Characterizing the Distribution of a STING agonist and its Metabolites in Mouse Liver
by MALDI Imaging Mass Spectrometry

Fang Xie¹, Tracy Gales¹, M.A. Ringenberg², Amaya I. Wolf³, M. Reid Groseclose¹

1. Bioimaging, GSK, Collegeville, PA 19426, USA
2. Non-clinical Safety, GSK, Collegeville, PA 19426, USA
3. Immunology Research, GSK, Collegeville, PA 19426, USA
Running Title: MALDI IMS in investigating drug distribution and metabolism

Correspondence: Fang Xie, Bioimaging, GSK, Collegeville, PA 19426, USA

Email: fang.x.xie@gsk.com

Number of text pages: 14

Number of tables: 1

Number of figures: 4

Number of references: 15

Number of words in Abstract: 157

Number of words in Introduction: 741

Number of words in Discussion: 995

**Abbreviations**

MALDI, matrix-assisted laser desorption/ionization; IMS, imaging mass spectrometry; STING, stimulator of interferon genes; CASI, continuous accumulation of selected ion; IHC, immunohistochemistry.
Abstract

A STING (stimulator of interferon genes) agonist GSK3996915 under investigation in early discovery for hepatitis B was orally dosed to a mouse model for understanding the parent drug distribution in liver, the target organ. MALDI imaging mass spectrometry (IMS) was used to quantify the distribution of GSK3996915 in liver collected from mice administered a single oral dose at 90 mg/kg. GSK3996915 was detected with a zonal distribution localized in the portal triad and highly concentrated in the main bile ducts, indicating clearance through biliary excretion. High spatial resolution imaging showed the distribution of the parent drug localized to the cellular populations in the sinusoids including the Kupffer cells. Additionally, a series of drug-related metabolites were observed to be localized in the central zones of the liver. These results exemplify the potential of utilizing MALDI IMS for measuring not only quantitative drug distribution and target exposure, but also drug metabolism and elimination in a single suite of experiments.

Significance Statement

An integrated imaging approach utilizing MALDI IMS, immunohistochemistry (IHC), and histology was used to measure MALDI IMS complemented with other imaging techniques such as immunohistochemistry addressed the question of target exposure at the cellular level. Localized quantification of the parent drug in the target organ and identification of potential metabolites in the context of tissue histology were also achieved in one experimental suite to support characterization of pharmacokinetic properties of the drug in the early discovery stage.
Introduction

Applications of imaging mass spectrometry (IMS) in pharmaceutical research and development have flourished in the last decade. IMS combines the label-free and multiplex features of mass spectrometry with the imaging capability to visualize the two-dimensional (or even 3D) distribution of molecules in the histopathological context. This unique feature has enabled wide utilization to support pharmaceutical research and development, including drug metabolism and pharmacokinetics, formulation and delivery systems, target engagement and pharmacodynamics, and toxicity mechanism investigations (reviewed in Schulz et al., 2019 and Swales et al., 2019). Despite the continuous increase in published studies of applying IMS in pharmaceutical research and development, there are limited examples of applying IMS to characterize drug metabolism, especially at the early discovery stage. According to a recent review of IMS application in pharmaceutical R&D (Schulz et al., 2019), among the 24 drugs studied using matrix-assisted laser desorption/ionization (MALDI) IMS, only 3 of them included assessment of drug metabolites. In these three studies, two were conducted at the development stage when preliminary information of metabolites was available and MALDI IMS was applied to determine the distribution of the known metabolites (Goodwin et al., 2016; Signor et al., 2007). Only the study published by Shahidi-Latham S et al. (Shahidi-Latham et al., 2012) described a whole-body mass spectrometric imaging that utilized MALDI IMS to measure the distribution of reserpine and its metabolite(s) distribution in Sprague-Dawley rats, including several previously unreported metabolite.
In our current study, a STING (stimulator of interferon genes) agonist GSK3996915 (Figure S1) was under early discovery for treatment of chronic hepatitis B virus infection. The development of compounds that modulate STING, a master regulator of DNA-mediated innate immune activation has been the focus of intense research for the treatment of cancer and infectious diseases as well as vaccine adjuvants. We have shown previously that STING agonists bind competitively with cGAMP and induce dose-dependent secretion of IFN-β following incubation with human peripheral blood mononuclear cells (hPBMCs) (Ramanjulu et al., 2018). Subsequent chemistry efforts identified small molecule STING agonists optimized for oral administration including GSK3996915. Additionally, while previous agonists were identified to stimulate both human and mouse STING, GSK3996915 was able to elicit a potent response specifically in human functional assays. In order to perform in vivo studies in mice, a transgenic mouse model expressing human STING was generated by inserting an expression vector with full-length human knock-in STING cDNA into the second exon of the mouse STING locus. Successful homologous recombination in mouse ES cells was validated and confirmed with PCR sequencing and Southern blot analysis. Human STING expression and localization in target cells and tissues was further confirmed by immunohistochemistry analysis. Functional cell assays using bone marrow derived macrophages were utilized for validation to test IFN-β response to various specific STING ligands. The model resolved the species-specific role of STING and allows study of human STING modulators (manuscript in preparation).

