

# Metabolite Profiling Using Human Hepatocyte Co-cultures and UHPLC-QTOF-MS with Data Independent MS/MS

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## OVERVIEW

### Purpose

To investigate a high throughput workflow for metabolite profiling using a novel human *in vitro* system and advanced UHPLC-MS/MS techniques to accurately predict human metabolite profile *in vivo*.

### Methods

Diclofenac, linezolid, and ziprasidone (ZIP) (@ 10  $\mu$ M) were incubated with human HepatoPac™ co-cultures at 37 °C in a 24-well format. Samples were collected at 0, 4, 48, and 168 h.

An AB Sciex API 5600 TripleTOF mass spectrometer equipped with a Shimadzu Nexera UHPLC was used to analyze incubation samples using TOF-MS and SWATH.

### Results

Major and most of minor metabolites reported in human AME studies were identified in human hepatocyte co-cultures for diclofenac, linezolid, and ziprasidone. Four new hydroxydiclofenac glucuronides and a new ziprasidone metabolite M6a were also identified in human hepatocyte co-cultures.

## INTRODUCTION

The identification of human derived metabolites is an imperative step in drug discovery and development process. Typical studies are conducted in human *in vitro* systems and metabolites are identified utilizing HPLC-MS/MS. Human hepatocyte co-culture is a new *in vitro* system, which is comprised of a mixture of hepatocytes and fibroblast cells, capable of remaining viable and highly functional for up to 4 weeks. In this presentation, the application of high resolution mass spectrometry (HRMS), ultrahigh-pressure liquid chromatography (UHPLC), full scan acquisition, and Sequential Window acquisition of All Theoretical fragment ion spectra (SWATH™) was used to profile and characterize metabolites of diclofenac, linezolid, and ziprasidone following incubations in human hepatocyte co-cultures.

## METHODS

### Incubation and Sample Preparation

Diclofenac, linezolid, and ziprasidone (@ 10  $\mu$ M) were incubated with 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  $\mu$ L 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 TOF-MS and SWATH acquisition. The mass spectrometric data were mined with Metabolite Pilot™ software (Version 1.6) using mass defect filtering, isotope pattern filtering, and background subtraction.

### Metabolite Profiling of Diclofenac in Human Hepatocyte Co-culture Incubation

1. The major human circulating and excreta metabolites, four (4) acyl glucuronides, 4'-hydroxy and 5-hydroxy diclofenac, were identified in human hepatocyte co-cultures as major or significant component (Figure 1).
2. Diclofenac quinone imine identified as an intermediate in human AME study was identified in human hepatocyte co-cultures (Figure 1).
3. Four (4) new hydroxydiclofenac glucuronides were also identified in human hepatocyte co-cultures (Figure 2).

