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An ex vivo fermentation screening platform to study drug metabolism by human gut microbiota

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Non-standard abbreviations: 5-ASA, 5-aminosalicylic acid; BCS, Biopharmaceutics Classification System; DMSO, dimethylsulfoxide; HPLC, High-performance liquid chromatography; HRMS, High-resolution mass spectrometry; NMR, Nuclear Magnetic Resonance; SIEM, Standard Ileal Efflux Medium; qPCR, quantitative PCR

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ABSTRACT

Colon microbiota-based drug metabolism has received little attention thus far in the process of drug development, whereas the role of gut microbiota in clinical safety and efficacy of drugs has become more clear. Many of these studies have been performed using animal studies, but the translational value of these data with respect to drug pharmacokinetics, efficacy, and safety is largely unknown. To investigate human colon microbiota-mediated drug metabolism, we applied a recently developed *ex vivo* fermentation screening platform, in which human colonic microbiota conditions are simulated. A set of 12 drugs (omeprazole, simvastatin, metronidazole, risperidone, sulfinpyrazone, sulindac, levodopa, dapsone, nizatidine, and acetaminophen) was incubated with human colon microbiota under strictly anaerobic conditions and samples were analyzed using HPLC-UV--HRMS analysis. The human microbiota in the fermentation assay consisted of bacterial genera regularly encountered in human colon and fecal samples and could be reproducibly cultured in independent experiments over time. In addition, fully anaerobic culture conditions could be maintained for 24 hours of incubation. Five out of the twelve included drugs showed microbiota-based biotransformation after 24 hours of incubation in the *ex vivo* fermentation assay, which were sulfasalazine, sulfinpyrazone, sulindac, nizatidine, and risperidone. We demonstrated that drug metabolites formed by microbial metabolism can be detected in a qualitative manner and that the data are in accordance with earlier reported *in vivo* metabolism. In conclusion, we here present a research tool to investigate human colon microbiota-based drug metabolism that may be applied to enable translatability of microbiota-based drug metabolism.

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INTRODUCTION

Within drug development, drug candidates are extensively tested for efficacy and safety. One of the major concerns for drug efficacy and safety is the metabolism of the drug and rate in which it is eliminated from the body. Whereas the major site of metabolism in the human body is the liver, where drugs and their metabolites are subsequently eliminated from the body through biliary excretion and successive fecal secretion via the GI-tract, recent insights elucidate the influence of gut microbiota on the metabolism of key therapeutics. Since the colon is identified by its high density and unique collection of bacteria, viruses and fungi (the microbiota) (Qin et al., 2010), the prospect of microbial interactions with drugs in the colon is rather high. Nevertheless, human colon microbiota-based drug metabolism has received little attention thus far. Previous research with laboratory animals showed the metabolic capacity of the gut microbiome to metabolize various nutrients and drugs (Spanogiannopoulos et al., 2016, Wallace et al., 2010), and a recent paper by Wilkinson et al provides a nice overview of clinical examples of the impact of gut microbiota on drug metabolism and therapeutic efficacy (Wilkinson et al., 2018).

While consideration of the role of microbiota in drug metabolism has been mostly focused on the conversion of drugs into inactive metabolites, the gut microbiome may also be targeted to enhance clinical response, for example by targeting microbiome-encoded enzymes for activation of pro-drugs or alteration of drug metabolism into active metabolites in order to enhance clinical response. An example of activation of a pro-drug by intestinal microbiota is sulfasalazine, designed to be cleaved by gut microbiota into the active metabolites amino salicylic acid (5-ASA) and sulfapyridine (Williams et al., 2011, Peppercorn and Goldman, 1972). Whereas 5-ASA is responsible for many of its local beneficial effects in patients with inflammatory bowel disease, sulfapyridine appeared to be highly absorbed into the systemic circulation causing known side effects such as headache, nausea, and fever (Peppercorn and Goldman, 1972). In order to obtain more insights into the potential consequences of microbiome-based biotransformation of compounds for drug pharmacokinetics, efficacy and safety, especially for those drugs for which we expect higher dose levels in the colon, it is important to thoroughly investigate human colon microbiota-based drug transformation.

