CHEM 4422 Dr Stone

Microsome Metabolism Assay

Liver microsomes can be used to model and predict the metabolism of a drug when it is orally ingested (3). Microsomes are made by homogenizing liver tissue and then removing all of the cellular machinery except for the fraction that contains the enzymes that metabolize foreign molecules (e.g.cytochrome p450, CYP450). These enzymes catalyze the addition of hydroxyl groups (OH) to xenobiotic molecules. This makes the foreign molecules more water soluble and thus easier to be excreted by the kidneys. The CYP450 enzymes contain an iron in the center that gives up electrons in this process. In order for the enzyme to work on another molecule, the enzyme must get some electrons to get reset to its active state. Another enzyme is present in the microsomes, cytochrome reductase (CytRed), and this enzyme transfers electrons from NADPH (Nicotinamide adenine dinucleotide phosphate) to the CYP450. To keep the levels of NADPH high for the regeneration of the CYP450, another enzyme and substrate must be added: glucose-6-phosphate and glucose-6-phosphate dehydrogenase will regenerate NADPH.

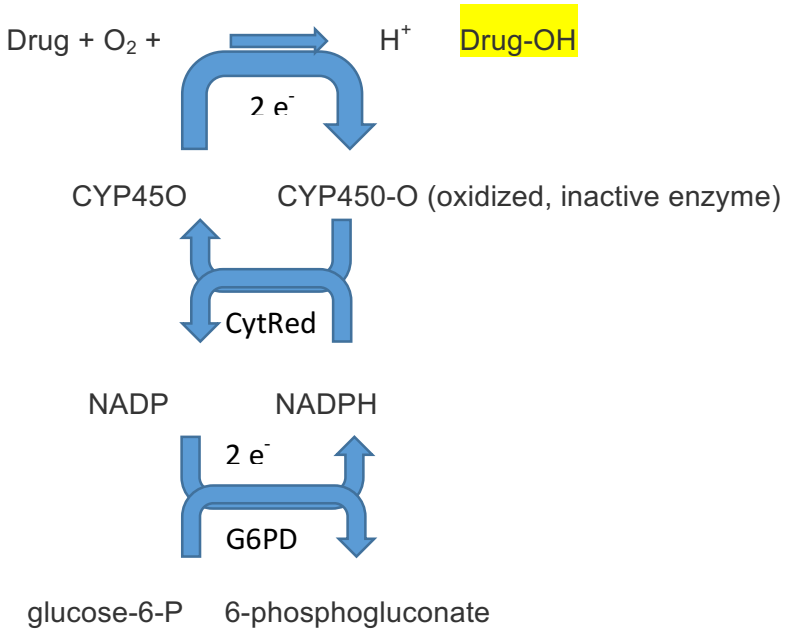

Figure 1: Microsomal assay for stability of drugs. CYP450 is an enzyme present in liver microsomes. After each reaction cycle, the CYP450 requires cytochrome reductase (CytRed) and NADPH to be regenerated, both of these are in liver microsomes. Liver microsomes can not regenerate NADPH after it is oxidized to NADP, so glucose-6-phosphate and glucose-6-phosphate dehydrogenase (G6PD) are added to the assay mixture.

For this assay you will need the following solutions:

Stock solutions:

Phosphate buffer

5 mL of 1M monobasic phosphate

5 mL of 1M dibasic phosphate

These will be used to make a 100mM phosphate buffer, pH 7.4. You will need to determine how much of each 1M solution is needed to make 1.0 mL of the 100mM pH 7.4 phosphate buffer.

Other solutions

NADP 30mM

Glucose-6-phosphate 300 mM

Magnesium Chloride 30 mM

Drug (provided) 10mM

Glucose-6-phosphate dehydrogenase (provided) 3×10^4 units/mL

Microsomes (provided) 25 mg/mL

The reaction mixture will contain the following

Ingredient	Final Conc
NADP	1mM
Glucose-6-P	10mM
G6PD	1 unit/mL
$MgCl_2$	4 mM
microsomes	1.25 mg/mL
drug	100uM

You will need to determine the volume of each reactant that is needed. The total volume will be 300 uL. Do these calculations **before** coming to class.

Each group will test one drug and have an appropriate control. You will need to determine the control **before** you come to class.

Basic Incubation:

Add all of the solutions, **except the drug**, to an Eppendorf tube, vortex.. Put in a shaker at 37C for 5 min.. Remove, vortex.. Add drug.. Vortex.. Remove 50uL aliquots and put into new Eppendorf tubes. Vortex all tubes. Put 5 tubes in a shaker at 37C. The remaining tube is the "zero" time point. Remove a tube and work up the sample at 10, 20, 40, 80 and 160 min.

For analysis by HPLC, **work up** each sample using the following procedure:

Add 250 uL of cold Acetonitrile and vortex.Let sit in the fridge for 15 min. Centrifuse at 15K rpm for 15 min. Carefully remove the supernatant and place in an HPLC vial for analysis.

HPLC analysis:

Use a C-18 column and an 5%-95% acetonitrile (0.1% TFA) gradient over 10 min. Return to 5% acetonitrile over 5 min and run 5% for 5 min. Measure the absorbance of the metabolites at 278nm and 298 nm.

Standard curve: Using 10 mM stock solution, you will need to make a standard curve. You will need to determine how to do this. The stock solution is limited, so use as little as possible. Do these calculations before coming to class.

Naritomi , Y., Terashita, S., Kimura, S., Suzuki, A., Kagayama, A., Sugiyama, Y. Prediction of Human Hepatic Clearance from in Vivo Animal Experiments and in Vitro Metabolic Studies with Liver Microsomes from Animals and Humans, Drug Metabolism and Disposition 29 (2001) 1316-1324.