Title: Effect of cimetidine on metformin pharmacokinetics and endogenous metabolite levels in rats

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Renal transport, organic cation transport, pharmacokinetic, metabolomics, drug-drug interactions.
ABBREVIATIONS:

OCT: Organic cation transporter
MATE: Multidrug and toxic extrusion transporter
DDI: Drug-drug interaction
PK: Pharmacokinetics
FDA: Food and Drug Administration
EMA: European Medicines Agency
PMDA: Pharmaceuticals and Medical Devices Agency
CL\textsubscript{R}: Renal clearance
AUC: Area under the curve
OAT: Organic anion transporter
THTR: Thiamine transporter
OATP: Organic anion transporting polypeptide
SLC: Solute carrier
i.p.: Intraperitoneal
i.v.: Intravenous
SD: Sprague-Dawley
LC-MS/MS: Liquid chromatography-tandem mass spectroscopy
QC: Quality control
MRM: Multiple reaction monitoring
CV: Cone voltage
IC\textsubscript{50}: Half-maximum inhibitory concentration
TPSA: Total polar surface area
ABSTRACT

Tubular secretion is a primary mechanism along with glomerular filtration for renal elimination of drugs and toxicants into urine. Organic cation transporters (OCTs) and multidrug and toxic extrusion (MATE) transporters facilitate the active secretion of cationic substrates including drugs such as metformin and endogenous cations. We hypothesized that administration of cimetidine, an Oct/Mate inhibitor, will result in increased plasma levels and decreased renal clearance of metformin and endogenous Oct/Mate substrates in rats. A paired rat pharmacokinetic study was carried out, where metformin (5 mg/kg, intravenous) was administered as an exogenous substrate of Oct/Mate transporters to six Sprague-Dawley rats with and without cimetidine (100 mg/kg, intraperitoneal). When co-administered with cimetidine, metformin area under the curve increased significantly by 3.2-fold, and its renal clearance reduced significantly by 73%. Untargeted metabolomics was performed to investigate the effect of cimetidine on endogenous metabolome in the blood and urine samples. Over 8,000 features (metabolites) were detected in the blood, which were shortlisted using optimized criteria, i.e., a significant increase ($p$-value <0.05) in metabolite peak intensity in the cimetidine-treated group, reproducible retention time, and quality of chromatogram peak. The metabolite hits were classified into three groups that can potentially distinguish inhibition of i) extra-renal uptake transport or catabolism, ii) renal Octs, and iii) renal efflux transporters or metabolite formation. The metabolomics approach identified novel putative endogenous substrates of cationic transporters that could be tested as potential biomarkers to predict Oct/Mate transporter mediated drug-drug interactions in the preclinical stages.
SIGNIFICANCE STATEMENT

Endogenous substrates of renal transporters in animal models could be used as potential biomarkers to predict renal drug-drug interactions in early drug development. Here we demonstrated that cimetidine, an inhibitor of organic cation transporters (Oct/Mate), could alter the pharmacokinetics of an exogenous substrate (i.e., metformin) and endogenous cationic substrates in rats. Several putative endogenous metabolites of Oct/Mate transporters were identified using metabolomics approach, which could be tested as potential transporter biomarkers to predict renal drug-drug interaction of Oct/Mate substrates.
INTRODUCTION
The kidneys are the major organs for drug elimination, especially for drugs that have minimum hepatic metabolism (Varma et al., 2009). Renal clearance ($\text{CL}_R$) is the net result of three concurrent processes, i.e., glomerular filtration, tubular secretion, and reabsorption. Glomerular filtration is a passive process that involves the removal of unbound chemicals from blood, whereas tubular secretion is a transporter-mediated process. Transporters are predominantly localized in the basolateral and apical membranes of the proximal tubules (Yin and Wang, 2016). Organic cationic transporters (OCTs) are the solute carrier (SLC) transporters with three isoforms (OCT1, OCT2, and OCT3), which are expressed in different organs in humans. OCT2 (SLC22A2) is predominantly expressed in human kidneys and is responsible for transporting cationic compounds from blood into renal tubular cells. The cationic substrates are then secreted from renal tubular cells to urine through multidrug and toxin extrusion (MATE1 and MATE2-K) transporters expressed at the apical membrane (Müller et al., 2018a). Although humans express OCT2, MATE1, and MATE2-K in the kidneys, rodent kidneys express Oct1, Oct2, and Mate1 (Lechner et al., 2021).

The activity of renal transporters can be perturbed by drug-drug interactions (DDIs) leading to alteration in drug pharmacokinetics (PK). For example, DDI of cimetidine and metformin is mediated by renal transporters in humans (Wiebe et al., 2020). Cimetidine is a nonspecific inhibitor for OCT2 and MATE transporters in the kidneys, whereas metformin is a substrate for the same cationic transporters. Metformin exists as a cation species at physiological pH, remains in its unbounded form in plasma, and excreted unchanged in the urine (Maideen et al., 2017). $\text{CL}_R$ of metformin is approximately $510 \pm 120$ mL/min (i.e., ~5-fold higher than its glomerular filtration clearance), indicating that the active tubular secretion is the major route for metformin elimination (Gong et al., 2012). Renal excretion of metformin from the blood into renal epithelial cells is primarily mediated by OCT2, which is then excreted from the renal tubular cells into the lumen of renal proximal tubules by MATE transporters (Gong et al., 2012). Coadministration of cimetidine has been reported to increase
plasma concentration and decrease $\text{CL}_R$ of metformin suggesting its in vivo OCT2/MATE inhibitory potential (Somogyi et al., 1987).

The United States Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) have issued guidelines for the investigation of transporter inhibition potential of new drugs by using transporter probe drugs in clinical studies. However, the use of an exogenous probe for evaluation of transporter-mediated DDI potential is only possible through a prospective clinical study at a later phase of drug development, which adds significant costs and may raise safety concerns in case of a severe DDI between a test drug and exogenous transporter substrate (Tornio et al., 2019). To address these challenges, endogenous biomarkers are proposed to replace the need for exogenous probe drugs for early assessment of clinical DDI during Phase I trials (Miyake et al., 2019). Endogenous transporter biomarkers can minimize pill burden, provide a minimally invasive procedure to evaluate DDI, be cost-effective, and allow studying transporter activity in specific populations (Rodrigues et al., 2018). Further, transporters are becoming increasingly relevant during the preclinical phase of drug development due to their involvement in drug toxicity and tissue concentration (Sasseville et al., 2014). Drug companies can minimize the risk of transporter-mediated DDI by analyzing blood and urine levels of transporter biomarkers during routine preclinical PK and toxicological studies prior to the first-in-human studies (Shen et al., 2019). However, the endogenous substrates or biomarkers of renal transporters in preclinical species, especially in rodents are not characterized.

