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Microsomal stability assay for human and mouse liver microsomes - drug metabolism

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We use this protocol and it's working

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Disclaimer

Abstract

Microsomal stability is a key parameter in drug discovery and development to support understanding of a compound's susceptibility to liver metabolism.
This assay assesses a compound's susceptibility to metabolism by liver enzymes, primarily cytochrome P450 (CYP) enzymes. Using either human or mouse microsomes.
The data analysis provides elimination constant, half-life, and intrinsic clearance . These are determined as results using linear regression analysis.

In this protocol, we measure (human or mouse) liver microsomal stability assay for 12 test articles in 96-well plate format. Microsomal incubations are carried out in 5 aliquots of 30 µL (one for each time point). The liver microsomal incubation medium comprises phosphate buffer (100 mM, pH 7.4), $MgCl_2$ (3.3 mM), NADPH (3 mM), glucose-6-phosphate (5.3 mM), glucose-6-phosphate dehydrogenase (0.67 units/mL) with 0.415 mg of liver microsomal protein per mL. In the negative control reactions, the NADPH-cofactor system is substituted with phosphate buffer. Test compounds (2 µM, final acetonitrile concentration 1.6 %) are incubated with microsomes at 37°C, shaking at 100 rpm. Five time points over 40 minutes are analyzed (0, 7, 15, 25, and 40 min). The reactions are stopped by adding 5 volumes of acetonitrile with internal standard to incubation aliquots, followed by protein sedimentation by centrifuging at 5500 rpm for 5 minutes. Supernatants are analyzed using the HPLC-MS/MS system coupled with a tandem mass spectrometer.
The elimination constant (k_{el}), half-life ($t_{1/2}$), and intrinsic clearance (Cl_{int}) are determined in a plot of ln(AUC) versus time, using linear regression analysis:

$$k_{el} = -slope \qquad t_{1/2} = \frac{0.693}{k} \qquad Cl_{\text{int}} = \frac{0.693}{t_{1/2}} \times \frac{\mu l_{incubation}}{mg_{microsomes}}$$

Materials

Reagents and preparations:

- 100 mM (1x) Potassium phosphate buffer (PPB), pH 7.4
- 33 mM $MgCl_2 \times 6H_2O$ solution
- Glucose-6-phosphate dehydrogenase (G6PDH) solution, 250 U/mL
- Human or mouse liver microsomes, 20 mg/mL
- Compounds stock solutions

Safety warnings

Always wear appropriate PPE for this protocol
Refer to Material Safety Data Sheets for additional safety and handling information.

Reagents and preparations

1 Prepare [M] 20 millimolar (mM) stocks of reference and test compounds in DMSO.

- Then, dissolve DMSO stock in acetonitrile (AcN) to [M] 125 micromolar (µM) , and mix by vortexing. Place AcN stock solutions as shown in **Fig. 1**.
- **Diclofenac** and **propranolol** are used as reference compounds in the assay with human microsomes, **imipramine** and **propranolol** - with mice microsomes.

