# *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

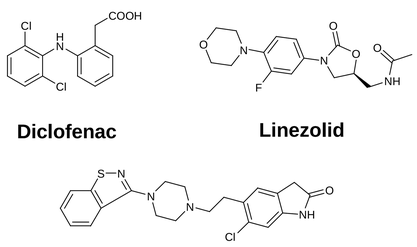
### Results

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

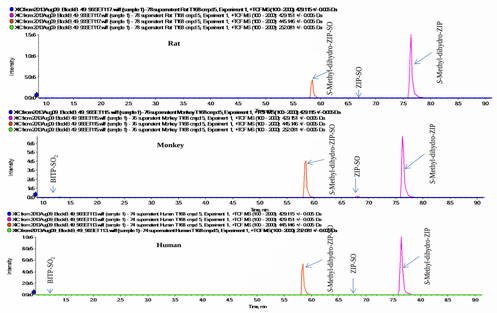
1. Two major human circulating and excreta metabolites, PNU-142586 and PNU-142300,[9] were identified in both animal and human hepatocyte co-cultures as major or significant (**Table 3**).
2. Metabolite profiles were qualitatively similar across all species tested, with three morpholine ring-opened products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

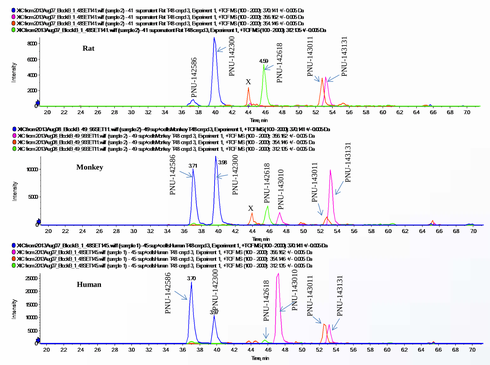
| Compound Name | Major *In Vivo* Human Metabolites | Hepatocyte Co-Cultures | | |
|---|---|---|---|---|
| | | Rat | Monkey | Human |
| Ziprasidone (ZIP) | Ziprasidone sulfoxide (ZIP-SO) | Yes | Yes | Yes |
| | *S*-Methyldihydroziprasidone (*S*-Methyl-dihydro-ZIP) | Yes | Yes | Yes |
| | *N*-Dealkylziprasidone *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

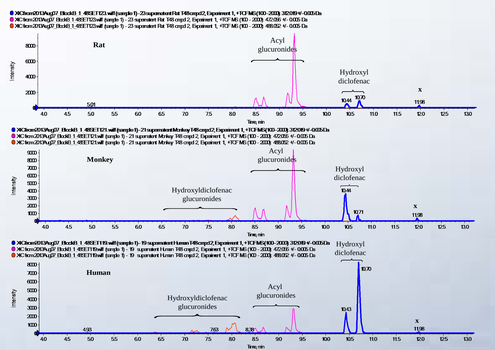
### Figure 2. Major Metabolite Profiles of Ziprasidone in Hepatocyte Co-Cultures of Rat, Monkey, and Human




### 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

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