In Vitro ADMET Laboratories, LLC

Plated Hepatocyte Relay Assay (PHRA) for the estimation of intrinsic hepatic clearance and metabolite profile of slowly metabolized compounds

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Introduction

The current practice of metabolic stability screening of new chemical entities (NCE) has led to the collection of chemicals that are resistant to metabolism by in vitro metabolic experimental system such as human liver microsomes and human hepatocytes.

The evaluation of metabolic fate and estimation of in vivo intrinsic clearance of these “slowly metabolized” compounds is a technical challenge, as in general, little metabolism can be observed using the routine short-term incubations with human liver microsomes, S9, or human hepatocyte suspensions.

To overcome this challenge, a relay long term metabolism assay has been developed with primary cultured hepatocytes.

Materials & Methods

Materials:

Plateable Cryopreserved Human Hepatocytes (IVAL, Columbia, MD): Pool of hepatocytes from four donors – HH1007, HH1023, HH1031, and HH1036
Collagen Coated 96 well plates (CellAffix™; APSciences Inc., Columbia, MD)
Universal Cryopreservation Recovery Medium (UCRM; IVAL) for the recovery of cryopreserved hepatocytes
Universal Primary Cell Plating Medium (UPCM; IVAL) for the initial plating of the hepatocytes
Hepatocyte Incubation Medium (HQM; IVAL) for assay

Methods:

Plated Hepatocyte Relay Assay (PHRA): The relay assay consists of firstly culturing plateable cryopreserved human hepatocytes (pooled from four donors) on day 1 for approximately 4 hrs to allow attachment, followed by incubation with the chemicals to be evaluated for a 24 hr. period.

On day 2, the incubation medium from day 1 is transferred to a new culture of human hepatocytes prepared from the same lots used on day 1 followed by another 24 hr of incubation (1st relay). The process is repeated on days 3 (2nd relay), 4 (3rd relay), and 5 (4th relay). With this novel relay method, a chemical can be evaluated for metabolism by human hepatocytes for up to 5 days.

Data Analysis:

Compounds’ parent accurate mass was used to do quantification.
Peak area was normalized by internal standard.
Concentration after each relay was corrected for loss of drug in plated hepatocytes post-pooling according to Li Di’s paper.
Percent remaining at each time points were compared to that of Time 0.
Following equations were used to calculate the CL_{int} and Cl_h.
CL_{int} = ln2*(1/T1/2 min)*(mL incubation/0.5 Mcells)*(120 M cells/g liver)*(21g liver/kg) = ml/min/kg
CL_h=Q*fu*Cl_{int}/(Q+(fu*Clint), (fu=1;human Q (ml/min/kg): 21).

Procedures

Day 1

1) Thaw and plate human hepatocytes (Plate 1) and allow 4 h for attachment
2) T= 0 hr: Replace medium to that containing chemicals to be evaluated (remove an aliquot as no hepatocyte control)
3) Incubate for 24 h

Day 2 (1st Relay)

1) Thaw fresh vials and plate hepatocytes to prepare the first relay plate (Plate 2).
2) T= 24 h: Pool the media containing the same chemical in all the wells in Plate 1. Remove aliquots for LCMS analysis.
3) Replace medium in Plate 2 with the pooled medium for the 1st Relay (T= 24 to 48 h). Incubate for 24 h.
4) Repeat procedures for Days 3 (2nd Relay; T=48 to 72 hr). 4 (3rd Relay; T=72 to 96 h) and 5 (4th Relay: T= 96 to 120 h)

Sample Treatment and LC/MS Analysis

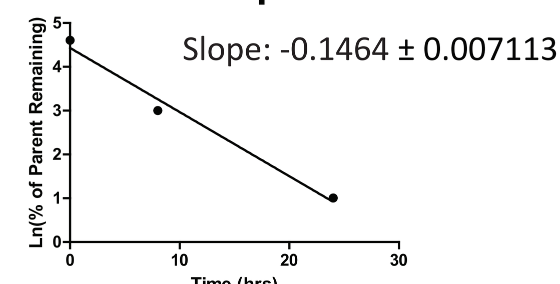
1) Supernatants (200 µL) were thawed at room temperature.
2) Samples were vortexed and centrifuged at 3220 g (Eppendorf centrifuge) for 10 minutes.
3) The supernatants (150 µL) were transferred to a new plate and injected to HPLC coupled with AB Sciex triple TOF 5600 system

HPLC system	Waters Acquity System Waters Corp. (Marlborough, MA)
HPLC column	Acquity, UPLC BEH C18 1.7 µm, 2.1 x 50 mm Waters Corp.
Column temperature	55°C
Mobile Phases:	A. 0.1% formic acid (ESI +) or 10 mM ammonium acetate, pH 6 (ESI -) B. Acetonitrile (ACN)

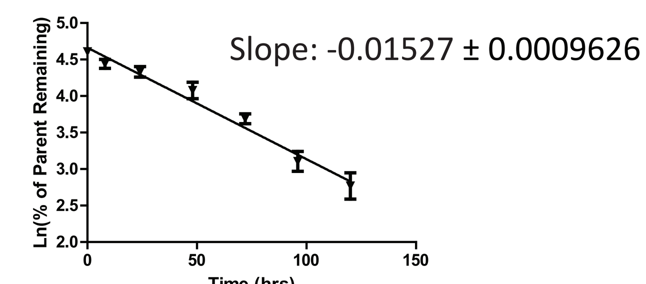
Time (min)	Flow Rate (mL/min)	A (%)	B (%)
0	0.45	95	5
0.2	0.45	95	5
3	0.45	10	90
3.5	0.45	10	90
3.6	0.45	95	5
4	0.45	95	5