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Over the last couple of years, there has been a tendency that newly developed drugs are characterized by low solubility and/or permeability, thereby belonging to Biopharmaceutics Classification System class II-drugs (BCS class II-drugs) or BCS class III or –IV drugs, but hardly to BCS class I-drugs, which are highly soluble and highly permeable drugs (Ku, 2008). This tendency is mainly caused by increased structural complexity of newly designed drugs. As a consequence, the solubility and absorption of these drugs is impaired resulting in prolonged residence in the gastrointestinal tract after oral administration, subsequently resulting in higher drug concentrations in the colon. However, not only orally administered drugs may end up in the colon, but also intravenously administered drugs can reach the intestines and colon via biliary excretion, for example after hepatic glucuronidation (Pellegatti, 2012, Spanogiannopoulos et al., 2016)

Recently, a fermentation screening platform with an anaerobic culturing method simulating colonic microbiota conditions has been developed (Ladirat et al., 2014, Ladirat et al., 2013). In this screening platform, human colon microbiota, retrieved from human stool samples and representing microbiota as found in the colon, are incubated. This platform has been demonstrated to be a very fast and efficient *ex vivo* animal-free screening platform to study interactions at the level of human colonic microbiota, such as screening for the efficacy of prebiotics and/or xenobiotic-mediated alterations of human microbiota activity or composition. In this paper we describe the application of this *ex vivo* fermentation screening platform to study human colon microbiota-based drug metabolism. Human stool samples of various healthy adults were used to create a stable pool of microbiota representing the composition of human colon microbiota (Chen et al., 2012, Gagniere et al., 2016). We focused on the reproducibility of the composition of the microbiota pool in independent experiments over time, verification of the anaerobic culture conditions during experiments, and the incidence of microbiota-based drug metabolism. For the latter, twelve marketed drugs of various BCS classifications and containing chemical substituents amenable to metabolism by anaerobic gut microbiota (Table 1) were incubated in this platform. We screened for colon microbiota-based drug metabolism and investigated whether it was in accordance with metabolites observed in earlier reported *in vivo* studies.

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MATERIAL AND METHODS

Drugs

The following drugs were obtained from Pfizer (Groton, Ct, USA): omeprazole, simvastatin, metronidazole, risperidone, sulfinpyrazone, sulindac, levodopa, dapsone, nizatidine, and acetaminophen (**Table 1**). Zonisamide and sulfasalazine were obtained from Sigma-Aldrich. The drugs were selected based on their BCS-classification, chemical structure, widespread prescription, and/or previously reported metabolism detected in human or animal faeces. All drugs were diluted in dimethylsulfoxide (DMSO) and were incubated with human colon microbiota using the fermentation screening platform, followed by LC-HRMS and HPLC-analysis.

Human colon microbiota

Stool samples were derived from seven apparently healthy volunteers, as defined by the following criteria: 1) No use of any antibiotics in the past two months; 2) No use of pre- or probiotics in the past week; 3) No bowel-problems in the past three months; 4) No diarrhea; 5) No use any medication in the past two weeks (excluding vitamin pills). All volunteers were Caucasian individuals, ranged from 25 - 45 years old and were subject to a European lifestyle and nutrition. As per clinical protocol, subject identities were kept strictly anonymous so other demographic characteristics, such as sex, age, race, and body weight are unknown. The stool samples were collected in anaerobic jars by applying self-contained gas generating systems (AnaeroGen 3.5L, Thermo Scientific) establishing an oxygen depleted environment essential for obligate anaerobic microorganisms. The collected stool samples were anaerobically pooled and fermented for approximately 40 hours in a fed-batch fermenter. This resulted in a homogenized, scaled up standardized microbiota pool, which could be used repeatedly after subdivision. This pool was stored as human colon microbiota samples as previously described (Ladirat et al., 2013, Minekus et al., 1999).

Ex vivo fermentation screening platform: experimental set-up and sampling

Experiments were performed according to previously published methods with minor adaptations (Ladirat et al., 2013). In brief, the human colon microbiota were pre-cultured overnight (37 °C, 300 rpm) under

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anaerobic conditions in Standard Ileal Efflux Medium (SIEM) to stimulate bacteria growth after storage (-80 °C, 12% glycerol), as previously published (Minekus et al., 1999). This medium contained per liter: 4.5 g sodium chloride (NaCl), 2.5 g potassium hydrogen phosphate (K_2HPO_4), 0.45 g calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 0.5 g magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 0.005 g ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 0.05 g ox bile, 0.01 g haemin, 0.4 g cystein, 0.6 g pectin, 0.6 g xylan, 0.6 g arabinogalactan, 0.6 g amylopectin, 5 g starch, 2 ml Tween 80, 3 g bactopectone and 3 g casein, constituted with 1 ml of a vitamin mixture containing per litre: 1 mg menadione, 2 mg D-biotin, 0.5 mg vitamin B-12, 10 mg pantothenate, 5 mg nico-tinamide, 5 mg para-aminobenzoic acid, and 4 mg thiamine. All components were obtained from Tritium Microbiology (Veldhoven, The Netherlands). After preparation, the SIEM medium was stored for a maximum of 48 hours in an anaerobic jar at 4 °C before usage.