Metabolomics is an emerging technique to identify endogenous substrates of drug transporters through analysis of metabolites in biological samples after perturbed transporter function (Thakur et al., 2023). An ideal transporter biomarker should be sensitive, selective, and independent of confounding factors associated with its formation, degradation, diet, and other transporter-independent factors (Chu et al., 2017). The growing interest in using transporter biomarkers and the availability of good analytical tools have led to the identification of biomarkers for various clinically
relevant drug transporters. Coproporphyrins I and III, heme synthesis byproducts, have been successfully used as biomarkers for hepatic organic anion transporting polypeptide 1B1 and 1B3 (OATP1B1 and OATP1B3) (Lai et al., 2016; Shen et al., 2017). Taurine and 6β-hydroxycortisol have been proposed as potential candidate biomarkers for organic anion transporters (OAT1) and (OAT3), respectively (Imamura et al., 2014; Tsuruya et al., 2016). Although several potential endogenous substrates have been identified for various transporters, the quest for sensitive and selective transporter biomarkers is still ongoing. For example, N1-methylnicotinamide (NMN), a proposed endogenous biomarker for human OCT/MATE, showed a decreased CLR in healthy volunteers treated with pyrimethamine (OCT/MATE inhibitor), but no change in plasma concentrations (Ito et al., 2012a). Thiamine (vitamin B1) has also been proposed as Oct1/Oct2 endogenous probe in mice, where a 5.8-fold increase in the plasma levels and 79% decrease in CLR has been reported in Oct1/Oct2 double-knockout mice compared to wild-type mice (Kato et al., 2015). However, thiamine has limited data supporting its utility as an Oct2 endogenous biomarker because of the involvement of other transporters in its elimination like thiamine transporters 1 and 2 (THTR1 and THTR2) (Brown, 2014; Chu et al., 2017). Additionally, CLR of thiamine can drop significantly in thiamine deficiency conditions due to its complete renal reabsorption (Weber et al., 1990).

The objective of this study was to identify endogenous substrates that can be used as potential biomarkers for renal cationic transporters. A cimetidine metformin PK DDI study was conducted in six Sprague-Dawley (SD). We hypothesized that cimetidine-mediated Oct/Mate inhibition will increase blood levels and decrease CLR of the exogenous probe (metformin) and endogenous substrates of Oct/Mate transporters. Untargeted metabolomics was utilized to identify potential Oct/Mate biomarkers in rats by comparing endogenous substrate levels in rat blood and urine samples with and without cimetidine treatment.

MATERIALS AND METHODS

Chemicals and Reagents
Metformin, cimetidine, and metformin-d6 were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). LC-MS grade methanol, acetonitrile, water, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Ethanol was procured from KOPTEC (King of Prussia, PA). Hydrochloric acid (HCl) was purchased from EMD Millipore Corporation (Billerica, MA).

Animals
Six SD rats (three males and three females, 11–13 weeks, 244–503 g) were purchased from Charles River Laboratories, Inc. (Hollister, CA) and housed for one week in a temperature- and humidity-controlled room with a 12-h light/dark cycle with free access to food and water. The experimental protocol and procedures were approved by Washington State University Institutional Animal Care and Use Committee.

Animal Study Protocol
Metformin and cimetidine were solubilized to form a clear solution for the dosing. Metformin was prepared as a 1 mg/mL solution in saline, whereas cimetidine was prepared in saline containing 10% ethanol adjusted to pH=6 using 1N HCl. The animal study was a two-arm paired study, where the same rats were used in two arms with one week washout period. The rats were anaesthetized with isoflurane and the tail vein was cannulated with a 22-gauge needle. In the first arm (metformin alone), six rats received vehicle (5 mL/kg, 10% ethanol in saline) as an intraperitoneal injection (i.p.) 15 min before 5 mg/kg intravenous (i.v.) metformin bolus injection. In the second arm (metformin and cimetidine), the same six rats received 100 mg/kg cimetidine (i.p.) 15 min before 5 mg/kg metformin injection.

The blood samples (25 µL) were collected in heparinized tubes via the tail vein at pre-dose, 0.17, 0.5, 1, 2, 4, 6, and 8 h and mixed with 25 µL heparinized water (containing 30 IU heparin). Urine samples were collected at 0–4 h and 4-8 h intervals in metabolic cages. Additionally, pre-
dose urine samples were collected 2 h before metformin treatment in the first arm. Blood and urine samples were stored at -80 °C until analysis by liquid chromatography-tandem mass spectroscopy (LC-MS/MS). Food was withheld during the study (0-8 h) with ad libitum access to water.

Metformin and Cimetidine Analysis in Blood and Urine Samples

Blood Analysis:
Metformin concentration in blood samples was measured against a standard curve following precipitation with acetonitrile containing 500 ng/mL of metformin-d6 as an internal standard. 400 µL acetonitrile containing metformin-d6 was added to the diluted blood sample (blood: water, 50:50 v/v), vortexed for 5 min, and centrifuged at 10,000 xg for 10 min. The supernatant (380 µL) was transferred into a new tube and dried using a vacuum dryer (Eppendorf, Germany). The dried sample was reconstituted with 75 µL of acetonitrile: water (10:90, v/v), vortex for 5 min and centrifuged at 16,000 xg for 15 min. Finally, 5 µL of the supernatant was diluted to 100 µL with acetonitrile: water mix (10:90, v/v), and 1 µL was injected into LC-MS/MS for metformin and cimetidine analysis.

