Suspension Hepatocyte Stability Incubations Protocol

Equipment and Reagents needed:

- Standard tissue culture treated 24-well plates
- Micropipette set
- Multi-channel pipettor
- Refrigerated bench top centrifuge
- 37°C Incubator
- Hepatocytes: ≥1 vial based on assay needs
    - At least 5.0 x 10^6 cells are needed per compound, assuming 5 time points, +controls and viability checks
- Hepatocyte incubation medium, depending on assay needs:
    - INVTROGRO CP (contains serum)
    - INVITROGRO HT (contains serum)
    - INVITROGRO HI (serum free)
    - INVITROGRO KHB (serum free)
    - CyrostaX Hepatocyte Incubation Medium (serum free)
- Test compound(s) and solvent
- Stop solution

Experimental Design and Technical Tips:

Purpose: to monitor test compound stability over time using human primary hepatocytes as a metabolic matrix.

- Typical incubation reactions contain 0.25 x 10^6 cells per well (250µL total) in a 24-well format.
- Common time points tested include 0, 10, 60, 120, and 240 min. (the 0-time point, non-incubated is 100% of starting compound).
    - Each time point should use a dedicated plate.
    - 3 replicate wells per time point.
- Positive controls: 7-Ethoxycoumarin (1.0 µM) or Midazolam (1.0 µM) in duplicate at 0 and 60 min.
- Negative control is vehicle only.
- Make sure the organic solvent used for test compound stocks is ≤ 0.1% total volume.
- Stop reaction, acetonitrile is commonly used as the stop solution.
    - Spike stop solution with internal standard for Metabolite ID (14C 7-ethoxycoumarin).
- Spin samples at 920 x g for 10 minutes at 10°C to separate protein precipitates from solubilized metabolite(s).
- For analytical assessment, there will be 19 wells/tubes per reaction, plus viability checks at each time point (zero substrate samples).

General Protocol

1. Warm 10mLs of incubation medium to 37°C
    a. This is enough medium for 1 test compound with 5 time points.
2. Prepare an appropriate volume of incubation media with test compound and keep at RT.

General Protocol Continued

3. Pipette 150 µL medium containing test compound into wells.
    a. For other well plate formats, 3/5 of the total well volume will be incubation media and test article.
4. Incubate plate at 37°C for 15 minutes.
5. Add stop reagent to 0 time point wells/plate (250µL or 1 volume).
6. Thaw, centrifuge, and dilute hepatocytes to 2.5 x 10^6/mL in sterile, warmed incubation media.
    a. Keep cells in 37°C incubator until needed, cells can be thawed prior to adding test article into plates.
7. Dispense 100 µL of hepatocytes into each well for a final concentration of 0.25x10^6 hepatocytes/well.
    a. For other well plate formats, 2/5 of the total well volume will consist of dilute hepatocytes.
8. Harvest 0 time point by adding cells to stop solution and collect samples.
9. Move plate(s) into 37°C culture incubator
    a. When the medium is bicarbonate buffered for non-atmospheric CO_2 concentrations, a CO_2 incubator (typically 5%) should be used.
10. Rock plates at 150 RPM during incubation.
11. At appropriate times, add 1 volume of stop solution and collect the contents of the wells.
12. Spin down samples at 920 x g for 10 minutes at 10°C and run supernatant on MS/MS.

Viability wells are on a separate plate with only incubation media; no test article is added unless toxicity data is also collected at the same time.