Metabolic Stability Assay Using Human Hepatocyte Co-cultures and Integrated Qualitative/Quantitative High Resolution Mass Spectrometry

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OVERVIEW

Purpose

To investigate Sequential Window Acquisition of all Theoretical fragment ion spectra (SWATH™) based integrated qualitative and quantitative (qual/quant) assay for simultaneous metabolic stability and metabolite profiling assessments.

Method

• An AB Sciex API 5600 TripleTOF mass spectrometer equipped with a Shimadzu Nexera UHPLC was used to analyze human hepatocyte co-cultures using TOF-MS and SWATH scans.

• Lorazepam, propranolol, ranitidine, and zoniporide were incubated in 96-well human hepatocyte co-culture plates. Samples were collected at 0, 1, 4, 24, 48, 72, 120, and 168 hours, and buspirone was added as IS. Metabolic stability data were generated using peak-area-ratios of the analyte-to-IS using MultiQuant™ software. The same raw data files were mined for preliminary metabolite information using MetabolitePilot™ software.

Results

Metabolic Stability:
Long term hepatocyte co-cultures enabled measurement of *in vitro* intrinsic clearance values for low clearance drugs.

Metabolite Identification:
Major metabolites were detected following a 7 day incubation with 1 µM drug .

INTRODUCTION

Traditional metabolic stability methodology using suspended hepatocyte for drug candidates screening is limited in their ability to accurately predict clinical outcomes. Hepatocyte co-culture platform is a bioengineered, *in vitro* system with a defined cyto-architecture that provides sustained hepatic functions for at least four weeks[1]. In this presentation, we investigated human hepatocyte co-cultures model using an integrated qualitative/quantitative high resolution mass spectrometry approach to assess metabolic stability using TOF-MS and Sequential Window Acquisition of All THeoretical fragment ion spectra (SWATH) for non-targeted metabolite MS/MS analysis. The assay was evaluated using propranolol, lorazepam, ranitidine, and zoniporide, at clinically relevant concentration (1 µM) in 96-well format.

METHODS

Metabolic Stability Study Design

Substrates:

1. Propranolol (CYP2D6, 1A2, glucuronosyl transferase)
2. Lorazepam (glucuronosyl transferase)
3. Zoniporide (aldehyde oxidase)
4. Ranitidine (FMO, P450, only 6% of dose metabolized; active tubular secretion). Renal elimination accounts for in vivo clearance.

Substrate Concentration: 1 µM
• 96-Well HepatoPac™ Micro-patterned co-cultures of Hepatocytes (~5000 cells/well)
• 96-Well stromal cells control (~15,000 cells/well)
Medium: 64 µL/well
Incubation Time: 0, 1, 4, 24, 48, 72, 120, 168 hours
Sample Preparation: 60 µL sample aliquots are collected and added to 60 µL of ACN containing IS. The mixtures are vortex-mixed for 2 min, and centrifuged at 3000 rpm for 10 min before analysis.
Analysis: Nexera UHPLC-API 5600 (HRMS)

Figure 1. Structures of the Tested Substrates

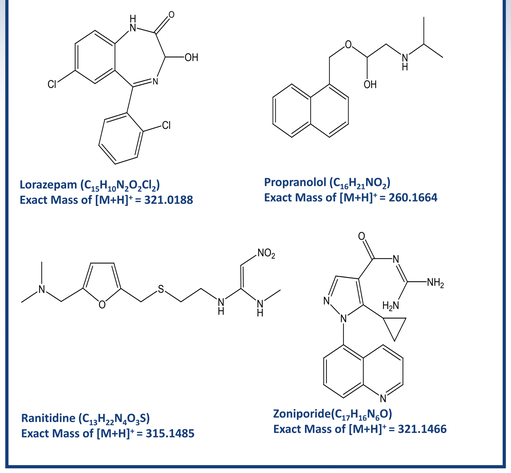


Figure 2. Human HepatoPac™ Cell Cultures (Propranolol Incubations)