Stock concentrations of test compounds (dissolved in DMSO) were further diluted in modified SIEM to a final concentration of 50 µM and 100 µM in the screening platform (96 - 2 mL deep wells plates). Final DMSO concentration was 1% in all incubations. The pre-cultured pooled human colon microbiota (0.1% v/v) were incubated for 24 hours (pH 5.8; 300 rpm) in fully anaerobic chambers (Don Whitley A45 Scientific Anaerobic Workstation) at 37 °C. The total samples were split through centrifugation into supernatant containing medium (used for subsequent LC-HRMS or HPLC analysis) and the pellet with microbes (used for subsequent qPCR analysis). Incubations with only microbiota in SIEM and incubations with only SIEM served as negative background controls for the HPLC and LC-HRMS analyses. Incubations with test compounds in SIEM (without microbiota) served as negative control for microbiota-based metabolism in order to determine compound stability or non-specific degradation of the test compounds due to culture conditions during the assay. Incubations with colon microbiota and the prebiotic fiber inulin served as positive control for the outgrowth of anaerobic *Bifidobacterium* as analyzed by qPCR, confirming fully anaerobic culture conditions during incubations. In addition, the log-file of the oxygen level within the anaerobe incubator was used as an supplementary indicator for the maintenance of anaerobic conditions during incubations. All experiments and controls were performed in triplicate.

For NMR experiments a larger scale incubation has been performed in which risperidone was incubated in a volume of 50 mL SIEM plus microbiota for 24h. Follow-up steps are as described above. The

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mixture was processed using a previously described approach (Walker et al., 2014) and used for NMR analysis.

Quantification of *Bifidobacterium* levels using qPCR

To verify whether the anaerobic conditions were maintained during the incubations, the presence and abundance of the anaerobic *Bifidobacterium* bacteria was analyzed. These species are one of the major genera of bacteria that make up the microbiota of the human colon and are very sensitive to oxygen, since low concentrations of oxygen are already lethal for these bacteria. The outgrowth of *Bifidobacterium* at different time points was analyzed via qPCR analysis. For the qPCR, total DNA was isolated from the microbial pellet samples according to previously described procedures (Ladirat et al., 2013, Crielaard et al., 2011). All *Bifidobacterium* species were targeted with the primers: F_allbif_IS (10 μ M) (GGG ATG CTG GTG TGG AAG AGA) and R_allbif_IS (10 μ M) (TGC TCG CGT CCA CTA TCC AGT), Probe MSB-FAM (5 μ M) (P_all_bif, TCA AAC CAC CAC GCG CCA) (Haarman and Knol, 2005). DNA isolated from a mix of various species of cultured *Bifidobacteria* was used as a positive control. For the analysis, 5 μ l DNA sample, 12.5 μ l Diagenode PCR mixture(2x) and 1 μ l of the probe, the forward primer, and reverse primer was used. The samples were analyzed with the Applied Biosystems 7500 Fast Real-Time PCR system with the settings for a standard run with an adjustment of the amount of cycles to 45. The Δ Ct-values were converted into 10Log-values to create growth curves.

Analysis of colon microbiota composition

Microbial composition of the fecal samples collected from the 7 individuals (before and after pooling, and before and after the *ex vivo* fermentation screen for 24 hours), were analyzed by MiSeq sequencing, as previously published (Arnoldussen et al., 2017).

Analysis by UHPLC, HPLC and NMR

UHPLC-UV-HRMS

To an aliquot of terminated incubations samples (0.5 mL) was added acetonitrile (2.5 mL) and the mixture was spun in a centrifuge at 1700 g for 5 min. The supernatant was transferred to a 15 mL conical glass tube and subject to vacuum centrifugation. To the dried residue was added 0.1 mL water (omeprazole, nizatidine)

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or 1% aqueous formic acid (metronidazole, sulindac, sulfasalazine, zonisamide, levodopa, dapson, acetaminophen) or 1% formic acid in 10% acetonitrile in water (risperidone, sulfinpyrazone) or 20% acetonitrile in water (simvastatin) for analysis by UHPLC-UV-HRMS. Samples (0.01 mL) were injected on to a Thermo Orbitrap Elite equipped with an Accela UHPLC system comprised of a quaternary pump, photodiode array detector, a CTC Analytics autoinjector. Separations were effected using conditions listed in tables in the Supplemental Methods (Table S1-S7).

