# *In vitro* Species Comparison using Long-term Hepatocyte Co-Culture Models and Highly Sensitive UHPLC-Q-TOF-MS with SWATH Analysis

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## 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. Traditionally, this is performed with liver microsomes or hepatocytes; however, these short-term *in vitro* systems do not usually provide multi-generation metabolites. In this study, we incubated selected compounds (linezolid, ziprasidone, and diclofenac) that have diverse chemical structures that are subjected to a wide range of biotransformation pathways with hepatocyte co-culture models over an extended time period, and mined the metabolite data with UHPLC-Q-TOF-MS and Sequential Windowed Acquisition of THeoretical Fragments (SWATH) analysis to compare the *in vitro* metabolite profiles to those found in humans *in vivo*.

## Methods

Linezolid, ziprasidone (ZIP), and diclofenac (@ 10 µM) were incubated with rat, or monkey, and human HepatoPac™ co-cultures in a 24-well format. Incubations with stromal cells served as the negative control. The plates were placed inside a humidified incubator at 37°C over 168 hours. The enzymatic reactions were terminated by adding 400 µL of ice-cold acetonitrile solution that contained buspirone (internal standard) directly to the well at 0, 4, 48, and 168 h. The mixture was vortex-mixed, centrifuged, and the supernatants were analyzed by LC-MS/MS consisting of a Shimadzu Nexera™ UHPLC and Sciex TripleTOF™ 5600 high resolution mass spectrometer using SWATH based MS/MS$^{ALL}$ scans to mine the mass spectra data.

## Preliminary Data

In the incubation of ZIP with hepatocyte co-cultures, *S*-methyl-dihydro-ZIP was identified as the major metabolite in both rats and humans, while *S*-methyl-dihydro-ZIP and ZIP sulfoxide were the major metabolites in monkeys. In addition, five, six, and seven minor metabolites were identified in rats, monkeys, and humans respectively. The three major human circulating and excreta metabolites, *S*-methyl-dihydro-ZIP, ZIP sulfoxide, and *N*-dealkylZIP sulfone, were also identified in both animal and human hepatocyte co-cultures.

In the incubation of linezoid with hepatocyte co-cultures, metabolite profiles were qualitatively similar across all species, with four morpholine ring-opened products PNU-142586, PNU-142300, PNU-143010, and PNU-143131, and an oxidation product PNU-143011 as the major metabolites in humans. PNU-142586 and PNU-142300 were the major metabolites in monkeys, while PNU-142300, PNU-142618, and PNU-143011 were major metabolites in rats. Two major human circulating and excreta metabolites, PNU-142586 and PNU-142300, were also identified in both animal and human hepatocyte co-cultures.

In the incubation of diclofenac hepatocyte co-cultures, 4'-hydroxyl and 5-hydroxyl diclofenac were the major metabolites in humans, while four diclofenac acyl glucuronides were the major metabolites in rats and monkeys. In addition, four hydroxyldiclofenac glucuronides and a dehydrogenated diclofenac were also identified in animals and/or humans. Two major human circulating and excreta metabolites, 4'-

hydroxyl and 5-hydroxyl diclofenac, were also identified in both animal and human hepatocyte co-cultures.

Our preliminary data set indicated that metabolites of the three compounds identified in human hepatocyte co-cultures were also found in those of rats or monkeys. By comparing the *in vitro* data with human *in vivo* studies, it also revealed that the animal and human hepatocyte co-cultures generated all the major human circulating and excreta metabolites. Therefore, this approach of long-term hepatocyte co-cultures couple with highly sensitive UHPLC-Q-TOF-MS and SWATH analysis appears to be a promising methodology to de-risk unique human metabolites before drug candidate nomination.

**Novel Aspect**
Combined approach of long-term hepatocyte co-cultures with UHPLC-Q-TOF-MS and SWATH methodology for *in vitro* species comparison