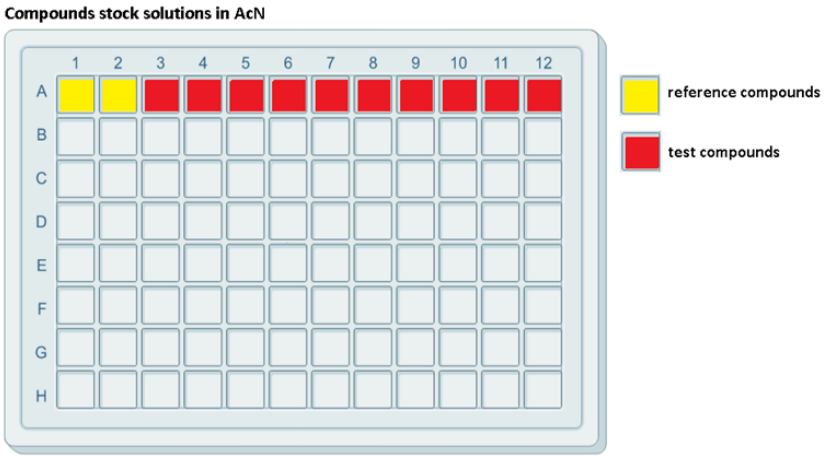

Fig. 1 Plate layout for compounds stock solutions in acetonitrile

2 **Quenching solution with internal standards**

Dissolve appropriate for LC-MS/MS analysis internal standards in ACN.

3 **NADPH tetrasodium salt, D-Glucose-6-phosphate sodium salt**

Weigh out 17.88 mg of NADPH tetrasodium salt (MW=833.35 g/mol) and 9.72 mg of D-Glucose-6-phosphate sodium salt (MW=282.12 g/mol) into separate tubes. Place tubes with powders at -20 °C .

Samples preparation

10m

4 Set the incubator-shaker at 37 °C and 100 rpm.

5 Place aliquots of microsomes and G-6-PDH On ice to thaw.

6 Prepare five 96-well plates and label them as **«Preincubation»**, **«K+/K-»**, **«0+/0-»**, **«40-»**, and **«Incubation»**.

- Place microtubes into plates as shown in **Fig. 2**.

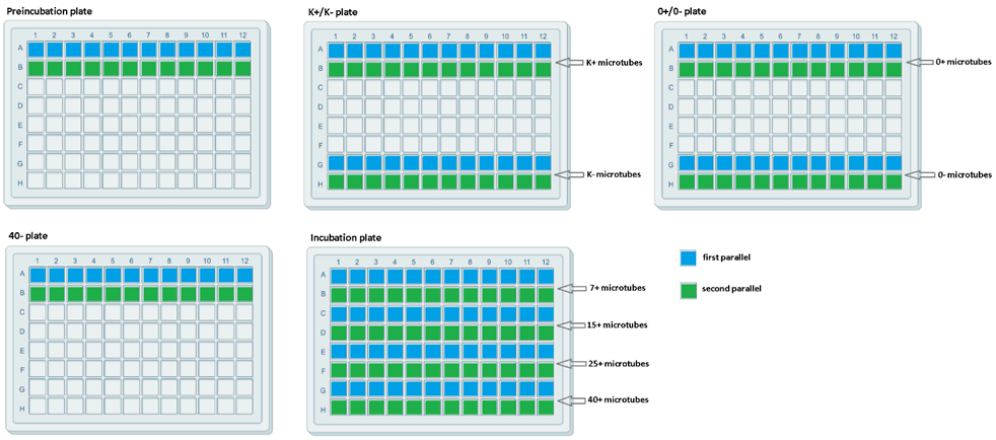

Fig. 2 Preparation of the 96-well plates with 0.75mL microtubes for the assay

7 Label tubes as **«K-»**, **«K+»**, and **«Microsomes»**.

Prepare the following solutions:

- **«K-».** Mix 1021 µL [M] 100 millimolar (mM) PPB and 325 µL [M] 33 millimolar (mM) $MgCl_2 \times 6H_2O$.
- **«K+»**. Mix 1388 µL [M] 100 millimolar (mM) PPB and 650 µL [M] 33 millimolar (mM) $MgCl_2 \times 6H_2O$ solution.
- **«Microsomes».** Fill the tube with 5785 µL [M] 100 millimolar (mM) PPB.

8 Aliquot 52 µL of «K-» solution into the corresponding microtubes of «K+/K-» plate, and then cover the microtubes with mates until starting the experiment.

9 Dispense the stop solution into the «0+/0-» plate, and then cover the microtubes with mates until the start of the experiment.

10 After thawing, add 216 µL of **human** or **mouse liver microsomes** into the tube labeled «Microsomes», mix manually, and place the tube On ice.

