**Abstract**

In the early stages of drug discovery, hepatic stability screening is an important and widely used method to assess the metabolic stability of new drug candidates. Here, we present a simplified high-throughput method for rapid stability screening using hepatocytes in a 96-well format for LC-MS determinations. A set of 24 known drugs was chosen that act as substrates for the major human hepatic P450 and UGT isoenzymes. The predicted hepatic clearance for these drugs was determined using cryopreserved, single donor hepatocyte preparations (In Vitro Technologies), and data were compared to literature values for in vivo systemic clearance. We were able to rank accurately compounds based on high, medium, and low stability by determining the loss of parent compound at a single time point of 2 hours in comparison to multiple time points assessment. This has resulted in profound savings of compound, time, and hepatocytes. Comparative studies with both single and pooled donor hepatocytes, as well as with fresh human hepatocytes, are underway currently.

**Introduction**

In recent years, freshly isolated hepatocytes, as well as cryopreserved hepatocytes, have been used for the prediction of metabolic clearance. 

To date, most of the methods conducted for hepatocyte incubations have made use of vials, tubes, or 24-well plates with reaction volumes of 0.5 mL or greater. We present here a simplified 100-µL, 96-well format for the assessment of hepatic stability and subsequent prediction of hepatic clearance.

Additionally, in recent months In Vitro Technologies has begun marketing multiple-donor pools of cryopreserved human hepatocytes. We were interested in comparing the predicted hepatic clearance values from our cryopreserved single donor hepatocytes (lot TPZ, In Vitro Technologies), a cryopreserved 10-donor pool (lot KIDN, In Vitro Technologies), and freshly isolated hepatocytes with observed in vivo systemic clearance values.

**Assumptions**

The prediction of in vivo systemic clearance from in vitro metabolic stability is dependent on several assumptions:

- In vitro enzyme kinetics are applicable to in vivo kinetics.
- Intrinsic clearance follows first-order kinetics.
- Metabolic clearance by the liver is the major route of clearance.

**Effect of reversible protein binding is not significant.**

**Methods**

In vitro stability in the presence of hepatocytes was conducted as follows. Fresh or cryopreserved hepatocytes were thawed if necessary, isolated from shipping media and diluted to a viable cell density of 1 x 10⁶ cells/mL, according to the supplier’s guidelines using KHB buffer (KHB, pH 7.3, Sigma) supplemented with ammonium (84 µg/mL), calcium chloride (1 mM), gymnemic (84 µg/mL), HEPES (20 mM), aspartic acid (4.2 mM) and sodium bicarbonate (2.2 mM). Viability was determined by trypan blue exclusion using a hemacytometer (3500 Hausser, VWR). A 10 mM DMSO stock solution of each drug was diluted to 2 µM using supplemented KHB buffer to create the working solution. A 96-well plate was pre-coated with each compound (i.e., 0, 30, 60, and 120 minutes). The plates were mixed at 300 rpm for 2 minutes at a rate of 100 µL per well (KBA MT S/4 Digital Microtiter Shaker, VWR) and immediately centrifuged at 2,000 g for 10 minutes using an Allegra microcentrifuge (Beckman Coulter). A 190-µL aliquot of the supernatant was then transferred from each well to a 96-well deep well plate (Corning). The plates were sealed using reusable plate mats.

**Quantitation**

Quantitation was performed using an ion trap LC-MS/MS method (Fugazi). Chromatographic separation was achieved using a YMC ODS AQ C18 column (2.1 x 30 mm, 3 µm, 180 Å) in conjunction with a 6-minute gradient using mobile phases A (80% acetonitrile containing 1% triphosphate) and B (90% acetonitrile in acetonitrile containing 1% triphosphate). Mass spectrometric detection of the analysis was accomplished using ESI- or APCI-ionization modes. Analyte responses were measured using extracted ion chromatograms of characteristic fragments from the [MH+H]+ ion. Calculations were performed using Excel 2000 (Microsoft).

**Results & Discussion**

- Similar predicted clearance values were generated using either 2 or 4 time point incubations for the cryopreserved single donor or 10-donor pool.
- Using either fresh or cryopreserved hepatocytes, to predict hepatic clearance led to a general underestimation of observed systemic clearance. Similar results have been reported previously.
- Both of the cryopreserved lots tested here, single donor and 10-donor pool, appear to have a CYP2D6 deficiency.
- The single donor of cryopreserved hepatocytes appears to be as predictive as using fresh hepatocytes in correctly categorizing these drugs by low, medium, or high clearance, when compared with observed in vivo systemic clearance values.

**Conclusions**

- A simplified 2-point, higher throughput 96-well assay can be used to rank accurately the predicted systemic clearance of compounds based on their clearances.
- Advantages
  - Analyte cost of about $20 per compound
  - Obtain a measure of variability using duplicate preparation
  - Analytical run time is cut in half (gain 24 hours instrument time each week)
  - Better assessment of compounds displaying medium or low clearance at 120-minute time point

**Path Forward**

Based on these results, we have decided to initiate a screening paradigm of initially evaluating compounds using a single donor of cryopreserved hepatocytes, followed by assessing compounds of interest with fresh hepatocytes on a monthly basis.

In this manner, we can screen compounds for metabolic stability using the same lot of hepatocytes and draw comparisons over time for similar chemical series. And we would also reduce the liabilities associated from screening with a single donor of cryopreserved hepatocytes.