The primary goal of this project was to determine the distribution of the drug in the target organ, liver, to better understand the target exposure and explore evidence of pathway engagement in support of a broader PK-PD assessment. In a pilot study in wild type mice,
the peak of IFN-β production in the serum and liver was at 4 hours following oral
administration of GSK3996915, whereas, the blood and liver concentrations of
GSK3996915 peaked at 45 minutes after the dosing. Therefore, in the current study, 45
minutes and 4 hours post-dosing were selected for sample collection. This drug
development program was in the early discovery stage and therefore metabolism was not
yet characterized. To achieve the primary goal, MALDI IMS was performed on liver to
measure hepatic drug and metabolite distribution compared alongside
immunohistochemistry (IHC) on a serial liver section to mark the target cellular
populations. We acquired full scan data on serial sections and detected a series of drug-
related ions based on accurate mass. Further characterization of the drug-related ions was
conducted by targeting specific regions of the liver tissues and acquiring MALDI MS/MS
fragmentation data to aid in metabolite structure elucidation. The results showed the
localized distribution of the parent drug in the liver and target exposure was assessed by
comparing the distribution with co-registered IHC images. More notably, the metabolic
pathways in the liver were annotated and the distribution of the detected metabolites were
mapped. This study exemplified the ability of MALDI IMS to provide insightful data and
address multiple fundamental questions in support of pharmaceutical research in a single
suite of experiments.

**Materials and Methods**

3.1. Materials and Reagents

HPLC grade methanol, ethanol, and trifluoroacetic acid (TFA) were purchased from
Fischer Scientific (Pittsburgh, PA). 2,5-Dihydroxybenzoic acid (DHB, purity 98%) was
purchased from Sigma Aldrich (St. Louis, MO) and purified by recrystallization. Andwin
Scientific Tissue-Tek Optimum Cutting Temperature (OCT) embedding media was purchased from Fischer Scientific (Pittsburgh, PA). The standard of the study compound GSK3996915 was provided by the medicinal chemistry department of GSK at the purity of 98.7%.

3.2. Tissue Sample Collection and Preparation

A STING-humanized model (HuSTING C57 HO) was used in the study. Six male mice (2–3-month-old) received a single oral dosing of a STING agonist (GSK3996915) at 90 mg/kg. Liver samples were collected at 45 minutes and 4 hours after the dosing (based on the time-course changes of pro-inflammatory cytokines and the pharmacokinetic profile in a pilot experiment), 3 mouse samples at each time point. The samples were snap-frozen in liquid nitrogen immediately after the collection and stored at -80°C until sectioning and analysis. The liver samples were sectioned at a thickness of 8 µm by a Leica Cryostat (Leica Biosystems Inc., Buffalo Grove, IL, USA) at -20 to -25°C. Liver samples from additional untreated male mice were collected and immersion fixed in 10% neutral buffered formalin for 48 hours and processed by routine histology methodology to 5 µm formalin-fixed paraffin-embedded (FFPE) sections. The study was conducted with the samples that were in accordance with the GSK policy on the Care, Welfare, and Treatment of Laboratory Animals and were reviewed by the Institutional Animal Care and Use Committee either at GSK or by the ethical review process at the institution where the work was performed. OCT was used to mount the tissue for cryosectioning.