For the blood calibration curve, metformin and cimetidine were serially diluted using methanol: water (80:20, v/v) to prepare 10X working concentrations. 5 µL from each working concentration was diluted in 45 µL matrix (25 µL blank blood plus 20 µL water) to achieve the final calibration curve concentrations in LC-MS vial of 0.5-953 ng/ml (metformin) and 0.5-965 ng/ml (cimetidine). Quality control (QC) samples were also prepared representing 9.9, 424, and 847 ng/mL of metformin and 10, 429, and 858 ng/mL of cimetidine in LC-MS vial. The calibration curve standards and QC samples were processed and analyzed similar to the blood samples.
Urine Analysis:

Urine samples were vortex mixed for 5 min. Urine sample (5 µL) was diluted 20-fold by 95 µL of acetonitrile containing 2.8 µg/mL of metformin-d6. The sample was vortex mixed for 2 min and centrifuged at 16,000 xg for 10 min. The supernatant (10 µL) was transferred to a tube containing 490 µL of 0.1% formic acid in water. 50 µL of the diluted sample was transferred into an LC-MS vial. For the urine calibration curve, metformin and cimetidine were serially diluted in methanol: water (80:20, v/v) to prepare final LC-MS vial concentrations of 1-572 and 2-1022 ng/mL, respectively. QC samples were prepared at 8.6, 257, and 515 ng/mL for metformin and 15.3, 460, and 920 ng/mL for cimetidine. Individual calibration standard or QC samples (5 µL) were processed similar to the urine samples from the PK study and transferred into LC-MS vials prior to analysis.

**LC-MS/MS conditions for blood and urine analysis**

The amount of metformin and cimetidine in the processed blood and urine samples, calibration curve standards, and QC samples were analyzed using LC-MS/MS (Waters Xevo-TQ-XS MS; Waters, Milford, MA) equipped with standard electrospray ionization source. Chromatographic separation of the analytes was achieved using microflow LC and Acquity UPLC® HSS T3 (1.8 µm, 1x100 mm) column. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was operated using the following gradient program: 0-1 min (5% B), 1-2.5 min (5% to 55% B), 2.5-4.5 min (55% to 70% B), 4.5-5.5 min (70% to 90% B), 5.5-6.5 min (90% B), 6.5-7 (90% to 5% B), and 7-9 min (5% B). The mobile phase flow rate was 50 µL/min and the injection volume was 1 µL. The LC-MS/MS was run in positive ionization (ESI+) and multiple reaction monitoring (MRM) modes with a cone voltage (CV) of 25 V. The MRM transitions were: metformin (m/z 130.1 → 60.0 and m/z 130.1 → 88.1), metformin-d6 (m/z: 136.1 → 77.1), and cimetidine (m/z: 253.1 → 95.2).
Data analysis for rat blood and urine samples

LC-MS/MS peak integration and quantification were performed using Skyline software 21.2.0.568. (University of Washington, Seattle, WA). The PK parameters of metformin were calculated by non-compartmental analysis of blood concentration versus time data using MATLAB® (R2021a; Natick, MA) and the area under the blood concentration-time curve from 0 to 8 h (AUC₀⁻₈₉) was calculated. The analyte concentrations in urine were converted to the amount excreted in urine (Ae) by multiplying it with the urine volumes. CLR was calculated by dividing Ae by AUC₀⁻₈₉ (Equation 1). The ratios of AUC₀⁻₈₉ and CLR were calculated in the presence and absence of cimetidine to evaluate the DDI.

\[
\text{Renal clearance (CLR)} = \frac{\text{Amount of metformin excreted unchanged in urine (Ae₀⁻₈₉)}}{\text{Metformin area under the curve (AUC₀⁻₈₉)}}
\]

Equation 1

Samples Preparation for Untargeted Metabolomics

Considering that the maximum concentration of cimetidine was observed between 0.5-2.0 h, 0.5 and 1 h blood timepoints were used for untargeted metabolomics. Forty µL of processed rat blood samples from each rat in both the arms were mixed into 320 µL acetonitrile containing 0.1% formic acid. The samples were vortexed for 5 min and centrifuged at 16,000 xg for 10 min. The supernatant was transferred to a different tube and stored overnight at -20 °C. The samples were centrifuged again at 16,000 xg for 10 min, and the supernatant was collected. The samples were evaporated in a vacuum dryer at 45 °C until completely dried. The dried samples were reconstituted with 0.1% formic acid in water. Finally, 1 µL was injected into nano-flow-based liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) for untargeted metabolomics analysis.

Similarly, rat urine samples from 0-4 h intervals were used for urine untargeted metabolomic analysis assuming the highest inhibition during this interval. The processed urine (100 µL) was added to 200 µL acetonitrile containing 0.1% formic acid. The sample was vortexed for 2 min
and stored overnight at -20 °C before centrifugation at 16,000 xg for 10 min. The supernatant
was transferred to a separate tube for evaporation in a vacuum dryer at 45 °C until completely
dried. The dried sample was reconstituted with 0.1% formic acid in water and 1 µL sample was
injected into nanoLC-MS/MS for untargeted metabolomics analysis.

**nanoLC-MS/MS conditions for untargeted metabolomics**

The blood and urine samples for the untargeted metabolomics experiment were analyzed using
nano-flow LC coupled to Thermo Scientific™ Q Exactive™ HF in data-independent acquisition
(DIA) mode. Chromatographic separation of analytes was performed using nanoflow LC and
Thermo Scientific PepMap™ RSLC C18 (75 µm x 25 cm, 2 µm particle size) column. The
mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in 80%
acetonitrile (B) was used in a gradient program: 0-5 min (0% B), 5 -35 min (5% to 40% B), 35-65
min (40% to 100% B), and 65-75 min (100% B). The mobile phase flow rate was 300 nL/min
and the injection volume was 1 µL.

**Data analysis of untargeted metabolomics**

The high-resolution mass spectrometry data were processed using XCMS online (Scripps
Research, La Jolla, CA) cloud-based bioinformatics platform to detect and align features. XCMS
analysis of these data provided ~8,000 features with distinct characteristics such as the
retention time, mass-to-charge (m/z) value, fold-change (with and without cimetidine), p-value,
and intensity of the integrated peak. The physicochemical properties (total polar surface area
(TPSA), pKa, molecular weight, fraction neutral or ionized, and Log P) of METLIN-predicted
compounds were predicted using ACD/Percepta® software (ACD/Labs, Advanced Chemistry
Development, Inc., Toronto, ON, Canada, version 2022.2.3, [www.acdlabs.com](http://www.acdlabs.com))
Statistical analysis

Statistical analysis was performed using GraphPad Prism (San Diego, CA) and Microsoft Excel (Redmond, WA). Statistical comparisons with and without cimetidine were made using paired two-tailed Student’s t-test for blood concentrations at different time points, the amount excreted in the urine, $\text{AUC}_{0-8\text{ h}}$, and $\text{CL}_R$. $p$-values of $< 0.05$ were considered statistically significant.