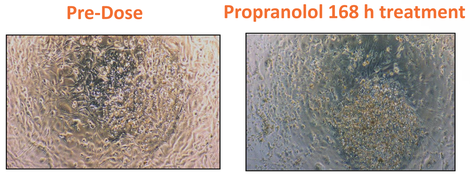


Figure 3. API 5600 Triple TOF Mass Spectrometer

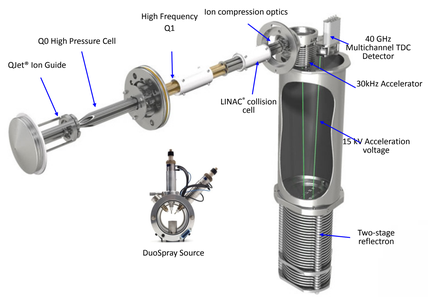


Table 1. Ultra High Pressure Liquid Chromatography and HRMS Parameters

UHPLC System	Shimadzu Nexera	
UHPLC Column	Acquity UPLC BEH C18, 2.1 x 50 mm, particle size 1.7 µm	
Column Temperature (°C)	40	
Injection Volume (mL)	10	
Flow Rate (mL/min)	600	
UHPLC Gradient Time (min)	**A**: 10 mM NH_4OAc, pH=5 with Formic Acid	**B**: ACN/Formic Acid (100/0.1 V/V)
0.0	95	5
1.0	95	5
3.0	5	95
4.0	5	95
4.1	95	5
5.0	95	5
TOF-MS	100-2000 for 100 ms	
SWATH MS/MS^{ALL} Range	200-800 Da	
SWATH Scans	25 ms per 25 Da window	
DP	80 V	
CE	35+/-15 V	
Total Cycle Time	750 ms	

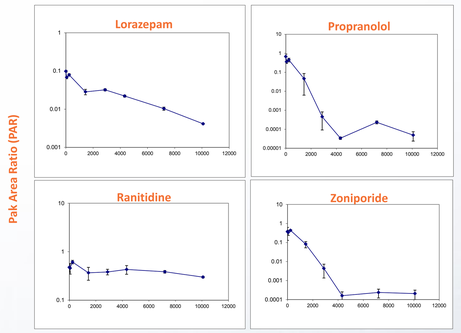
RESULTS

Figure 4. Integrated Qualitative and Quantitative Metabolic Stability Analysis

Advantages Using UHPLC-Q-TOF for Quantitative and Qualitative Bioanalysis

- No need for optimization or decide on a fragment, using the generic acquisition conditions
- Full scan MS preserves all the information about the sample (drug, metabolites, dosing vehicle, degradants, biomarkers, etc.)
- HRMS provides opportunities to use mass defect filter (MDF), isotope pattern filtering, background subtraction, etc. for metabolite detection.
- Provides an option for acquiring quantitative and qualitative information simultaneously.

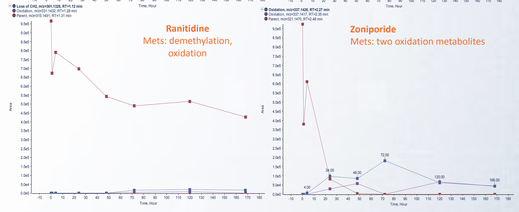
Figure 5. Metabolic Stability Results



	Estimated Hepatic Clearance Values CL_h (mL/min*kg) of Current Study	*In Vivo* Hepatic Clearance Values CL_h (mL/min*kg) From Literature
Lorazepam	5.5	1.1[2]
Propranolol	16.8	16.11[2]
Ranitidine	2.1	2.9[2]
Zoniporide	12.4	21[3]

Figure 6. Propranolol (1 µM) Metabolite Formation (MetabolitePilot™ Software)

Figure 7. Metabolite Formation Time Courses of Four Drugs from Single Incubations



CONCLUSIONS

Using lorazepam, propranolol, ranitidine, and zoniporide as model compounds to acquire metabolic stability and metabolite profile information simultaneously at clinically relevant concentration.

This approach is feasible when using high resolution TOF-MS in combination with data independent MS/MS scan.

The estimated hepatic clearance values of current study are in line with the observed *in vivo* CL_h values.

REFERENCE

1. WW Wang, SR Khetani, S Krzyzewski, DB Duignan, RS Obach Assessment of a micro-patterned hepatocyte co-culture system to generate major human excretory and circulating drug metabolites. *Drug Metab. Dispos.* 2010, 38(10),1900-1905.
2. RJ Riley, DF McGinnity, AP Austin A unified model for predicting human hepatic, metabolic clearance from in vitro intrinsic clearance data in hepatocytes and microsomes *Drug Metab. Dispos.* 2005, 33(9), 1304-1311.
3. D Dalvie, CH Zhang, WH Chen, T Smolarek, RS Obach, CM Loi Cross-species comparison of the metabolism and excretion of Zoniporide: contribution of aldehyde oxidase to interspecies differences. *Drug Metab. Dispos.* 2010, 38(4), 641-654.