can be used for metabolism-related studies. Pooled donors can represent the "average" human population or particular factors of research interest, such as age, BMI, or limited capabilities for certain CYP isoforms.

Important notes

- The following is a general procedure for metabolism studies in liver microsomes. It is recommended that the metabolism of a test article be measured under initial rate conditions. Since every test article is different, each will require separate optimization of microsomal protein concentration, test article concentration, and incubation times. Ideally, the amount of substrate consumed during the reaction should be 10% to 15% in order to measure initial rates of metabolite formation.
- Microsomes should be stored at -80°C until immediately prior to experiment. Microsomes can be refrozen twice without compromising enzymatic activity.
- Use universal safety precautions and appropriate biosafety cabinet when handling primary hepatocytes.

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Critical materials and reagents

- 100 mM Phosphate buffer, pH 7.4, prepared with ddH_2O
- 20 mM NADPH, solubilized in 100 mM phosphate buffer
- UDPGA, Alamethicin, and MgCl (if measuring UGT activity)
- Test article
- Organic solvent to stop reaction

Equipment

- 37°C water bath
- N_2 evaporator
- LC-MS/MS

Technical support

For questions related to this protocol, contact us at:

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Protocol

1. Prepare a 100X stock of the test article in solvent.
   - → If the test article is insoluble in water, then acetonitrile is the preferred solvent.
   - → Because organic solvents can inhibit CYP activities, always limit the final concentration of any organic solvent to <1%.
2. Thaw microsomes slowly on ice. Adjust concentration to 20 mg/mL, if necessary.
3. If you plan to measure UGT activity, see Note at the end of this protocol.
4. For a total of 190 µL, add the following:
   - → 183 µL of 100 mM buffer
   - → 2 µL of 20 mM NADPH (1mM final concentration)
   - → 5 µL microsomes (0.5 mg/mL final protein concentration)
5. Pre-incubate microsomes, buffer, and test article in water bath for 5 minutes.
   - → If you plan to incubate longer than 60 minutes in step 7, consider using a higher concentration of NADPH or a NADPH-recycling system (NRS), which consists of NADP, G6P, and G6PDH.
6. Initiate the reactions with the addition of 10 µL 20 mM NADPH.
7. Incubate up to 60 minutes at 37°C with gentle agitation.
8. Terminate reactions by the addition of 200 µL organic solvent (i.e. ethyl acetate).
9. Vortex samples, and centrifuge at approximately 3000 rpm for five minutes.
10. Withdraw the supernatant from the protein pellet.
11. Analyze according to your analytical method.
12. Controls include:
    - → Zero time point with test article
    - → Longest time point, without NADPH
    - → Heat-inactivated microsomes (45°C pretreatment for 30 min)
    - → Incubation with testosterone or other specific CYP substrate

Note: For the measurement of UGT activity, adjust final reaction volume in step 4 to include:

- → 5 mM UDPGA
- → 50 mg alamethicin/mg microsomal protein
- → 1 mM MgCl

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