RESULTS

Effect of cimetidine coadministration on metformin pharmacokinetics

Due to technical challenges in the rat dosing and sampling, complete blood and urine data could only be obtained from four rats. As expected, metformin blood concentration was higher in the cimetidine-metformin arm compared to the metformin alone arm at all time points. In particular, the increase in metformin concentration was significant at 0.5, 1, and 2 h by 2.5, 4.3, and 8.3-fold, respectively (Figure 1A). $\text{AUC}_{0-8\text{ h}}$ and $\text{CL}_R$ of metformin were 6.2 mg.h/L and 0.32 L/h (metformin arm) versus 20.3 mg.h/L and 0.09 L/h (metformin plus cimetidine arm), respectively (Figures 1B and 1C). Accordingly, metformin $\text{AUC}_{0-8\text{ h}}$ was significantly increased by 3.2-fold and its $\text{CL}_R$ was significantly decreased by 73% in the cimetidine arm with $p$-values equal to 0.003 and 0.029, respectively (Table 1). The amount of metformin excreted unchanged in urine did not significantly change between the two arms (Figure S1). The blood levels of cimetidine at different timepoints are shown in Figure S2.

Effect of cimetidine on endogenous metabolites

The analysis of 0.5 and 1 h blood samples revealed 7,178 and 7,927 features, respectively. Metabolomics cloud plot provided a visual representation of metabolites detected at 0.5 h samples before and after applying the first filtration criterion described below (Figures 2A and 2B) (Gowda et al., 2014). The cloud plot revealed that cimetidine treatment resulted in
significant changes in endogenous metabolome in the 0.5 h blood samples. The metabolomics data of 1 h blood samples showed a similar effect of cimetidine (Figures S3A and S3B). The metabolomics analysis of urine samples revealed 5,983 features, however, cimetidine administration had a minimum effect on urine metabolomics compared to the blood metabolomics data (Figures S3C and S3D).

The blood metabolomics data were filtered based on the following parameters: significantly increased metabolite levels in the cimetidine arm (1.9-10-fold, \(p\)-value <0.05) and retention time (<40 min). The upper limit of the increase in the biomarker levels (10-fold) was selected based on the reported magnitude of renal drug interactions considering at least 10% contribution of the glomerular filtration in metabolite renal clearance (Feng and Varma, 2016). These criteria yielded 91 and 81 unique features at 0.5 and 1 h, respectively. These features were further filtrated based on the quality of chromatographic peaks (signal-to-noise ratio > 5), which resulted in 29 (in 0.5 h sample), 22 (in 1 h sample), and 20 (common in 0.5 and 1 h samples) metabolites as putative biomarkers. Out of these, 11, 16, and 9 hits were predicted by METLIN at 0.5, 1 h, and both time points, respectively. These hits were analyzed by shortlisting only nitrogen-containing compounds (neutral or positively charged) with a mass error of less than 4 ppm (Figure 2C). The final list of the blood features was compared with the urine metabolomics data to identify the presence of these features in the urine. The urine metabolomics data were also analyzed similar to the blood data to identify features that were uniquely detected and significantly lower in the cimetidine-treated arm.

Seventy-one hits were detected in blood samples (0.5 and 1 h), of which, 36 features were identified by METLIN software, i.e., 24 features detected only in blood samples and 12 features commonly detected in blood and urine samples. One hit was detected exclusively in urine samples after applying the selection criteria (Figure 3A). Distribution of the predicted hits between blood (0.5 and 1 h) and urine samples were classified into three groups (Figure 3B). The first group (M1) represented features that were significantly increased in the blood samples.
but were not detected in the urine. Seven and 11 features were uniquely found at 0.5 and 1 h blood samples, respectively, while 6 hits were common in 0.5 and 1 h blood samples. The second group (M2) contained features that are detected in both blood and urine samples. The fold change in blood samples for the features in group M2 showed a significant increase in the concentration of putative metabolites in all blood samples after cimetidine inhibition. However, the fold change in the urine samples was not significant (Figure 3C). Three features, \textit{m/z} values of 206.0807, 233.0915, and 356.2420, were found in all samples (0.5 and 1h, and urine). Four and 5 features were common in the urine and blood samples, respectively. The third group (M3) represented one feature (\textit{m/z} 194.1176) that was significantly reduced in the urine samples, but not detected in the blood samples.

