In Vitro Species Comparison Using Long-Term Hepatocyte Co-Cultures Model and Highly Sensitive UHPLC-QTOF-MS with SWATH Analysis

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OVERVIEW

Purpose

To develop a reliable, quicker, and cost-saving *in vitro* method to accurately predict major human metabolite profile *in vivo* and to de-risk disproportional or unique human metabolites before a drug candidate nomination

Method

Using long-term animal and human hepatocyte co-cultures coupled with non-targeted MS/MS^{ALL} with SWATH acquisition by a UHPLC-QTOF system to generate metabolite profile information

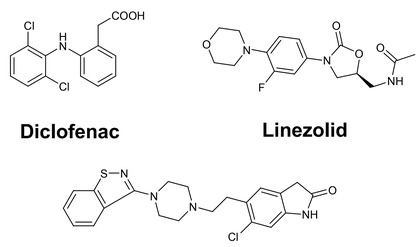
Results

Metabolites of the tested compounds identified in human hepatocyte co-cultures were also found in those of rat and/or monkey and the major human circulating and excreta metabolites of these compounds were also found in human and/or animal hepatocyte co-cultures. The proposed approach appears to be reliable.

INTRODUCTION

One of the main goals of *in vitro* species comparison studies is to assess whether there is adequate coverage from the preclinical species to humans with respect to disproportional and/or unique human metabolites. Also important is to accurately predict major human metabolite profile *in vivo*. Traditionally, this is performed with subcellular fractions and/or suspended hepatocytes; however, these short-term *in vitro* systems do not usually provide multi-generation metabolites.[1, 2, 3, 4] In this study, we incubated selected compounds of diverse chemical structures (linezolid, ziprasidone, and diclofenac, **Figure 1**) that were subjected to a wide range of biotransformation pathways with long-term hepatocyte co-cultures model over an extended time period, and mined the metabolite information from the mass spectra generated by an UHPLC-QTOF-MS system through non-targeted MS/MS^{ALL} with SWATH acquisition to compare the metabolite profiles across species and to the major metabolite profile found in humans *in vivo*.

Figure 1. Structures of the Tested Drugs



METHODS

Sample Preparation

Linezolid, ziprasidone (ZIP), and diclofenac (@ 10 µM) were incubated with rat, monkey, or human HepatoPac™ co-cultures at 37°C in a 24-well format. Incubations with stromal cells served as the negative control. The plates were placed inside a humidified incubator over 168 hours. The enzymatic reactions were terminated by adding 400 µL of ice-cold acetonitrile solution directly to the well at 0, 4, 48, and 168 h. The mixture was vortex-mixed, centrifuged, and the supernatants were analyzed by UHPLC-MS/MS.

UHPLC-HRMS and UHPLC-MS/MS Conditions

The system used for metabolite identification and profiling consisted of a Shimadzu Nexera™ UHPLC system (**Table 1**) and a TripleTOF™ 5600 high resolution mass spectrometer (AB Sciex) controlled by Analyst TF™ software (version 1.6). Mass spectrometric analysis was performed through MS/MS^{ALL} with Sequential Windowed Acquisition of all THeoretical Fragments (SWATH) acquisition (**Table 2**). The mass spectrometer data were mined with MetabolitePilot™ software (Version 1.6) using mass defect filtering, isotope pattern filtering, and background subtraction.

Table 1. Liquid Chromatography Conditions

UHPLC Column	ACQUITY UPLC BEH C18 2.1 x 100 mm 1.7 µm
Column Temperature	40 °C
Flow rate	600 µL/min
Injection Volume	10 µL
Mobile Phase A	10 mM CH_3COONH_4 in water, pH=5.0
Mobile Phase B	Acetonitrile containing 0.1% formic acid
UHPLC Gradient	5-5-40-50-95-95-5% of B @0.0-1.5-9.0-10.0-11.0-12.0-13.0-15.0 min

Table 2. TripleTOF™ 5600 Parameters

Parameter	Value
Collision Gas (CAD)	6 Psig N_2
Curtain Gas (CUR)	30 Psig N_2
Ion Source Gas 1 (GS1)	60 Psig N_2
Ion Source Gas 2 (GS2)	60 Psig N_2
Ion Spray Voltage (IS)	5500 V
Temperature (TEM)	550 °C
Declustering Potential (EP)	80 V
Full Scan TOF-MS Range	100-2000 Da
SWATH MS/MS ^{ALL} Range	250-950 Da
Accumulation Time	35 ms per 25 Da
Collision Energy (CE)	35 V
Collision Energy Spread (CES)	±15 V

