BD Biosciences

Assay Methods

Protocol: Cryopreserved Hepatocyte Metabolic Stability Assays

Isolated hepatocytes contain all the enzymes and co-factors needed for phase I and phase II drug metabolism, making them an excellent in vitro model for assessment of drug metabolic stability and metabolite profiling. The following protocol describes a method for evaluating the metabolism of a test compound using cryopreserved human and rat hepatocytes. A positive control assay is included in the protocol for the phase I (P450) and phase II (UDP-glucuronosyl transferases [UGT] and sulfotransferase [SULT]) metabolism of 7-ethoxycoumarin (7-EC). 7-EC is O-deethylated by P450, and conjugated by UGTs, and, to a lesser extent SULT, (about 15:1 glucuronidation vs. sulfation) in human hepatocytes. The reverse is true for rat hepatocytes where sulfation occurs to a much greater extent than glucuronidation.

Chemicals and Suppliers	
Chemical	**Supplier (Catalog No.)**
7-Ethoxycoumarin (7-EC)	Sigma Chemical Co. (E1379)
7-Hydroxycoumarin (7-EC metabolite standard)	Sigma Chemical Co. (U-7626)
7-Hydroxycoumarin Glucuronide (7-EC metabolite standard)	*BD Biosciences (451022)
7-Hydroxycoumarin Sulfate (7-EC metabolite standard)	*BD Biosciences (451024)
Dimethyl Sulfoxide (DMSO)	JT Baker (T15599)
ZnSO4	Sigma Diagnostics (14-4)
Barium Hydroxide	Sigma Diagnostics (14-3)
Williams E Media	Sigma Chemical Co. (N1878)
Krebs-Henseleit Buffer (KHB)	Sigma Chemical Co. (K-3753)

Materials:

- BD Gentest™ cryopreserved human or rat hepatocytes (BD Biosciences)
- BD™ hepatocyte purification kit (BD Biosciences, Cat. No. 454500)
- 37ºC water bath
- Low speed centrifuge
- Biosafety hood
- Vacuum pump
- Incubator at 37°C and atmospheric level capacity
- Multiwell plates (6-, 12-, 24-, 48-, or 96-well BD Falcon™ microplates)

Solutions:

A. KHB (Sigma Chem. Co., Catalog No. K-3753)
Prepared according to manufacturer's instructions

B. 1 M Fructose

C. 300 mM Glycine

D. 0.1 M 7-EC (dissolved in DMSO)

E. 7-EC metabolite standards dissolved in H_2O to a final concentration of 200 µM

F. Test compound (dissolved in DMSO or appropriate solvent)

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Procedure:

1. Prepare the test compound by dissolving in an organic solvent and diluting into KHB buffer (rat) or WEM (human). If an organic solvent is used to dissolve the test compounds, then the stock concentration should be high enough such that the final organic solvent concentration in the incubation is or the maximum. For example, final methanol and acetonitrile concentrations should be below 1%, while final DMSO concentrations should be below 0.2%.

   Note*: For optimal results with cryopreserved rat hepatocytes, prepare KHB buffer with 10mM fructose and 3mM glycine.

2. For test compound stocks dissolved in organic solvent, a convenient step is to further dilute the test compound in KHB buffer (rat) or WEM (human) to make a 2X concentrate (i.e. if the final assay concentration is to be 100 µM, then the 2X stock in KHB buffer would be 200 µM).

3. Prepare the positive control stock in DMSO as described above. The 7-EC/DMSO stock should be 0.1 M. The 2X concentrate of 7-EC in KHB buffer (rat) or WEM (human) is 200 µM, making the final assay concentration 100 µM.

4. Follow the hepatocyte thaw procedure and purification procedure enclosed with the BD™ hepatocyte purification kit in order to successfully recover the hepatocytes.

5. Resuspend the cells in KHB buffer (or KHB with 10mM fructose, 3mM glycine) (rat) or WEM (human) at a final concentration of about 0.5 x 10^6 cells per ml. A desirable final cell concentration for most applications is 0.25 x 10^6 cells/ml.

6. Mix equal volumes of test compound in KHB (rat) or WEM (human) and resuspend hepatocytes. The final volume will depend on the incubation vessel. For example, use 125 µL final volume per well in a 96-well plate and 200 µL per well in a 24-well plate.

7. Incubate in a humidified, 37°C, atmospheric pressure incubator for the desired length of time (e.g. 60 minutes). Multiple time points (10 minutes up to 120 minutes) are recommended for determining metabolic stability or metabolite profile of a test compound.

8. Quench the incubation with an equal volume of acetonitrile or other stop solutions that are compatible with the particular analytical method. The amount of stop solution added may also vary depending on the analytical method.

9. The positive control incubation should be quenched with $ZnSO_4$ and BaOH. The volume of each quench solution added should equal to 25% of the assay volume (e.g. 50 µL of each per 200 µL incubation volume).

   Note: The $ZnSO_4$ solution should always be added prior to the BaOH solution.

10. Transfer to 1.7mL micro centrifuge tubes and centrifuge at 14,000 rpm for 3 minutes to pellet debris.

11. Remove the supernatant for immediate analysis (e.g. LC/MS, HPLC) or store samples at -20°C.

12. **Analytical Method for Positive Control Assay (HPLC Method).**

a) A Waters 2690 or equivalent HPLC instrument should be used for separation of metabolites of 7-EC.

b) The HPLC column should be a C-18, 4.6 x 250 mm (5 µm). We recommend a Zorbax C-18 column (HP-Cat. No. 880975-902). The column temperature should be set to 45°C. The HPLC flow rate is 1 ml/minute.

c) Metabolite peaks are measured by UV detection at 320 nm or fluorescent detection of 320/380, gain of 100.

d) A typical injection volume is 100 µL of assay supernatant.

e) The HPLC mobile phases consist of the following: Mobile Phase A: 0.1% Trifluoroacetic Acid in H_2O, Mobile Phase B: 0.1% Trifluoroacetic Acid in acetonitrile

f) Initial HPLC conditions are 100% Mobile Phase A. Upon sample injection, Mobile Phase B is increased to 10% over a 15 minute period, followed by an increase to 60% over 10 minutes, and a final increase to 60% over a 10 minutes period to elute the 7-EC parent compound. The entire HPLC run time is 30 minutes.

g) Metabolites are quantitated by comparison to the peak areas of known amounts of authentic metabolite standards.

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