**Predicted cationic transporter substrate candidates**

Table 2 shows a list of predicted metabolites for each \textit{m/z} feature along with the fold change in blood and urine samples in the cimetidine-treated versus metformin alone arm. \textit{m/z} 206.0807 was detected in both blood (0.5 and 1 h) and urine samples, which was predicted to be gentianamine, swietenidin B, N-(3-hydroxypropyl) phthalimide, and methyl 1-methoxy-1H-indole-3-carboxylate (Figure 4A). Similarly, \textit{m/z} 233.0915 was classified in group M2, which corresponded with compounds such as oxabetrinil and robustine. \textit{m/z} 356.2420 was predicted to be a peptide analogue. The blood samples showed a significant increase in the treatment arm compared to the control arm for \textit{m/z} 233.0915 (Figure 4B) and 356.2419 (Figure 4C). \textit{m/z} 134.0598 from group M1 corresponds to indoxyl, oxindole, 4-hydroxy benzyl cyanide, 4-hydroxyindole, mandelonitrile, 2-methyl-4,5-benzoxazole, and 6-hydroxyindole. Metabolite with \textit{m/z} 162.0545 was detected in 0.5 h blood sample and urine sample (group M2) which was corresponds to 4,6-dihydroxyquinoline, 4,8-dihydroxyquinoline, quinoline-3,4-diol, quinolin-2,8-diol, 3-hydroxy-1H-quinolin-4-one, and 3-formyl-6-hydroxyindole as a putative compound. Similarly, \textit{m/z} 146.0597 was detected in 1 h samples as well as in the urine samples with
predicted compounds of oxyquinoline, 3-methylenoindole, isocarbostyril, quinolin-2-ol, 4-
hydroxyquinoline, 2(1H)-quinolinone, indole-3-carboxaldehyde, 4-formyl indole, isoquinoline N-
oxide, and 5-isoquinolinol. The single feature in group M3 (m/z 194.1176) was found only in the
urine samples with significant decrease by 50% in LC-MS intensity in cimetidine arm compared
to metformin-alone arm corresponding to 3,4-methylenedioxymethamphetamine, butamben,
salsoline, (R)-N-methylsalsolinol, (S)-N-methylsalsolinol, heliamine, isoprocarb, 2,3,5-
trimethacarb, 2-methylpropyl 2-aminobenzoate, butyl2-aminobenzoate, ethyl N-
ethylantranilate, 2-(ethylamino)-4'-hydroxy-propiophenone, isoproterenol, metaproterenol, and
methoxamine.
Physicochemical properties such as Log P, pKa, molecular weight, neutral/ionized fractions, and
TPSA of the predicted metabolites showed board ranges (Table S1), which is consistent with
the promiscuity of OCT substrates (Hendrickx et al., 2013). Some putative compounds were
positively charged at blood pH (7.4) such as N-succinimidyl [tris(2,4,6-trimethoxyphenyl)
phosphonio] acetate (m/z 671.2118), and cystamin (m/z 153.0519). Whereas other potential
compounds were partially positively charged at pH of 7.4, for example, pyrrolidino-[1,2E]-4H-
2,4-dimethyl-1,3,5-dithiazine (m/z 207.0986), tussilagine (m/z 244.0921), 2,5,6-trihydroxy-5,6-
dihydroquinoline (m/z 180.0650), and imiloxan (m/z 245.1277). Many predicted compounds with
m/z equal to 194.1176 (detected only in urine) like salsoline, isoproterenol, metaproterenol, and
methoxamine had a tendency to be positively charged in blood.

Discussion:
CL_R is the net effect of glomerular filtration, tubular secretion, and tubular reabsorption. OCTs
and OATs are the two primary transporters at the basolateral membrane of renal tubular cells
that are responsible for the active uptake of compounds from the blood and play a critical role in
the secretion of several commonly prescribed drugs such as antidiabetic (metformin),
antibacterial (cephalosporins), diuretics (furosemide), and chemotherapies (Morrissey et al.,
Renal transporters are also involved in transporter-mediated DDIs, where PK of a transporter substrate drug can be altered when administered with an inhibitor (precipitant) (Gessner et al., 2019). FDA requires pharmaceutical companies to assess renal transporter inhibition capability for new potential drugs by conducting prospective clinical DDI studies in a later stage of drug development (FDA, 2020). The utility of endogenous compounds as biomarkers to assess transporter function is currently a rapidly emerging area of research to address challenges associated with the traditional evaluation of potential DDI of new chemical entities by using exogenous probe drugs (Thakur et al., 2023).

In our study, we used cimetidine-metformin DDI as an approach to identify endogenous substrates of Octs and Mate transporters in rats. Metformin is an exogenous probe that is renally eliminated in rats through Oct and Mate transporters. For example, a study conducted in diabetic rats demonstrated that naringenin can enhance the renal uptake and excretion of metformin by increasing Oct2 protein expression (Mato et al., 2019). Another study observed an elevation in metformin systemic exposure and a reduction in its CLR when Oct-mediated renal excretion of metformin was inhibited by verapamil (Han and Choi, 2020). Further, the urinary excretion of metformin appears to be decreased as a result of reduced renal expression of efflux transporter Mate1 induced by the multiple administration of atenolol in rats (Ma et al., 2015).

Cimetidine is a known human OCT2 and MATE inhibitor that can alter the levels of exogenous and endogenous substrates for renal cationic transporters. The reported half-maximum inhibitory concentration (IC50) values of cimetidine against human OCT2 and MATE transporters are highly variable, i.e., the range of IC50 values for OCT2 and MATE is 6.6-135 µM and 1.5-47 µM, respectively (Lepist et al., 2014; Li et al., 2015; Yin et al., 2016; Müller et al., 2018b). Based on these IC50 data, we expected that the selected dose of cimetidine in this study can inhibit Octs (basolateral transporter) before reaching the apical side to inhibit Mate transporter,
particularly in the first 2 hours where cimetidine blood concentration was above IC₅₀ against both Oct and Mate transporters.

Although cimetidine is a more potent inhibitor for Mate transporters compared to Octs, inhibition of Mate transporters would increase the kidney tissue concentration of metformin and decrease CLᵣ without affecting the plasma levels assuming negligible passive diffusion of metformin due to its cationic nature. Indeed, it was reported that Mate inhibition by pyrimethamine had no significant effect on metformin plasma concentration in mice (Ito et al., 2010). Contrary, our results showed a significant increase in blood exposure and a corresponding decrease in CLᵣ for metformin suggesting Oct and Mate inhibition by cimetidine, which is consistent with the reported clinical cimetidine-metformin PK study (Wiebe et al., 2020).