RESULTS

Incubation of ZIP with Hepatocyte Co-Culture

1. Three major human circulating and excreta metabolites *S*-methyl-dihydro-ZIP, ZIP sulfoxide, and *N*-dealkyl ZIP sulfone,[3-8] were identified in both monkey and human hepatocyte co-cultures (**Table 3**). *S*-Methyl-dihydro-ZIP and ZIP sulfoxide were also found in rat.
2. *S*-Methyl-dihydro-ZIP and *S*-Methyl-dihydro-ZIP-SO were the major metabolites in rat, monkey, and human hepatocyte co-cultures (**Figure 2**).
3. Metabolites identified in human were also found in animals.

Incubation of Linezolid with Hepatocyte Co-Culture

1. Two major human circulating and excreta metabolites, PNU-142586 and PNU-142300,[9] were identified in both animal and human hepatocyte co-cultures as major or significant (**Table 3**).
2. Metabolite profiles were qualitatively similar across all species tested, with three morpholine ring-opened products PNU-142300, PNU-142586, and PNU-143010 as the major metabolites in human. PNU-142586, PNU-142300, and PNU-143131 were the major metabolites in monkey, while PNU-142300 and PNU-142618 were major metabolites in rat (**Figure 3**).
3. Metabolites identified in human were also found in animals.

Table 3. Generation of Major *In Vivo* Human Metabolites in Hepatocyte Co-Cultures of Rat, Monkey, and Human

Compound Name	Major *In Vivo* Human Metabolites	Hepatocyte Co-Cultures		
		Rat	Monkey	Human
Ziprasidone (ZIP)	Ziprasidone sulfoxide (ZIP-SO)	Yes	Yes	Yes
	S-Methyldihydroziprasidone (*S*-Methyl-dihydro-ZIP)	Yes	Yes	Yes
	N-Dealkylziprasidone *S*-oxide (BITP-SO)	*	*	*
	N-Dealkylziprasidone sulfone (BITP-SO_2)	No	Yes	Yes
Linezolid	*O*-Dealkylation/ring opening, carboxylic acid (PNU-142586)	Yes	Yes	Yes
	N-Dealkylation/ring opening, carboxylic acid (PNU-142300)	Yes	Yes	Yes
Diclofenac	4′-Hydroxydiclofenac	Yes	Yes	Yes
	5-Hydroxydiclofenac	Yes	Yes	Yes
	Acyl glucuronides	Yes	Yes	Yes

* Not searched due to its molecular weight outside of SWATH range

Figure 2. Major Metabolite Profiles of Ziprasidone in Hepatocyte Co-Cultures of Rat, Monkey, and Human

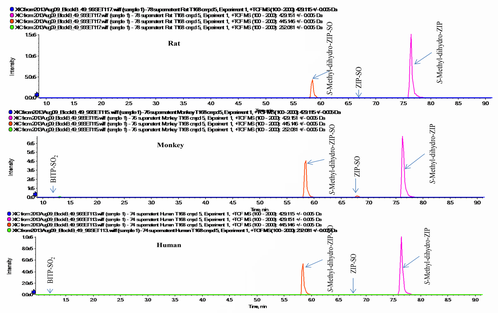


Figure 3. Major Metabolite Profiles of Linezoid in Hepatocyte Co-Cultures of Rat, Monkey, and Human

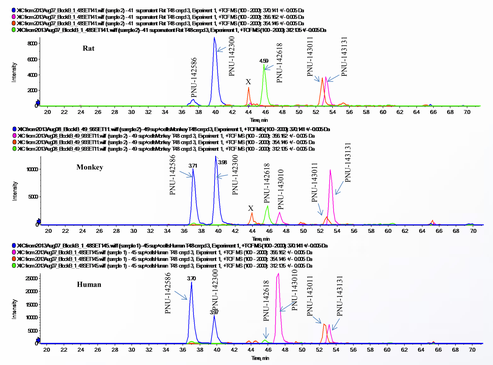
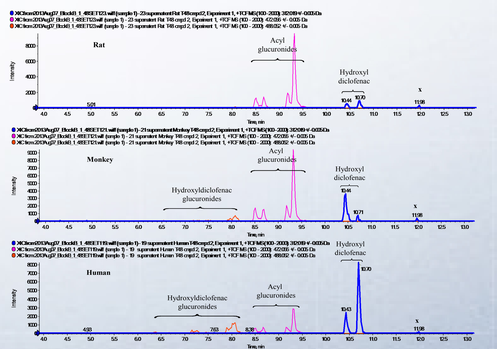