3.3. Immunohistochemistry and In-situ Hybridization

F4/80 (Cl:A3-1 clone) was purchased from BioRad Life Science (Hercules, California). An optimized dilution (1:400) was used to stain the formalin-fixed frozen sections on a
BenchMark ULTRA Advanced Staining System (Ventana Medical Systems, Oro Valley, AZ, USA). The stained slides were scanned on an Aperio CS2 scanner (Leica Biosystems, Deer Park, IL, USA). For multiplex immunohistochemical staining, FFPE sections from untreated mice were loaded on a Roche Ventana Discovery Ultra autostainer, deparaffinized, antigen retrieved with EDTA (pH 9.0), incubated with for 30 minutes in antibodies to STING (Cell Signaling Technologies, Danvers MA, Cat #13647S, 0.58 ug/ml) and the macrophage marker Iba1 (Wako, Japan Cat #019-19741,0.1 ug/ml), signal amplified using Roche secondary antibodies and chromogens, counterstained with haematoxylin and cover slipped.

3.4. MALDI Mass Spectrometry Imaging

Solutions covering a range of concentrations for GSK3996915 (0, 0.05, 0.25, 0.5, 1, 1.5, 2, 2.5, and 3 µg/mL) were prepared in 50:50 methanol:water. Prior to matrix application, 1 µL of each solution was deposited onto sections of control mouse liver tissue and allowed to air-dry. For each sample section for quantification, a slide with a control mouse liver section deposited with the standard solutions was prepared for simultaneous matrix application. Concentrations were converted to µg/g and calibration curves were generated as reported Barry (Barry et al., 2019b). The limit of blank (LOB) was calculated following Barry et al., 2019a.

The matrix solution consisted of 50 mg/mL DHB dissolved in methanol:water (50:50) with 0.1% TFA, was applied using an HTX TM Sprayer (Carrboro, NC) in 10 psi nitrogen pressure, 70 °C nozzle temperature, 1350 mm/min nozzle velocity, and 3 mm track spacing with a 1.5 mm offset. For the pixel size of 50 µm imaging, the matrix was
sprayed in 8 passes with a 0.1 mL/min flow rate. For the pixel size of 10 µm imaging, the matrix was sprayed in 16 passes with a 0.05 mL/min flow rate.

All MALDI IMS was performed using a Scimax 7T Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA) in positive ion mode. Targeted MS/MS imaging data for GSK3996915 was acquired by setting the quadrupole center mass to m/z 504.3, the quadrupole isolation window to ±10, and the CID voltage to 22 V. Spectra were acquired from m/z 300-550 with a data processing size of 256K resulting in an estimated spectral resolution of ~49,000 at m/z 400. This method efficiently generated a dominant fragment specific for GSK3996915 with a mass transition of m/z 504.253 to 383.183. This approach was used for measuring the distribution of GSK3996915 due to the presence of an interfering endogenous peak detected at m/z 504.257. Baseline resolution of GSK3996915 and the endogenous peak (without MS/MS) would have required the use of an ultra-high mass resolution acquisition method and resulted in exceptionally long acquisition times.

For assessment of the metabolite tissue distribution, full scan mode was used and spectra were acquired with a mass range of m/z 100-2000 with a data processing size of 1M providing an estimated spectral resolution of ~70,000 at m/z 400. For the GSK3996915 metabolites reported here, there was no evidence of interfering endogenous species based on comparison to naïve control liver tissues. Other key acquisition parameters including laser focus (small for 50 µm/pixel, minimum for 10 µm/pixel), data reduction (95%), laser shots (500 for 50 µm/pixel, 25 for 10 µm/pixel), and laser frequency (2 kHz) were held constant.
For identification of prospective metabolites of GSK3996915, MS/MS spectra were manually acquired from a region around the main bile duct captured in the liver sections where the highest drug concentrations localized. Spectra were acquired by centering the quadrupole mass on the ion of interest with a ±1 isolation window and acquiring 25 scans (sum) with a CID energy of 19 V.