Several potential biomarkers have been proposed for human OCT2/MATE in kidney such as creatinine, trimethylamine-N-oxide (TMAO), and NMN, but none of these biomarkers are ideal. For example, creatinine is a product of the nonenzymatic, spontaneous breakdown of creatine and creatine phosphate within muscle tissue (Brosnan et al., 2011). Apart from its endogenous production, creatinine can also be obtained from the consumption of cooked meat (Pimenta et al., 2016) and from creatine supplements commonly used in fitness training and bodybuilding (Velema and Ronde, 2011). CLᵣ of creatinine did not significantly change in healthy subjects after administration of cimetidine (Somogyi et al., 1987) considering that creatinine tubule secretion does not exceed 20% of the total creatinine CLᵣ (Breyer and Qi, 2010). TMAO is a metabolite of trimethylamine, formed through the metabolic processes of the gut microbiome from dietary sources such as choline and phosphatidylcholine (Velasquez et al., 2016). Although TMAO plasma concentrations doubled in Oct1/Oct2 knockout mice compared to the wild-type mice along with a decrease in CLᵣ, it is associated with high diurnal variability in humans (Miyake et al., 2017).
Further, although NMN showed a decrease in $CL_R$ in healthy individuals treated with trimethoprim (Müller et al., 2015), NMN levels can be influenced by a number of factors as it is a product of nicotinamide metabolism by the cytosolic nicotinamide N-methyltransferase (Bi et al., 2014) and a substrate of multiple human transporters like OCT2, MATE1, and MATE2-K (Ito et al., 2012a). Additionally, MNM is affected by circadian rhythm (Musfeld et al., 2001) and its $CL_R$ may be confounded by reabsorption into the renal tubules (Weber et al., 1991). We did not detect NMN in the untargeted analysis, which could be due to below detection levels of NMN in the diluted rat samples in our study. Considering the need to establish robust renal cationic transporter biomarkers, we utilized Oct/Mate inhibition by cimetidine to identify endogenous compounds in rat blood and urine that could serve as biomarkers for renal cationic transporters. For this purpose, blood and urine samples that represented high concentration timepoints of cimetidine were used to achieve in vivo Oct/Mate inhibition. Our data support that cimetidine maximum inhibition was obtained in the first 2 hours of the study, where a significant increase in metformin blood concentration was observed.

A robust untargeted metabolomics strategy was applied to measure metabolite levels with and without cimetidine co-administration. A 1.9-fold minimum cut-off was used when compared between the cimetidine-treatment versus control arms because this value was similar to metformin fold-change. The upper limit was set to 10 because renal DDI is generally mild with an expected fold-increase in plasma AUC not exceeding 4-fold (Feng and Varma, 2016) due to the involvement of glomerular filtration in the renal excretion. Metabolites with a fold difference higher than 10 are either likely metabolic products of cimetidine or metabolites that are generated when non-renal transporters are perturbed. Further, we selected the features that showed a retention time of less than 40 min in a 75 min gradient run as we anticipated that the cationic substrates would be relatively hydrophilic with retention times similar to metformin that eluted at 1 min. Only nitrogen-containing compounds were selected assuming that only neutral,
cationic, and quaternary amine compounds are substrates for OCT2 compared to zwitterions and acids (Hendrickx et al., 2013).

The metabolic features that were significantly increased in the blood (group M1) but not detected in the urine cannot be confirmed as substrates of renal transporters because of the lack of renal clearance data. These features could indicate inhibition of either extra-renal (e.g., hepatic) transporter or enzyme involved in the metabolism of the candidate feature by cimetidine. Whereas the features that were significantly increased in the blood but did not change in the urine (suggesting a decrease in \( \text{CL}_R \)) likely reflect cimetidine-mediated renal Octs inhibition (group M2). Finally, the feature that was significantly decreased in urine with cimetidine-treated samples could indicate inhibition of renal efflux transport or metabolite formation in the kidney (group M3). Taken together, features detected in group M2 are the most likely renal Oct substrates that can be tested for application in renal Oct-mediated DDIs.

Our study identified several metabolites as potential Oct/Mate substrates. These compounds belong to different categories such as oxindoles, herbicides, quinoline /quinolone, and peptides. Oxindoles are a class of hetero-aromatic organic compounds that occur naturally in the body fluids and tissues of mammals and are also synthesized as natural compounds by various plants (Cerchiaro et al., 2006). Oxindole derivatives are believed to be metabolized by liver enzymes in rats and result in the formation of oxindole metabolites such as 6-hydroxyoxindol (Beckett and Morton, 1966). There is limited information regarding the transporters responsible for the uptake or efflux of oxindoles. Some herbicides were identified as potential substrates for cation transporters. For example, oxabetrinil is a commercially available oxime herbicide (Jia et al., 2022). Quinoline and quinolone derivatives are also detected as potential hit in the metabolomics analysis. The quinoline and quinolone serve as building blocks for complex natural compounds, which are widespread in nature and can be extracted from various sources, including plants such as the cinchona tree’s bark, animals, and microorganisms (Heeb et al.,
The metabolomics data also identified several peptides as endogenous cationic substrates; however, these peptides might not be ideal candidates due to the possibility of involvement in renal reabsorption through peptide transporters (PEPT1 and PEPT2) (Wang et al., 2022). Additionally, some proposed peptides have large molecular weights, whereas only small organic cation molecules not exceeding 500 Dalton can be transported through cationic transporters (Hendrickx et al., 2013).

There are a few limitations of our study: i) cimetidine could modulate the activity of non-specific renal transporter as well as non-renal transporters. Ito et al., showed that cimetidine significantly inhibited human OCT1, OCT2, MATE1, and MATE2-K in cDNA-transfected HEK293 cells (Ito et al., 2012b). Similarly, Barth et al., revealed extensive inhibition for MATE and only partial inhibition for OCT2 on the levels of NMN, as a proposed biomarker for OCT/MATE, in plasma and urine with and without cimetidine administration (Barth et al., 2023). ii) Only four rats completed the PK study due to technical difficulty in dosing and sampling. iii) Finally, although stringent filtration criteria were implemented for narrowing down the metabolome features, it could have ended up losing some promising features. Nevertheless, the significant change in the metabolite levels as well as the use of multiple time points addressed the limitation of our pilot study.

In conclusion, several potential endogenous biomarkers of Oct/Mate transporters were identified using untargeted metabolomics. The identified endogenous transporter biomarkers are promising findings for predicting renal DDIs in the preclinical stages of drug development. A novel approach was proposed to predict and distinguish the inhibition at extra-renal uptake or catabolism, renal basolateral uptake (Oct1 and 2), and renal apical efflux or metabolite formation of cationic endogenous substrates. The ability of putative rat biomarkers identified in this study can be tested in the clinic to extrapolate animal data to humans in order to establish a robust panel of OCT2/MATE biomarkers for clinical applications.
Supplementary information:

Supplementary data is available.
ACKNOWLEDGMENTS:

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CONFLICT OF INTEREST

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Other authors declare no conflict of interest.
Data availability statement:

The authors declare that all the data supporting the findings of this study are contained within the paper.
AUTHOR CONTRIBUTIONS

Participated in research design: A.S.A, V.S.M., and B.P.