HPLC-UV

Sulfasalazine was used as a quality reference drug in all experiments to screen for anaerobic metabolism. Sulfasalazine is metabolized into 5-ASA and sulfapyridine by azoreductases of gut microbiota (Peppercorn and Goldman, 1972). The concentration of sulfasalazine and its metabolite sulfapyridine was determined based on the UV-absorption using HPLC, with an UV-detector, an autosampler and, a Waters Xbridge BEH C18 column (4.6 x 150 mm). The absorption peaks were identified and quantified using Lablogic Laura software (South Yorkshire, UK). The incubated samples were filtered (10 min, 1690g, 45 μ m), diluted with acetonitrile (10%) and stored at -80 °C until further use. The samples were diluted with acetonitrile (30%) and injected (10 μ l) onto the HPLC column. Acetonitrile with 0.1% formic acid was used as a mobile phase with a flow rate of 0.6 ml/min⁻¹ at 10 °C.

NMR

All samples were dissolved DMSO-d₆ “100%” and placed in a 1.7 mm NMR tube (0.04 ml) under a dry argon atmosphere. ¹H and ¹³C spectra were referenced using residual DMSO-d₆ (¹H δ =2.50 ppm relative to TMS, δ =0.00, ¹³C δ =39.50 ppm relative to TMS, δ =0.00). NMR spectra were recorded on a Bruker Avance 600 MHz (Bruker BioSpin Corporation, Billerica, MA) controlled by Topspin V3.2 and equipped with a 1.7 mm TCI Cryo probe. 1D spectra were recorded using an approximate sweep width of 8400 Hz and a total recycle time of approximately 7 s. 2D data were recorded using the standard pulse sequences provided by Bruker. Post-acquisition data processing was performed with either Topspin V3.2 or MestReNova V9.1. Quantitation of NMR isolates was performed by external calibration against the ¹H NMR spectrum of a 5 mM benzoic acid standard using the ERETIC2 function within Topspin V3.2.

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RESULTS

Microbial composition and stability

Microbiota composition was analyzed in the stool samples collected from seven healthy adult volunteers, before and after pooling (**Figure 1**), showing the presence of all microbial phyla regularly encountered in the human microbiome (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*) in the inoculation material. These data showed that despite the rather large interindividual variation in the microbiota composition of all pre-fermented stool samples of the 7 individuals, the microbial diversity of the pooled sample was representative for the individual samples and for the human *in vivo* situation (**Figure 1A-H**). Pooled stool samples were fermented to increase the samples size, aliquoted and stored $<-70^{\circ}\text{C}$ upon usage. Although the fermentation and storage process inevitably affected the colon microbiota composition, the overall diversity of the microbiota composition of the pooled sample was still maintained, demonstrated by the microbial composition of the pooled sample at the beginning and end of the *ex vivo* fermentation screen (**Figure 1I and 1J**). Limited intra- and inter-experimental variation in microbial composition was observed, demonstrating the robustness of the assay under highly controlled laboratory conditions (**Supplemental Figure S1**).

Maintenance of anaerobic culture conditions

Maintenance of strict anaerobic culture conditions during incubations was verified by analyzing the outgrowth of the strictly anaerobic *Bifidobacteria* over 24 hours in the absence and presence of the probiotic fiber inulin. Proper growth-curves were observed for all experiments (**Supplemental Figure S2**), showing an outgrowth of *Bifidobacteria* of more than 1 log, indicating that anaerobic conditions allowing for active growth of strictly anaerobic bacteria were indeed maintained throughout all incubations. These data were confirmed by the data log of the oxygen concentrations within the incubator, showing no deviations during the experiments.

