# Metabolite profiling and characterization by ion mobility LC-timsTOF Pro PASEF

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## Introduction

Rapid and accurate identification and characterization of drug metabolites play a critical role in preclinical and clinical development stages to assist lead compound structure optimization, screening drug candidates, and finding active or potentially toxic metabolites. In this work, a DDA non-targeted LC-timsTOF Pro PASEF (the accumulation parallel serial fragmentation) metabolomics workflow was conducted to profile and characterize drug metabolites (Figure 1). Metabolites were postulated by utilizing BioTransformer[1], a knowledge and machine learning based approach to small molecules metabolism. Metabolite predict structures were elucidated by in silico fragmentation, MS/MS spectral library, comparison of acquired to reference or predicted CCS values using a CCS prediction algorithm. Together, each of these steps forms a fully integrated workflow that utilizes the fourdimensional data to ensure low level drug metabolites can be annotated.

### Methods

A time-series experiment was conducted by spiking pooled human liver microsomes (HLM, Sigma) and Canadine (TRC) into a pre-incubated NADPH regeneration system at  $37^{\circ}C$ ; 100 µL of reaction solution at 0, 5, 15, 30, 45, 60, 90 and 120 min was aliquoted; the reactions were stopped by adding cold acetonitrile; all samples were centrifuged at 12,000 rpm at 4°C for 10 min; the supernatant was transferred into sample insert vial and 5  $\mu$ L was injected (n=3) for each of the two biological replicates. Analysis was performed by LCtrapped ion mobility (TIMS) using an Elute UHPLC connected to a timsTOF Pro system (Bruker) with PASEF data acquisition and ESI positive mode. The resulting four-dimensional data (m/z, RT, mobility, and MS/MS) was processed using DataAnalysis 6.1 and MetaboScape 2023 (Bruker), where raw data was automatically recalibrated for mass and mobility.

Figure 1. In vitro HLM-Drug metabolism by LC-timsTOF Pro PASEF

### **Results and Discussion**

PASEF in timsTOF Pro provides very fast MS/MS acquisition speed at full sensitivity following ion mobility separation and enables very low abundant metabolites could be picked up for fragmentation. About 81 transformations of the predicted Canadine metabolites were listed in Figure 2 based on Cytochrome P450 Phase I biotransformation metabolism by BioTransformer.

Dred	ction	-
Prea	Cana	s dine
~ ~	Cana	dine
Ú	Cana	adine
•	C	anadi
	С	anadi
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-	Cana	adine
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~	Cana	dine
	C	anadi
	C	anadı

Met BioTransformer



#### nd for metabolites highlighted in box Mass [Da] Formula AO Reactio Hydroxylation of heteroalic CYP1A2, CYP2C9, CYP2D 355.14197 C20H21NO5 355.14197 C20H21NO5 Aliphatic hydroxylation of c CYP2C19, CYP2D6 355.14197 C20H21NO5 Hydroxylation of heteroalic CYP1A2 353.12632 C20H19NO5 xidation of secondary alco CYP1A2, CYP2C9, CYP2C 371.13689 C20H21NO6 Hydroxylation of heteroalic CYP1A2, CYP2C9, CYP2D 371.13689 C20H21NO6 Aliphatic hydroxylation of c CYP2C19, CYP2D6 C<sub>19</sub>H<sub>19</sub>NO<sub>4</sub> m/z 326.1387) Canadine 371.13689 C20H21NO6 Aromatic hydroxylation of 1 CYP1A2, CYP2C9, CYP2C $C_{20}H_{21}NO_4$ 341.12632 C19H19NO5 CYP1A2, CYP2C9, CYP2C D-Dealkylation m/z 340.1543 N-Oxidation of alicyclic terl CYP1A2, CYP2C19, CYP2I 371.13689 C20H21NO6 371.13689 C20H21NO6 N-Oxidation of aliphatic ter CYP1A2, CYP2D6, CYP3A 371.13689 C20H21NO6 Dxidation of nitrogen in str. CYP2C9, CYP3A4 341.12632 C19H19NO5 CYP1A2, CYP2C9, CYP2C D-Dealkylation 371.13689 C20H21NO6 Aliphatic hydroxylation of c CYP2C19, CYP2D6 371.13689 C20H21NO6 Hydroxylation of heteroalic CYP1A2, CYP2C9, CYP2D 371.13689 C20H21NO6 Aliphatic hydroxylation of c CYP2C19, CYP2D6 371.13689 C20H21NO6 Hydroxylation of heteroalic CYP1A2 371.13689 C20H21NO6 Allylic hydroxylation CYP1A2, CYP2C19, CYP2I 355.14197 C20H21NO5 CYP1A2, CYP2C19, CYP2I Allylic hydroxylation 355.14197 C20H21NO5 Hydroxylation of heteroalic CYP1A2 355.14197 C20H21NO5 Allylic hydroxylation CYP1A2, CYP2C19, CYP2I 355.14197 C20H21NO5 N-Oxidation of alicyclic terl CYP1A2, CYP2C19, CYP2I C<sub>18</sub>H<sub>19</sub>NO<sub>4</sub> C<sub>19</sub>H<sub>21</sub>NO<sub>4</sub> N-Oxidation of aliphatic ter CYP1A2, CYP2D6, CYP3A 355.14197 C20H21NO5 m/z 328.1543 m/z 314.1387 355.14197 C20H21NO5 Oxidation of nitrogen in str. CYP2C9, CYP3A4 Hydroxylation of heteroalic CYP1A2, CYP2C9, CYP2D 355.14197 C20H21NO5 355.14197 C20H21NO5 Aliphatic hydroxylation of c CYP2C19, CYP2D6 Aliphatic hydroxylation of c CYP2C19, CYP2D6 355.14197 C20H21NO5 CYP1A2, CYP2C9, CYP2C 325.13141 C19H19NO4 O-Dealkylation C₂H₄ 311.11576 C18H17NO4 D-Dealkylation CYP1A2, CYP2C9, CYP2C 0 - CH2 341.12632 C19H19NO5 Hydroxylation of heteroalic CYP1A2 0 - CH2 341.12632 C19H19NO5 Allylic hydroxylation CYP1A2, CYP2B6, CYP2C 0 - CH2 341.12632 C19H19NO5 Aliphatic hydroxylation of c CYP2B6, CYP2C19, CYP2I 0 - CH2 341.12632 C19H19NO5 Hydroxylation of heteroalic CYP1A2, CYP2C9, CYP2D 0 - CH2 341.12632 C19H19NO5 Aliphatic hydroxylation of c CYP2B6, CYP2C19, CYP2I 0 - CH2 Hydroxylation of heteroalic CYP1A2 341.12632 C19H19NO5 0 - CH2 341.12632 C19H19NO5 Allylic hydroxylation CYP1A2, CYP2B6, CYP2C C<sub>20</sub>H<sub>21</sub>NO<sub>5</sub> m/z 356.1493 $C_{20}H_{21}NO_5$ 0 - CH2 N-Oxidation of alicyclic terl CYP1A2, CYP2C19, CYP2I 341.12632 C19H19NO5 m/z 356.1493 0 - CH2 N-Oxidation of aliphatic ter CYP1A2, CYP2D6, CYP3A 341.12632 C19H19NO5 0 - CH2 341.12632 C19H19NO5 Oxidation of nitrogen in str. CYP2C9, CYP3A4 0 - CH₂ Hydroxylation of heteroalic CYP1A2, CYP2C9, CYP2D 341.12632 C19H19NO5 0 - CH2 341.12632 C19H19NO5 Aliphatic hydroxylation of c CYP2B6, CYP2C19, CYP2I 0 - CH2 341.12632 C19H19NO5 Aromatic hydroxylation of I CYP1A2, CYP2B6, CYP2C 🗸