Conducted experiments: A.S.A. and V.S.M.


Wrote or contributed to the writing of the manuscript: A.S.A., V.S.M., A.T., D.K.S., and B.P.
References:


Ito S, Kusuhara H, Yokochi M, Toyoshima J, Inoue K, Yuasa H, and Sugiyama Y (2012b) Competitive inhibition of the luminal efflux by multidrug and toxin extrusions, but not basolateral uptake by organic cation transporter 2, is the likely mechanism underlying the pharmacokinetic drug-drug interactions caused by cimetidine in the kidney. \textit{J Pharmacol Ex Ther} 340:393–403.


Figure legends

**Figure 1:** Effect of cimetidine on the mean blood concentration-time profile (A), blood AUC$_{0-8}$ h (B) and renal clearance CL$_R$ (C) of metformin (green circle = metformin alone and red circle = metformin + cimetidine). The data were compared by two-tailed paired *t*-test: *p*-value $<$ 0.05 (*), and $<$ 0.01 (**). Complete data were only obtained for four rats due to technical challenges in the dosing and sampling.

**Figure 2:** Untargeted metabolomics was performed by analyzing high-resolution LC-MS data using XCMS Online software (The Scripps Research Institute, La Jolla, CA). Interactive cloud plot of endogenous metabolites representing metabolic features that were elevated (green) and decreased (red) in the blood sample (0.5 h) treated with metformin + cimetidine as compared to metformin alone before (A) and after (B) applying the first screening step (C). *p*-value is represented by the color intensity and the fold-change is indicated by the radius of each feature. Screening criteria applied to blood untargeted metabolomic features are summarized (C).

**Figure 3:** Comparison of metabolomics features that were altered by cimetidine treatment in the blood and urine samples. Venn diagram of the number of hits detected in blood untargeted metabolomics at 0.5 h and 1 h samples: A) all features detected in the blood and urine after applying the selection criteria, B) distribution of all hits predicted between blood and urine, C) bar chart of the fold change in the blood and urine samples in cimetidine-metformin arm as compared to metformin-alone arm. *p*-value $<$ 0.05 (*).

**Figure 4:** LC-MS signal intensities of three representative differentially expressed (*p*-value $<$0.05) metabolic features (A = m/z 206.0807, B = m/z 233.0915, and C = m/z 356.2420) in 0.5 h and 1 h blood samples after metformin alone versus metformin plus cimetidine treatments.
A) \( m/z \ 206.0807 \) corresponds to gentianamine, swietenidin B, N-(3-hydroxypropyl)phthalimide or methyl 1-methoxy-1H-indole-3-carboxylate. B) \( m/z \ 233.0915 \) corresponds to oxabetrinil or robustine. C) \( m/z \ 356.2420 \) corresponds to a peptides, Ala-Arg-Lys, Lys-Arg-Als, Ala-Lys-Arg, Arg-Ala-Lys, Lys-Ala-Arg, or Arg-Lys-Als.
### Table 1: Comparison of pharmacokinetic parameters of metformin alone and metformin plus cimetidine in rats (n=4).

<table>
<thead>
<tr>
<th>PK Parameters</th>
<th>$\text{AUC}_{0-8\ h}$ (mg*h/L)</th>
<th>$\text{CL}_R$ (L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin Arm</td>
<td>6.2</td>
<td>0.32</td>
</tr>
<tr>
<td>Metformin plus cimetidine Arm</td>
<td>20.3</td>
<td>0.09</td>
</tr>
<tr>
<td>Fold increase or %Decrease</td>
<td>~3.2</td>
<td>73%</td>
</tr>
<tr>
<td>$p$-value</td>
<td>0.003</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Two-tailed paired *t*-test
Table 2: List of mass-to-charge (m/z) values and corresponding predicted compounds by METLIN online software that were significantly elevated in the blood with cimetidine treatment irrespective of changes in the urine levels.