Figure 1. Structures of the Diclofenac Metabolites

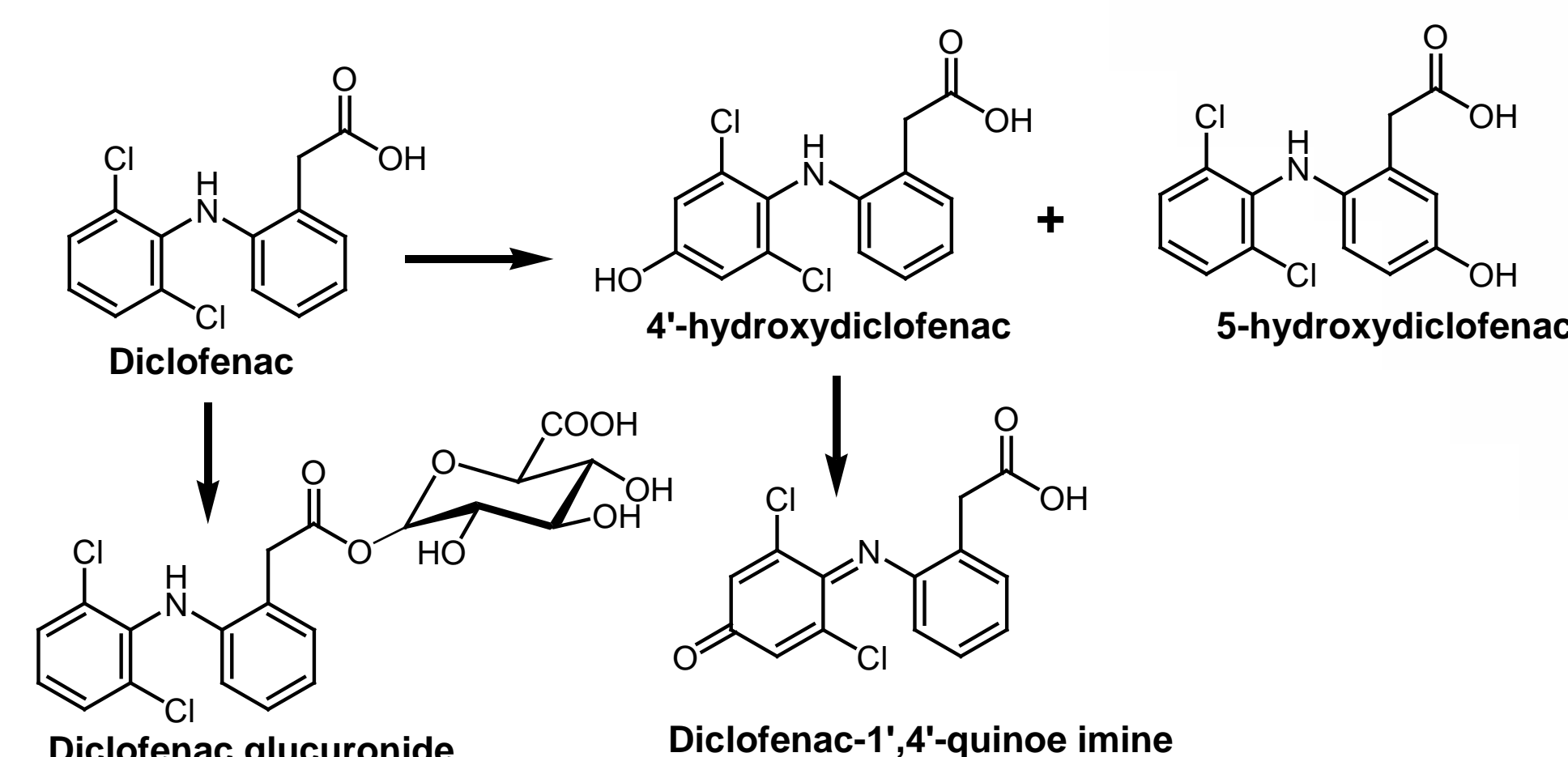
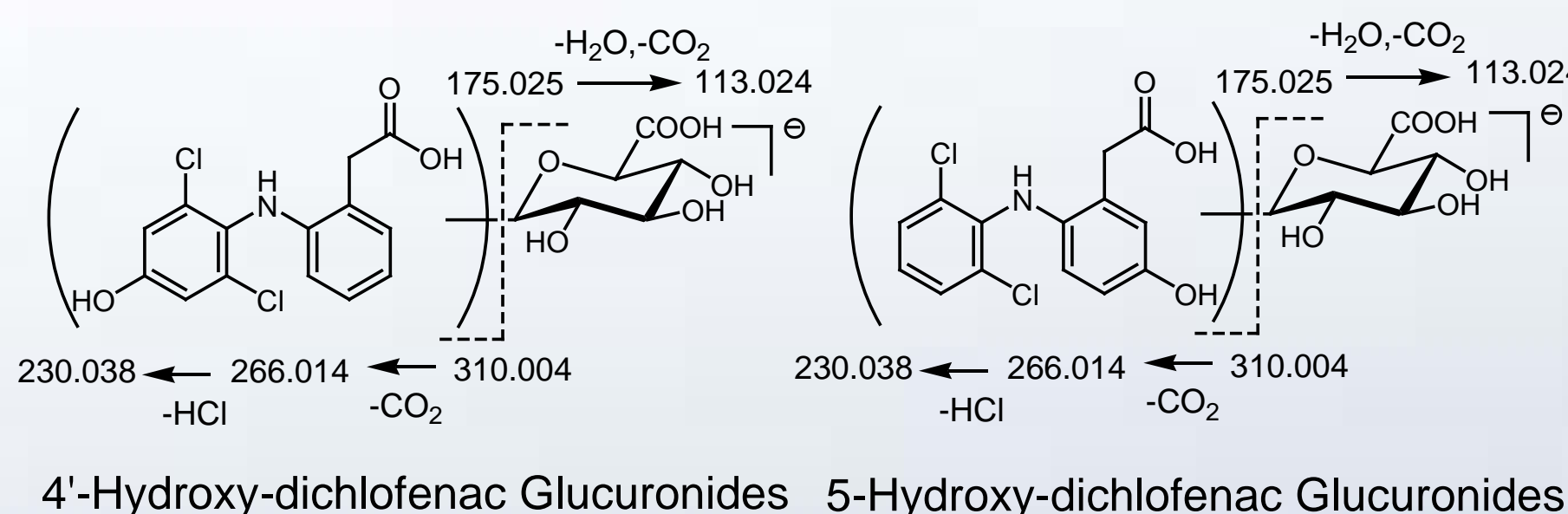