#### Figure 2. Canadine biotransformation and metabolic pathway in human liver microsomes



Peak finding of Canadine drug metabolism was performed in MetaboScape with the T-ReX ® 4D algorithm, which automatically extracts and aligns features based on mass accuracy, isotope pattern, MS/MS and CCS information for each feature enabled confident annotation by using SmartFormula, Analyte List, Spectral Library etc. Based on Canadine metabolic pathway, a "Target List" was generated to annotate the data which matches well with the Biotransformer annotation (see Figure 3). The extracted ion chromatogram confirms the O-demethylation Canadine M1 (m/z 326.1387) and demethylation Canadine M3 (m/z 314.1387), and the O,O'demethyl Canadine metabolite M2 (m/z 328.1543) and oxidated Canadine M4 (m/z 356.1493) which end two isomers at different retention time. The extracted ion mobilogram displays different ion mobilities of Canadine and its metabolites where the oxidated Canadine M4 ends multiple isomers.



Figure 3. EIC and EIM of Canadine and its metabolites

#### References

[1] Djoumbou-Feunang et al.; J. Cheminform, 2019:11:2

# Conclusion

- in vitro HLM drug metabolism sample analysis workflow by LC-timsTOF Pro PASEF was presented.
- Predicted metabolites could be annotated by BioTransformer together other tools in MetaboScape.
- Ion mobility data provides further confident in metabolite fast profiling and characterization.



	_	BPC +All M
· · ·		EIC C <sub>20</sub> H <sub>21</sub> NO <sub>4</sub> [M+H] + (339), Mono-Isotopic, All M
		EIC C <sub>19</sub> H <sub>19</sub> NO <sub>4</sub> [M+H] + (325), Mono-Isotopic, All M
		EIC C <sub>19</sub> H <sub>21</sub> NO <sub>4</sub> [M+H] + (327), Mono-Isotopic, All M
		EIC C <sub>18</sub> H <sub>19</sub> NO <sub>4</sub> [M+H] + (313), Mono-Isotopic, All M
		EIC C <sub>20</sub> H <sub>21</sub> NO <sub>5</sub> [M+H]+ (355), Mono-Isotopic, All M
		A
12.5	15.0	17.5 20.0 Time [min
		EIM 340.1544±0.1 +All MS, 7.0-7.3 mir
		EIM 326.1386±0.1 +All MS, 6.3-6.5 min
		EIM 326.1387±0.1 +All MS, 6.7-6.9 min
		EIM 328.1548±0.1 +All MS, 5.8-6.1 min
		EIM 314.1398±0.1 +All MS, 5.0-5.1 min
		EIM 356.1490±0.1 +All MS, 6.4-6.6 min
		EIM 356.1491±0.1 +All MS, 6.6-6.7 min
1.0	1.1	1.2 1.3 Mobility, 1/Ko [V·s/cm <sup>2</sup>

LC-timsTOF for drug metabolism