Results

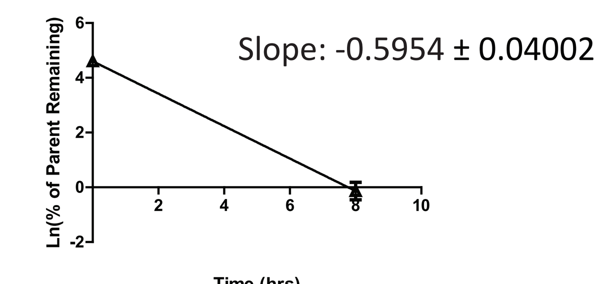
Risperidone



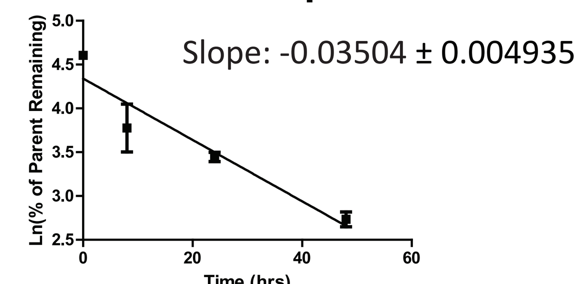
Meloxicam



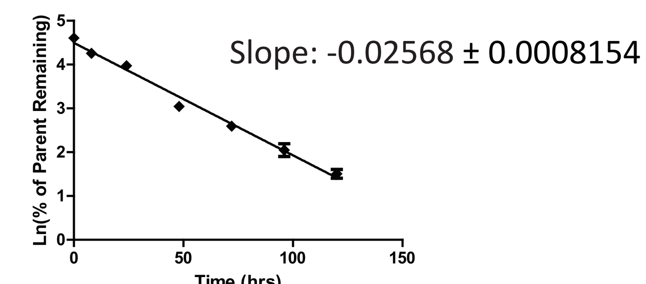
Diclofenac



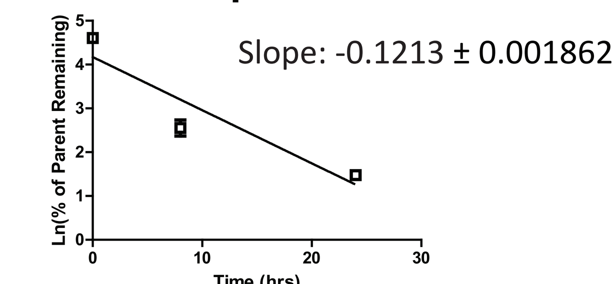
Clozapine



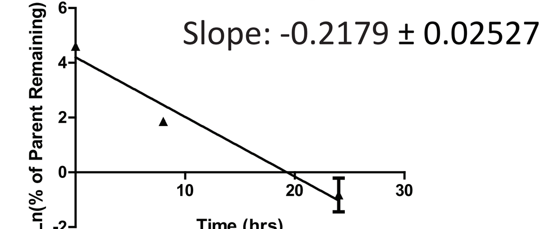
Tolbutamide



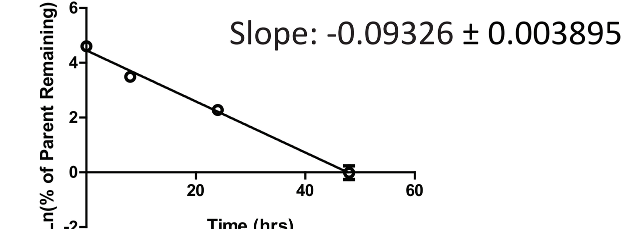
Ziprasidone



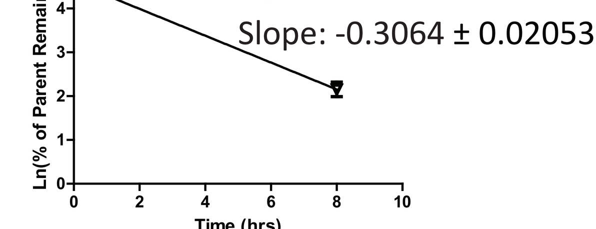
Glimepiride



Riluzole



Ibuprofen



Compound	CL_{int} (ml/min/kg) in vitro	CL_h (ml/min/kg) in vitro	$Cl_{nonrenal, blood}$ (ml/kg) in vivo
Risperidone	12.3	7.75	5.16
Clozapine	2.94	2.58	2.9
Glimepiride	18.3	9.8	1.12
Meloxicam	1.29	1.2	0.12
Tolbutamide	2.15	1.96	0.31
Riluzole	7.84	5.7	2.05
Ziprasidone	10.2	6.9	CL: 11.7
Diclofenac	49.8	14.8	7.67
Ibuprofen	25.7	11.6	1.5

Clozapine, riluzole only first 48 hrs data
Ibuprofen in “–” mode, no IS normalization
Incubation volume: 0.1mL

Summary

The Plated Hepatocyte Relay Assay (PHRA) was validated with 10 model drugs with in vivo hepatic intrinsic clearance ranged from 0.081 mL/min/kg (warfarin; slowly metabolized) to 7.67 mL/min/kg (diclofenac; rapidly metabolized).

Linear time-dependent parent disappearance was observed, with the calculated hepatic clearance values falling comparable to the known in vivo values.

Conclusions

The results suggest that PHRA can be used for the estimation of in vivo hepatic clearance of slowly metabolized compounds that cannot be readily evaluated using routine methods. The assay can also be used for the metabolite profiling and phenotyping of slowly metabolized compounds.

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