11 Aliquot 231 µL of «Microsomes» solution into the «Incubation» plate. Then, add 6.5 µL [M] 125 micromolar (µM) AcN-stock solutions of compounds, and mix by pipetting. 10m

- Cover microtubes with mates and place the plate in the incubator-shaker for 00:10:00 to pre-warm the solution before the experiment.

12 Within 10 minutes perform the following steps:

12.1 Dissolve 17.88 mg of NADPH in 325 µL of [M] 100 millimolar (mM) PPB, mix by pipetting;

12.2 Dissolve 9.72 mg of D-Glucose-6-phosphate in the same way.

12.3 Add 325 µL of both NADPH and D-Glucose-6-phosphate solutions into the «K+» tube;

12.4 Add 16 µL of G-6-PDH into the «K+» tube, gently mix by pipetting;

12.5 Aliquot 104 µL of «K+» solution into the corresponding microtubes of «K+/K-» plate.

12.6 Fill in the «0+/0-» plate with the desired amount of quenching solution.

Incubation 45m

13 After 10 minutes of pre-incubation, remove the «Preincubation» plate from the incubator-shaker, and mix samples by pipetting.

14 **«K-» samples.** Transfer 71 µL of pre incubation solution into the first parallel of «K-» microtubes, mix by pipetting, and aliquot solution into the corresponding «40-» and «0-» microtubes. Perform the same operation with another parallel.

15 **«K+» samples.** Transfer 142 µL of preincubation solution into the first parallel of «K+» microtubes, mix by pipetting, aliquot solution into the corresponding «0+» microtubes (start the timer from this moment), and into the microtubes of «Incubation» plate (7-, 15-, 25-, 40-min time points).

- Perform the same operation with another parallel and record the time shift between the 0-min time points of the first and second incubation parallels.

16 Cover «0+/0-» microtubes with caps, vortex plate and place in the refrigerator. Cover «7», «15», «25», «40», and «40-» microtubes with mats, and place in the incubator-shaker.

17 At each time point, remove corresponding microtubes from the incubator-shaker and add quenching solution to stop the reaction.

18 After the total incubation time, centrifuge the final plates at 5500 rpm, 00:05:00. 5m

The final plates should look as shown in **Fig. 3**.

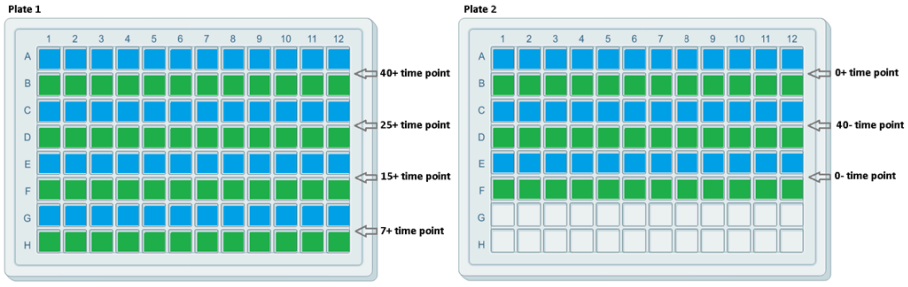

Fig. 3 Sample layout on the final plate

19 Analyze the samples using HPLC-MS/MS in the following order (start the analysis from the samples with theoretically lower concentrations of the tested compound): 40m

00:40:00 time point→25 min→15 min→7 min→0 min→40- min →0-.

Data Analysis

20 The elimination constant (kel), half-life (t1/2), and intrinsic clearance (Clint) are determined in a plot of ln(AUC) versus time, using linear regression analysis:

$$k_{el} = -slope \qquad t_{1/2} = \frac{0.693}{k} \qquad Cl_{\text{int}} = \frac{0.693}{\text{t}_{1/2}} \times \frac{\mu l_{incubation}}{mg_{microsomes}}$$

Equations used