All ion images were generated using FlexImaging v5.0 (Bruker Daltonics, Billerica, MA) from the raw data. The MS/MS data were manually analyzed using Compass DataAnalysis v5.0 and tentative fragment ions were identified using the SmartFormula annotation tool.
Results

Distribution of the Parent Drug. Targeted MALDI IMS (CASI mode) was used to investigate the overall distribution of the parent drug across liver tissue sections at a pixel size of 50 µm. Figure 1B shows the distribution of the parent drug in one liver tissue section from each animal. The images indicated that the distribution of the drug was not homogenous in the liver. By comparing the ion images to the H&E staining on an adjacent serial section (Figure 1A) and overlaying the ion images with the H&E staining post MALDI IMS acquisition (Figure 1C), it can be visualized that at 45 minutes postdose, higher drug signals were detected periportal relative to the centrilobular regions. Additionally, an area of high intensity signal for GSK3996915 was detected colocalized with a main bile duct captured on one of liver tissue sections (Figure 1B, M349). At 4 hours postdose, the overall signal intensities in all samples substantially decreased compared with the 45-minute samples; however, in the main bile duct captured on a liver tissue section from M355 (Figure 1B) high intensity signal for GSK3996915 was still observed. A dilution series of the standard spotted on a control liver section was used to build the calibration curve for quantification (Supplemental Figure 2). Moreover, a section from a different lobe of each liver sample (T2) was also imaged (Supplemental Figure 3) and quantified. Table 1 summarizes the average concentration of GSK3996915 estimated in each tissue and in the main bile ducts captured in sections from animals M349 and M355.

High Spatial Resolution Distribution of the Parent Drug. To specifically investigate the engagement of the drug with the target cell population, an area of a serial section from each liver sample collected at 45 minutes postdose was imaged with high spatial...
resolution (pixel size of 10 µm). Figure 2 displays the ion image detected for GSK3996915 in an area of a liver section from M348. The highest intensity signals for GSK3996915 were localized to the bile ducts in the portal triads with lower intensity signals detected primarily in the sinusoids, which is also where Kupffer cells are localized as shown in Figure 2F. Moreover, in a separate experiment, the dual IHC staining of STING and Kupffer cells of liver sections from the same mouse model verified that STING was expressed in Kupffer cells in sinusoids and endothelial cells lining the central veins, but not in hepatocytes (Figure 2G).

**Distribution of Drug Metabolites.** To assess the presence of metabolites of GSK3996915, full-scan imaging data was acquired on a separate set of liver tissue sections serial to the sections analyzed with targeted CASI mode imaging of GSK3996915. Filtering of the dataset using molecular formula prediction peak annotation revealed a list of potential drug-related ions, that were highly localized to the main bile ducts captured in M349 and M355. On-tissue MALDI MS/MS of putative metabolite peaks collected directly from the region of the main bile duct in M349 provided the preliminary structure identification of the detected metabolites. Figure 3 shows the proposed metabolic pathway of the five detected metabolites identified by accurate mass and MS/MS (Supplemental Table 1). Figure 4 is the ion images of the detected metabolites on representative sections at 45 minutes postdose. The overlaid ion images of the parent drug ion and the metabolite ion indicated that except in the main bile duct captured on M349, the distribution of the parent drug and the metabolites were unique, with parent drug localized to the portal zones and the metabolites localized to the central zones.
Discussions