Figure 4. Major Metabolite Profiles of Diclofenac in Hepatocyte Co-Cultures of Rat, Monkey, and Human



Incubation of Diclofenac with Hepatocyte Co-Cultures

1. The major human circulating and excreta metabolites, four acyl glucuronides, 4′-hydroxyl and 5-hydroxyl diclofenac,[10,11] were identified in both animal and human hepatocyte co-cultures as major or significant (**Table 3**).
2. Four diclofenac acyl glucuronides were the major metabolites in all species at 4 h. At 48 h, acyl glucuronides were the major metabolites in rat and monkey, while acyl glucuronides and 4′-hydroxyl and 5-hydroxyl diclofenac were major metabolites in human (**Figure 4**). At 168 h, 4′-hydroxyl and 5-hydroxyl diclofenac were the major metabolites in monkey and human, while acyl glucuronides and 4′-hydroxyl and 5-hydroxyl diclofenac were major metabolites in rat.
3. In addition, multiple hydroxyldiclofenac glucuronides and a dehydrogenated diclofenac (detected in negative mode) were also identified in animal and/or human.
4. Metabolites identified in human were also found in animals.

CONCLUSIONS

- Major human circulating and excreta metabolites of the three compounds were found in human hepatocyte co-cultures.
- Metabolites of the three compounds identified in human hepatocyte co-cultures were also found in those of rat and/or monkey.
- The non-targeted MS/MS^{ALL} with SWATH acquisition enables a comprehensive qualitative and quantitative analysis of all components within the dynamic range interrogated. High resolution MS and MS/MS spectrum of every analyte in the sample reduce potential for interferences, therefore provide high quality data. The ability of re-interrogation of the MS data of all analytes allows the update of metabolite profile information without additional experiments.
- This approach of long-term hepatocyte co-cultures coupled with non-targeted MS/MS^{ALL} with SWATH acquisition by UHPLC-QTOF-MS provides a reliable, quicker, and cost-saving method to accurately predict major human circulating and excreta metabolites as well as to compare metabolite profiles across species in order to de-risk unique or disproportional human metabolites before drug candidate nomination.

REFERENCE

1. Anderson S et al., Predicting circulating human metabolites: how good are we? Chem Res Toxicol, 2009;22:243-256.
2. Dalvie D et al., Assessment of three human in vitro systems in the generation of major human excretory and circulating metabolites. Chem Res Toxicol, 2009;22:357-368.
3. Wang WW et al., Assessment of a micropatterned hepatocyte coculture system to generate major human excretory and circulating drug metabolites, DMD, 2010; 38(10):1900-1905.
4. Ballard TE et al., Generation of major human excretory and circulating drug metabolites using a hepatocyte relay method. DMD, 2014;42:899-902.
5. Prakash C et al., Metabolism and excretion of a new antipsychotic drug, ziprasidone, in humans. DMD, 1997; 25(7): 863–872.
6. Prakash C et al., Metabolism and excretion of the novel antipsychotic drug ziprasidone in rats after oral administration of a mixture of 14C- and 3H-labeled ziprasidone. DMD, 1997; 25(1): 206–218.
7. Prakash C et al., Characterization of the novel benzisothiazole ring-cleaved products of the antipsychotic drug ziprasidone. DMD, 1997; 25(7): 897–901.
8. Prakash C et al., Characterization of a novel metabolite intermediate of ziprasidone in hepatic cytosolic fractions of rat, dog, and human by ESI-MS/MS, hydrogen/deuterium exchange, and chemical derivatization. DMD, 2005; 33(7): 879–883.
9. Slatter JG et al., Pharmacokinetics, metabolism, and excretion of linezolid following an oral dose of [14C]linezolid to healthy human subjects. DMD, 2001; 29(8):1136-1145.
10. Stierlin H et al., Biotransformation of diclofenac sodium (Votaren) in animals and in man, I: Isolation and identification of principal metabolites. Xenobiotica, 1979;9:601-610.
11. Stierlin H et al., Biotransformation of diclofenac sodium (Votaren) in animals and in man, I: Quantitative determination of the unchanged drug and principal phenolic metabolites, in urine and bile. Xenobiotica, 1979;9:611-621.