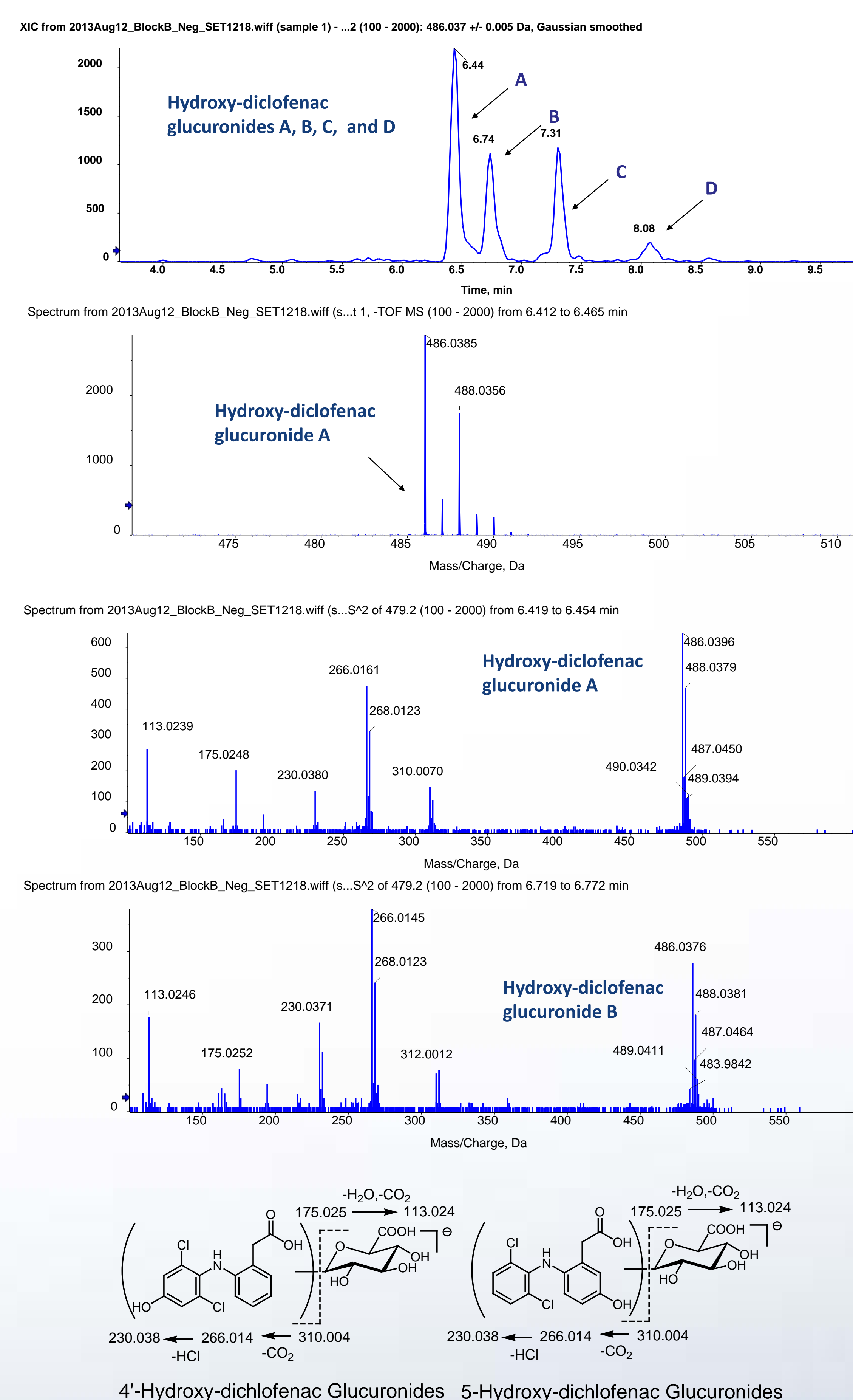