The use of MALDI IMS to understand the tissue distribution of therapeutic agents can provide unique spatial information to better define pharmacokinetics of drug candidates. The majority of published studies focus on correlating the distribution of drug molecules to the anatomical and/or histological features of a tissue (reviewed by Spruill et al., 2022). In contrast, reports of investigating drug distribution to single cells by MALDI IMS have been scarce, mainly due to hardware limitations of spatial resolution and the associated sensitivity challenges. However, characterizing the drug distribution at cellular level can be critical in some cases to address the question of target exposure and engagement. In the current study, the drug target STING is not homogeneously distributed in the target organ liver. STING is mainly expressed and activated in hepatic non-parenchymal cells including Kupffer cells, sinusoidal endothelial cells, and hepatic stellate cells (Chen et al., 2021), whereas, the major liver cell population hepatocytes do not express STING. Western blot results highlighted that the highest expression of STING was in the Kupffer cells compared with endothelial cells and stellate cells (unpublished data). Therefore, it is important to differentiate the distribution of the drug in different cellular populations, especially between hepatocytes and the non-parenchymal cells, in addition to imaging the drug distribution at the zonal level of liver. Through high spatial resolution MALDI imaging (10 µm/pixel) along with immunohistochemistry staining of the Kupffer cell marker on a serial section, we were able to conclude that the drug was highly localized in the bile ducts of the portal zone and the sinusoids rather than the hepatocytes, providing increased confidence of STING target exposure.
The quantitative capability of MALDI IMS has advanced rapidly. Application of mimetic tissue models (Barry et al., 2019a) or dilution series (Barry et al., 2019b) allows pixel by pixel quantification of treated drugs on tissue sections and comparable accuracy to LC-MS-MS quantification in terms of average concentrations. The pixel-by-pixel quantification uniquely offers localized quantification of any region of interest on tissue sections. In our study, we showed that high drug concentrations exceeding 300 µg/g (determined by linear extrapolation beyond the highest calibration point) in major bile ducts was present at 4 hours following a single oral dose at 90 mg/kg, even though the drug concentrations outside the main bile ducts significantly decreased to less than 10 µg/g at 4 hours compared to 45 minutes. The results indicated biliary excretion of the drug molecule and the quantitative results could support further modelling of the pharmacokinetics. Additional studies are warranted to validate the time-course clearance of the drug through biliary excretion and more accurately quantify the drug concentrations in the bile.

Liver is known to be a primary site of drug metabolism that can convert parent prodrugs to active metabolites or active drugs to inactive forms for elimination or even produce toxic intermediates (reviewed by Almazroo et al., 2017). Drug induced liver injury (DILI) has been a crucial issue in drug discovery and development. Imaging mass spectrometry offers a powerful tool to determine the temporal and spatial distribution of drugs and metabolites in tissue including the liver in support of investigating relationship between reactive metabolites and DILI (reviewed by Spruill et al., 2022). In the study reported by Grove et al. (Grove et al., 2019), MALDI IMS was applied to map the distribution of amodiaquine (AQ) and its metabolites in rat liver to understand AQ-induced idiosyncratic DILI. The study detected the metabolites of AQ including de-ethyl-AQ (DEAQ) and the
glutathione adduct (AQ-SG) with AQ localized near the portal triad and the metabolite DEAQ localized near the central veins. The imaging results also showed depletion of glutathione in the centrilobular region to form AQ-SG. These results suggested that AQ was bioactivated to quinonimine in the centrilobular to deplete glutathione forming AQ-SG as the initial step in AQ-induced liver injury. Other examples include employing MALDI IMS to visualize acetaminophen and its metabolites in mouse liver to investigate acetaminophen-induced liver toxicity (Sezgin et al., 2018) and employing electrospray IMS to image diclofenac and its metabolites in mouse liver and kidney (Mesa Sanchez et al., 2022). In all these examples, the metabolic pathways of the parent drugs were well characterized prior to the imaging experiments. IMS was conducted in a more targeted way to investigate the distribution of the parent drugs versus the metabolites. In our current study, the drug molecule was at the early discovery stage and the metabolism information was not available. However, the full scan data of high spectral resolution generated by MALDI IMS along with molecular formula prediction and ROI targeted on-tissue MS/MS analysis allowed us to identify/annotate drug-related metabolites. The distribution of the metabolites was unique from the distribution of the parent drug as the metabolites were observed to be localized to the centrilobular regions, with the exception of the major bile duct captured on the section where both parent drug and the metabolites were highly concentrated. This distribution pattern is consistent with what was reported in Grove’s study (Grove et al., 2019) and is aligned with the hepatic distribution of cytochrome P450 enzymes given the P450s are more localized in the central zones than the portal zones (Lindros, 1997). The metabolites and their distribution detected by MALDI IMS can be illuminating for the subsequent metabolism studies of the project,
including in vitro incubation with hepatocytes and LC-MS-MS analysis of liver homogenates to provide definitive metabolite identification.

Taking together, in the current study, we have demonstrated that by applying MALDI IMS on serial sections at different modes (CASI and full scan), and further integrating with immunohistochemistry, questions of drug exposure at target cellular population, drug concentrations at specific anatomical/histological structures, and metabolism of the parent drug can be addressed in one suit of experiments. The study also exemplified the potential of MALDI IMS as an emerging tool at the early stage of drug discovery to quantify drug distribution, understand target exposure, and spatially characterize drug metabolism that can shed light of drug pharmacokinetics and future investigation.