<table>
<thead>
<tr>
<th>m/z</th>
<th>MATLIN predicted compounds</th>
<th>Fold increase (blood, 0.5 h)</th>
<th>Fold increase (blood, 1 h)</th>
<th>% Decrease (urine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>134.0598</td>
<td>Indoxyl, oxindole, 4-hydroxybenzyl cyanide, 4-hydroxyindole, mandelonitrile, 2-methyl-4,5-benzoazoxazole, 6-hydroxyindole</td>
<td>2.7</td>
<td>3.5</td>
<td>ND</td>
</tr>
<tr>
<td>233.0915</td>
<td>Oxabetrinil, robustustine</td>
<td>3.1</td>
<td>1.9</td>
<td>ND</td>
</tr>
<tr>
<td>179.0674</td>
<td>Erucin, 1-thiocyanato-4-(methylthio)butane</td>
<td>3.2</td>
<td>2.2</td>
<td>ND</td>
</tr>
<tr>
<td>206.0807</td>
<td>Gentianamine, swietenidin B, N-(3-hydroxypropyl)phthalimide, methyl 1-methoxy-1H-indole-3-carboxylate</td>
<td>2.3</td>
<td>5.9</td>
<td>NS</td>
</tr>
<tr>
<td>207.0986</td>
<td>1,3-Isocyanato-6-(methylthio)hexane, pyrrolidino-[1,2E]-4H-2,4-dimethyl-1,3,5-dithiazine</td>
<td>2.7</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>236.0708</td>
<td>Viridicatol, 3-hydroxy-4-(3-hydroxyphenyl)-1H-quinolin-2-one, 2-(4-hydroxybenzyl)isoindole-1,3-dione, 2-acetamido-6H-dibenzo[b,d]pyran-6-one, N-(6-oxo-6H-dibenzo[b,d]pyran-3-yl)acetamide</td>
<td>2.3</td>
<td>2.1</td>
<td>ND</td>
</tr>
<tr>
<td>146.0597</td>
<td>Oxyquinoline, 3-methylenoxindole, isocarboxystyril, quinolin-2-ol, 4-hydroxyquinoline, 2(1H)-quinolinone, indole-3-carboxaldehyde, 4-formyl indole, isoquinoline N-oxide, 5-isoquinolinol</td>
<td>ND</td>
<td>3.2</td>
<td>NS</td>
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<tr>
<td>239.1609</td>
<td>Dihydrozeatin</td>
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<td>335.1241</td>
<td>Cappariloside A</td>
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<td>2.3</td>
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<td>162.0545</td>
<td>4,6-Dihydroxyquinoline, 4,8-dihydroxyquinoline, quinoline-3,4-diol, quinolin-2,8-diol, 3-hydroxy-1H-quinolin-4-one, 3-formyl-6-hydroxyindole</td>
<td>2.1</td>
<td>ND</td>
<td>NS</td>
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<tr>
<td>164.0702</td>
<td>3-Methylidioxyindole, 4-oxo-1-(3-pyridyl)-1-butanone, 5-hydroxy-3,4-dihydrocarbostyril, p-acetaminobenzaldehyde</td>
<td>2.2</td>
<td>ND</td>
<td>NS</td>
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<tr>
<td>244.0921</td>
<td>N-Hexanoyl-L-homoserine lactone, ecgonine methyl ester, tussilagine, neotussilagine, cytaranbide, cytidine</td>
<td>5.1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>251.1019</td>
<td>Ethyl 2,3-dioxo-1,2,3,4-tetrahydro-4-quinolinecarboxylate</td>
<td>2.6</td>
<td>ND</td>
<td>NS</td>
</tr>
<tr>
<td>180.0650</td>
<td>(Z)-N-[2-(5-Dihydroxyphenyl)ethenyl]-formamide (cis erbastin), N-formyl-2-(4-hydroxyphenyl)acetamide, adrenochrome, 2,5,6-trihydroxy-5,6-dihydroquinoline, methyl N-formylanthanilate, 1-(4-methoxyphenyl)-2-nitroethylene, 5,8-dihydroxy-3,4-dihydrocarbostyril</td>
<td>3.9</td>
<td>ND</td>
<td>NS</td>
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<tr>
<td>193.0830</td>
<td>Berteroin, 7-cyano-7-deazaguanine</td>
<td>2.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>238.1067</td>
<td>2',4'-Dimethoxyacetacetanilide, 3-hydroxy-carbofuran, 2,3-dihydro-1,4-benzodioxin-2-ylmethyl dimethylcarbamate</td>
<td>1.9</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
252.1099  Deoxyadenosine, 3'-deoxy-adenosine, 5'-deoxyadenosine, cordycepin
308.2210  Dihydrocapsaicin
247.1070  Gamma-fagarine, pteleine
245.1277  Imiloxan, D-5-oxo-N-(trans-2-phenylcyclopropyl)-L-2-pyrrolidinecarboxamide, 1,2-bis(6-methylpyridin-2-yl)ethane-1,2-diol
176.0702  Gentianine, 5-hydroxyindoleacetaldehyde, N-acetyllindoxyl, (indol-3-yl)glycolaldehyde, 2-methylquinoline-3,4-diol, 3-methyl-quinolin-2,8-diol, 3-hydroxy-2-methyl-1H-quinolin-4-one, hydroxymethyl indol-3-yl ketone, 1-methoxy-1H-indole-3-carboxaldehyde
148.0753  2-Naphthylamine, 1-naphthylamine, 6-methylquinoline, quinaldine, indole-3-carbiniol, 3-methylloxindole, 2-indanone oxime, 1-(2-furanylmethyl)-1H-pyrole, 4-(2-furanylmethylene)-3,4-dihydro-2H-pyrole, 3-(2-furanylmethyl)-1H-pyrole
159.0273  Hypoxanthine
671.2118  N-succinimidy [tris(2,4,6 trimethoxyphenyl) phosphonio] acetate
238.0703  N-(fur-2-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine
318.1801  N-Isobutyl-2,4,8,10,12-tetradecapentaenamide
153.0519  Cystamine
228.0625  Gentianamine, swietenidin B, N-(3-hydroxypropyl)phthalimid, methyl 1-methoxy-1H-indole-3-carboxaldehyde
247.9998  2,3-Dihydroxy-6,7-dichloroquinoxaline, 6,7-dichloroquinazoline-2,3-dione
393.0817  Allithiamine
550.2854  His Gln Gin Arg, His Gln Gin Arg, His Arg Gin Gln Gln Gin Arg Gin Arg Gin Gin Gin Arg Gin Gin Gin Gin Arg Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin Gin 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metaproterenol, methoxamine

All predicted metabolites were significantly higher in the blood samples in cimetidine arm compared to metformin alone arm with \textit{p-value} < 0.05. ND (not detected) represents m/z values that were not detected in XCMS Online. NS (not significant) represents no significant change (decrease or increase) in the in m/z feature intensity in cimetidine arm compared to metformin alone arm.
Figure 1

A. Metformin blood concentration (mg/mL) over time (h).

B. Metformin area under the curve (AUC) for different conditions.

C. Metformin renal clearance comparison among different rats.
Figure 2

A: Toggle Upregulated  Toggle Downregulated

B: Toggle Upregulated

C:

- 7178 MS features (0.5 h)
- 7927 MS features (1 h)

<table>
<thead>
<tr>
<th>Total features</th>
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<td>Features detected from the raw data by XCMS Online software</td>
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<th>First screening step</th>
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<tr>
<td>1. Elevated</td>
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<td>2. p-value &lt; 0.05</td>
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<tr>
<td>3. Fold Change (1.9-10)</td>
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<td>4. Retention time &lt; 40 min</td>
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<table>
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<tr>
<td>5. Good chromatographic peak</td>
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<td>7. Tendency to be neutral or positively charged</td>
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<tr>
<th>Third screening step</th>
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<tr>
<td>8. Signal to noise ratio &gt; 5</td>
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<tr>
<td>9. Peak symmetry</td>
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</tbody>
</table>
Figure 3

A

Total blood
METUN blood
blood+urine
urine only

35
24
12
1

B

M1
0.5 h
7
6
1 h
11

M2
3

M3
1
Urine

C

Fold Change

Blood fold change
Urine fold change

206.0807
223.0915
356.2420
162.0545
164.0702
180.0650
362.0833
146.0597
176.0702
182.0836
228.0827
240.0170
24
Figure 4

A

B

C

0.5 h

1 h

0.5 h

1 h

0.5 h

1 h

Metformin

Metformin + Cimetidine