Figure 2. XIC, Protonated Molecules, Product Ion Spectra, and Structures of New Hydroxydiclofenac Glucuronides from Incubation of Diclofenac in Human Hepatocyte Co-Cultures



## RESULTS

### Metabolite Profiling of ZIP in Human Hepatocyte Co-culture Incubation

1. Three (3) major human circulating and excreta metabolites *S*-methyl-dihydro-ZIP(M9), OX-AA (M4), and *N*-dealkyl ZIP sulfone (M1), were identified in human hepatocyte co-cultures (Figure 3).
2. Minor human metabolites M2, M3, M3a, M4a, M5, M6, M7, M8, and M10 identified in human AME study were identified in human hepatocyte co-cultures (Figure 3).
3. A new minor metabolite M6a (Figure 4) was identified in human hepatocyte co-culture.

Figure 3. Structures of Ziprasidone Metabolites

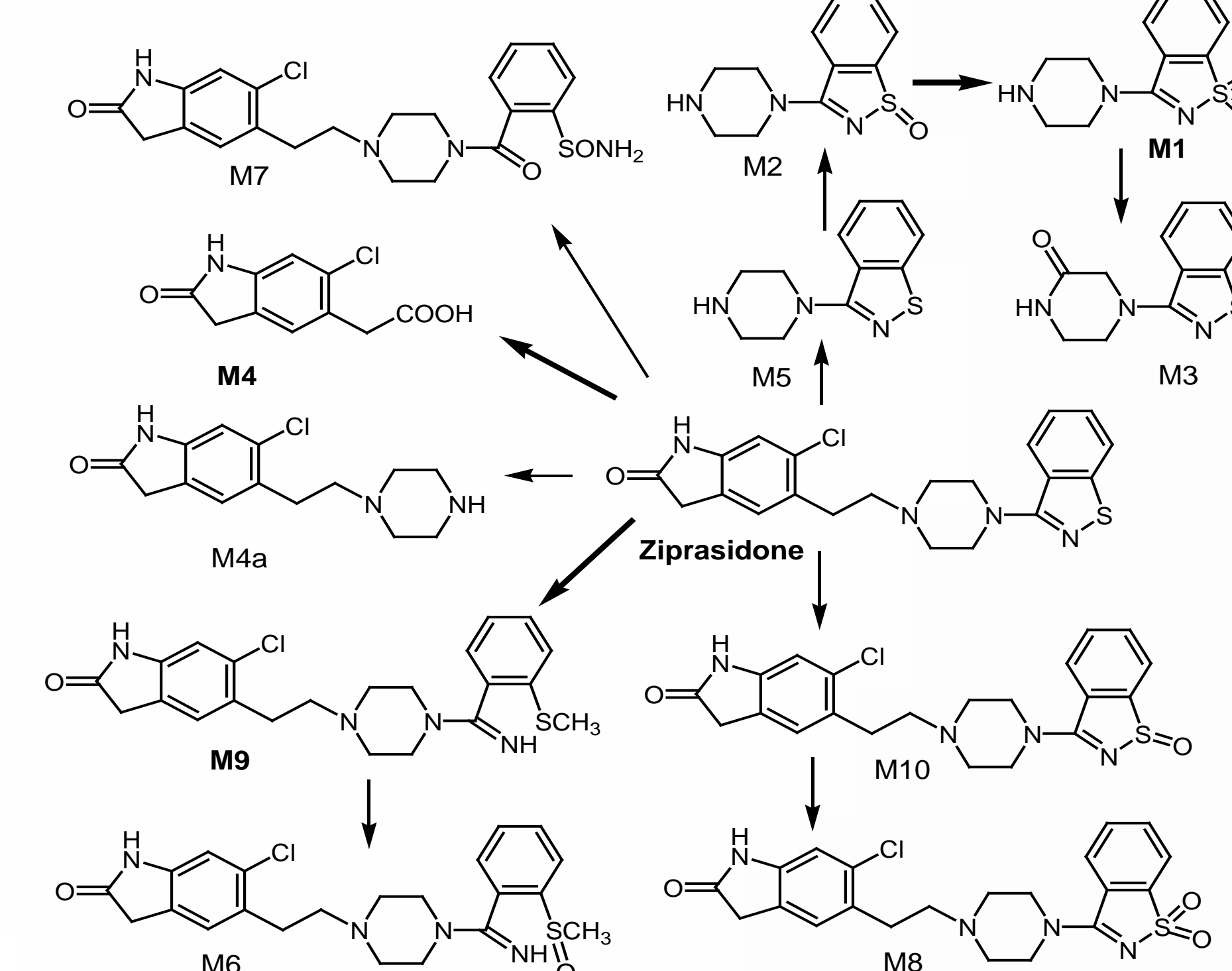
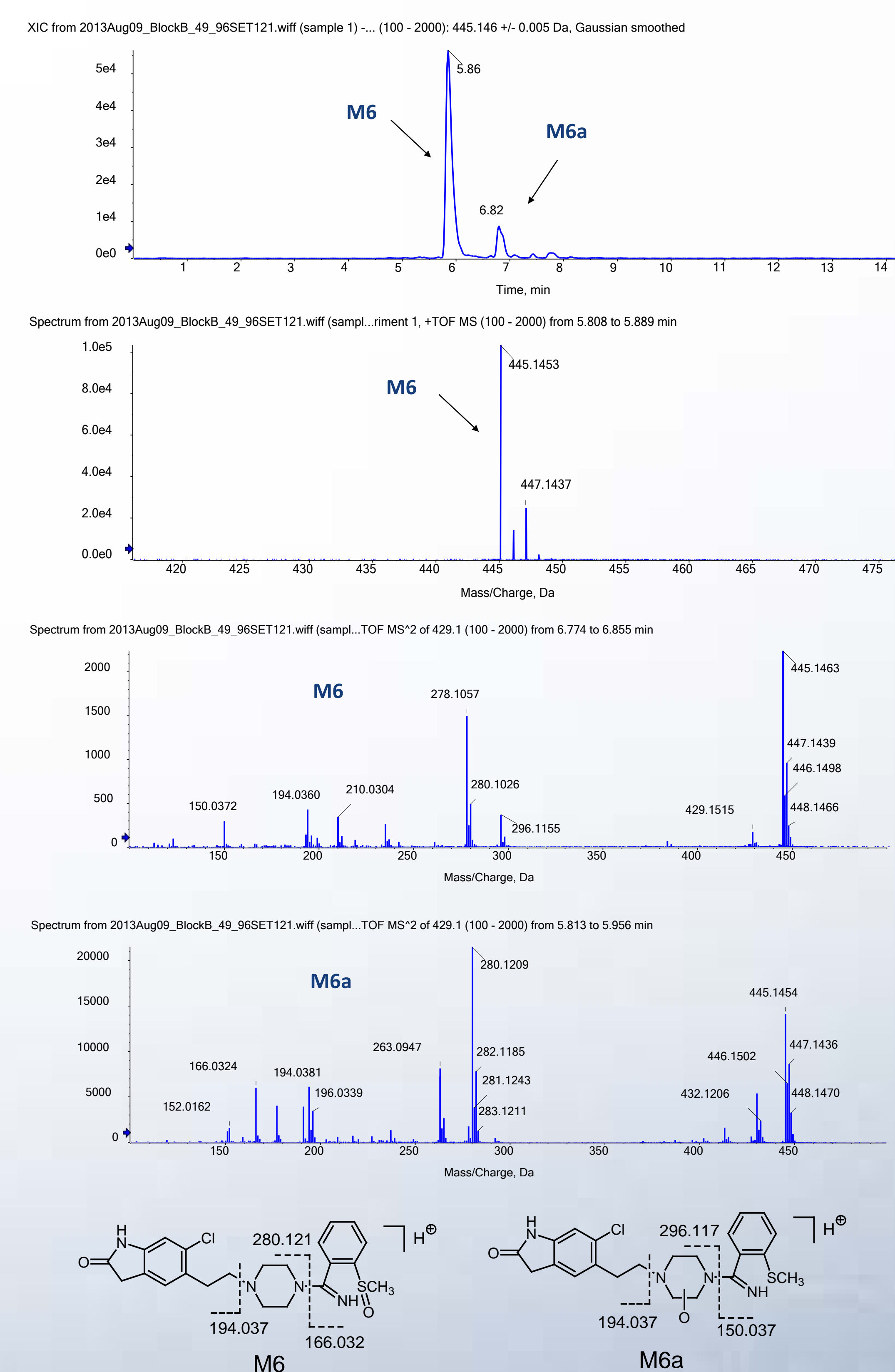