Acknowledgements

We thank Jared Spletstoser and Joshi Ramanjulu for the support of this work.

Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contributions

Participated in research design: Xie, Gales, Ringenberg, Wolf, Groseclose

Conducted experiments: Xie, Gales, Groseclose

Contributed new reagents or analytic tools: Wolf
Performed data analysis: Xie, Gales, Ringenberg, Groseclose

Wrote or contributed to the writing of the manuscript: Xie, Gales, Wolf, Ringenberg, Groseclose


Reference


Footnotes

This work received no external funding.

No author has an actual or perceived conflict of interest with the contents of this article.
Figure Legends

**Fig.1.** Mouse liver tissue sections (A) stained by H&E (T1S2); (B) analyzed by MALDI IMS in CASI mode (T1S1) at 50 µm spatial resolution showing the representative ion images for GSK3996915 fragment (m/z 383.183); (C) ion images of GSK3996915 fragment (m/z 383.183) overlaid with post-MALDI H&E staining with main bile duct annotated.

**Fig.2.** Mouse M348 live sections (A) analyzed by MALDI IMS in CASI mode (T1S3) at 10 µm spatial resolution showing the representative ion images for GSK3996915 fragment (m/z 383.183); (B) ion images of GSK3996915 fragment (m/z 383.183) overlaid with the optical image; (C) ion images of GSK3996915 fragment (m/z 383.183) overlaid with post-MALDI H&E staining; (D) zoomed-in image of the boxed area in (B); (E) zoomed-in image of the boxed area of (C); (F) IHC staining of F4/80 cell population on a comparable area in (D) and (E) on a serial section T1S4; (G) dual immunohistochemical staining of STING (purple) and Iba1 (yellow, Kupffer cell/macrophage marker) on FFPE liver sections with STING staining observed in Kupffer cells (large arrows, yellow and purple combine to orange-red color), endothelial cells (arrowheads) and no STING staining was observed in hepatocytes (H, thin arrows).

**Fig.3.** Proposed metabolic pathway of GSK3996915 in mouse liver.

**Fig.4.** Ion images of GSK3996915 and detected metabolites (M1-M5) on mouse liver sections (T1S5) at 45 minutes after dosing at 50 µm spatial resolution (A) ion images of M1-M5 (B) ion images of M1-M5 overlaid with the ion image of the parent drug. ND, not detectable.
Table 1: Concentration of GSK3996915 in Liver Sections Quantified by MALDI IMS

<table>
<thead>
<tr>
<th>Time</th>
<th>Animal</th>
<th>Section</th>
<th>Average Concentration (µg/g)</th>
<th>Main Bile Duct Concentration (µg/g)*</th>
<th>Concentration Outside the Main Bile Duct (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45 minutes</td>
<td>M348</td>
<td>T1</td>
<td>47.31</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2</td>
<td>33.98</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T1</td>
<td>52.40</td>
<td>332.51</td>
<td>33.46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2</td>
<td>26.09</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M350</td>
<td>T1</td>
<td>17.91</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2</td>
<td>19.09</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4 hours</td>
<td>M354</td>
<td>T1</td>
<td>1.91</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2</td>
<td>2.73</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M355</td>
<td>T1</td>
<td>23.91</td>
<td>306.80</td>
<td>9.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2</td>
<td>8.16</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>M356</td>
<td>T1</td>
<td>5.43</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2</td>
<td>4.24</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*The calculated concentrations were extrapolated beyond the highest concentration of the calibration curve.
Figure 3

- M1: N-dealkylation, [M+H]+ 476.2216
- M2: Demethylation, Glucuronide Conjugation, [M+H]+ 666.2693
- M3: Oxidation, [M+H]+ 520.2478
- M4: N-dealkylation, oxidation, [M+H]+ 492.2165
- M5: N-dealkylation, demethylation, glucuronidation, [M+H]+ 638.2380
Figure 4

A

M1

1% - 15%

M2

1% - 10%

M3

5% - 15%

M4

7% - 15%

M5

5% - 15%

B

M1 Parent

1% - 15%

M2 Parent

1% - 10%

M3 Parent

5% - 15%

M4 Parent

7% - 15%

UD

2% - 15%