Figure 4. XIC, Protonated Molecules, Product Ion Spectra, and Structures of M6 and M6a from Incubation of ZIP in Human Hepatocyte Co-Cultures



### Metabolite Profiling of Linezolid in Human Hepatocyte Co-culture Incubation

1. Two (2) major human circulating and excreta metabolites, PNU-142586 (M4) and PNU-142300 (M6), were identified in human hepatocyte co-cultures as major or significant components (Figure 5)
2. Minor metabolites M2, M10, M11, M12, M13, M14, M15, M18, and M19a were also identified in human hepatocyte co-cultures (Figure 5).

Figure 5. Structures of Linezolid Metabolites

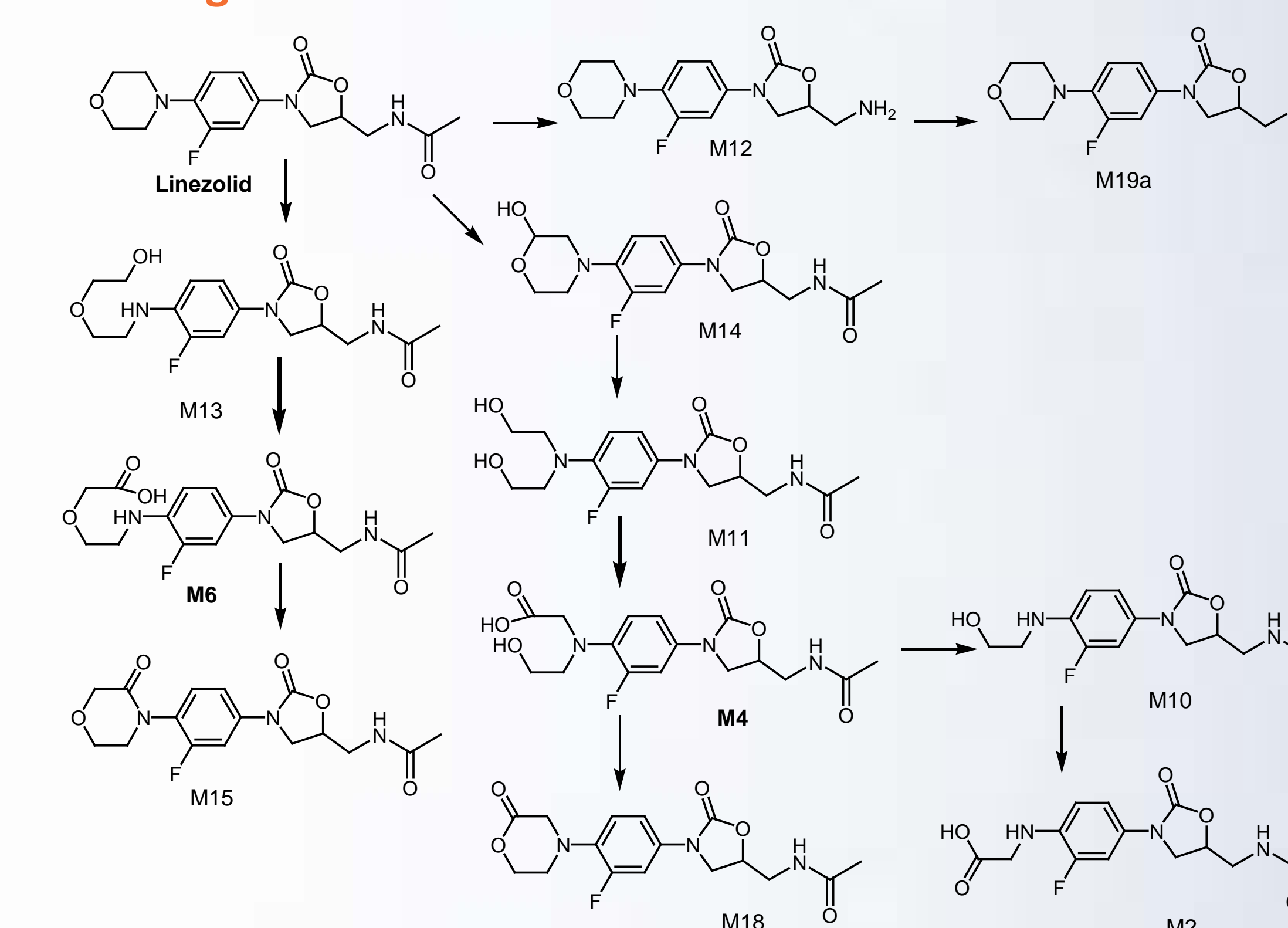


Table 1. Liquid Chromatography Conditions

UHPLC Column	Acquity UPLC BEH C18 2.1 x 100 mm 1.7 $\mu$ m
Column Temperature	40 °C
Flow Rate	0.6 mL/min
Injection Volume	10 $\mu$ L
Mobile Phase A	10 mM CH <sub>3</sub> COONH <sub>4</sub> in water, pH=5.0
Mobile Phase B	Acetonitrile /0.1% formic acid
UHPLC Gradient	5-40-50-95% mobile phase B @0-9-10-11 min

## CONCLUSIONS

1. Human hepatocyte co-cultures produced major and most minor metabolites found in human AME studies for the three compounds tested. New metabolites were also identified in human hepatocyte co-cultures.
2. The TOF-MS and data independent SWATH acquisition generated high quality MS and MS/MS spectra for all metabolites in a single UHPLC injection, and data could be mined retrospectively to avoid additional experiments.
3. The analysis of human hepatocyte co-cultures incubation samples using data independent MS/MS and metabolite ID software proved to be a higher throughput workflow for the identification of human derived metabolites.

## REFERENCE

1. Prakash C et al., Metabolism and excretion of a new antipsychotic drug, ziprasidone, in humans. *DMD*, 1997; 25(7): 863-872.
2. Slatter JG et al., Pharmacokinetics, metabolism, and excretion of linezolid following an oral dose of [<sup>14</sup>C]linezolid to healthy human subjects. *DMD*, 2001; 29(8):1136-1145.
3. Stierlin H et al., Biotransformation of diclofenac sodium (Voltaren) in animals and in man. I: Isolation and identification of principal metabolites. *Xenobiotica*, 1979;9:601-610.
4. Anderson S et al., Predicting circulating human metabolites: how good are we? *Chem Res Toxicol*, 2009;22:243-256.