Microbiome mediated biotransformation of drugs

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In order to study the significance of gut microbiota as a determinant of drug pharmacokinetics and metabolism (and according therapeutic response), the developed platform was applied to incubate a subset of 12 drugs (**Table 1**) with pooled human adult colon microbiota. Five out of the twelve included drugs showed microbiota-based biotransformation after 24 hours of incubation (nizatidine, risperidone, sulfasalazine, sulfapyrazone, and sulindac), whereas 8 hours after incubation no major biotransformation of these drugs was observed based on LC-HRMS or HPLC data (**Figure 2** and **Table 2**). After 24 hours of incubation with human colon microbiota in the fermentation assay, the prodrug sulfasalazine was completely reduced to sulfapyridine, whereas the cleavage product 5-ASA could not be detected (**Figure 3**). Sulindac and sulfapyrazone displayed a sulfoxide reduction to the thioethers due to colon microbial metabolism (**Figure 4 and 5**). Sulindac was completely converted by the colon microbiota within 24 hours into deoxysulindac. Nizatidine was also reduced by human colon microbiota, resulting in a nitroso-deoxygenated metabolite (**Figure 6**). The benzisoxazole N-O bond of risperidone was reduced and the resulting imine metabolite converted to the ketone, either spontaneously or possibly by the microbiota (**Figure 7**). After 24 hours of incubation with colon microbiota, the reduction of risperidone was complete. These 5 drugs appeared to be stable during incubation in the assay, as no degradation or metabolite formation was detected when incubated without microbiota in the assay (**Figure 3-7**). For the drugs acetaminophen, levodopa, metronidazole, dapsone, omeprazole, simvastatin, and zonisamide no specific metabolites were detected after incubation with human colon microbiota for 6 or 24 hours. Though, for levodopa and metronidazole a decline of the parent drug was detected after 24 hours incubation with human colon microbiota, no specific metabolites were found.

While HPLC-MS data are frequently obtained in drug metabolism experiments, in many cases NMR spectral data are needed in order to verify structures of metabolites. In the current study we have used microbiota-mediated metabolism of risperidone as an example to demonstrate that the fermentation assay could be scaled up to produce enough metabolite for NMR analysis. This was successful and 1D proton and 2D homonuclear and heteronuclear experiments confirmed the risperidone metabolite structure as the previously described ketone (**Figure 8**).

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DISCUSSION

In the current study we applied a previously developed microbial fermentation assay (Ladirat et al., 2013) for studying human colon microbiota-mediated biotransformation of drugs. For this, microbiota from stool samples, which were collected from seven healthy adult volunteers, were homogenized, pooled and scaled up to create a standardized microbiota pool representing human colon microbiota. Despite the rather large interindividual variation in the microbiota composition of all pre-fermented stool samples of the 7 individuals, the microbial diversity of the pooled sample was representative for the individual samples and for the human *in vivo* situation as has previously been published (Ladirat et al., 2013). In this latter publication, the representativeness of the human colon microbiota samples was studied by determining the presence and abundance of more than 200 bacterial targets of which *Bacteroides*, *Bifidobacteria*, *Enterobacteria*, *Clostridia* and *Lactobacillus* are examples of prominent bacteria species present in the human gut (Eckburg et al., 2005, Zoetendal et al., 2006). These data showed that the microbiota composition of the pooled pre-fermented samples was representative for the composition of human colon microbiota, since key species as found in the human colon were present and the microbial diversity of the pooled sample was representative for the individual samples. During fermentation, storage and assay incubation, the overall diversity of the human microbiota composition of the pooled sample was still maintained, demonstrated by the microbial composition of the pooled sample at the beginning and end of the *ex vivo* fermentation screen and the comparison of microbial composition at the end of experiments performed over time. The use of a pool in this manner is similar in practice to the use of pooled human hepatocytes or liver microsomes to study drug metabolism rates and pathways. Understanding interindividual variability is lost in such an approach, but once the data are generated using a pooled sample, follow up experiments to address interindividual variability can be pursued as warranted.

Maintenance of strict anaerobic conditions during the *ex vivo* fermentation assay is important for reliability of the obtained results, since in the colon the microbiota thrive in an anaerobic environment. Stringent anaerobic conditions were verified by proper outgrowth of the strictly anaerobic *Bifidobacterium* over 24 hours in the assay in the presence of the probiotic fiber inulin and by data log of the oxygen concentrations within the incubator .

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In order to study the potential significance of gut microbiota as a determinant of drug pharmacokinetics and metabolism, the developed platform was applied to incubate a subset of 12 drugs with pooled human adult colon microbiota. The drugs were selected based on their widespread prescription, BCS classification, and potential for microbial metabolism as for some has previously been reported in literature (**Table 1**). Five out of the twelve included drugs showed microbiota-based biotransformation after 24 hours of incubation in the *ex vivo* fermentation assay, which were sulfasalazine, sulfapyrazone, sulindac, nizatidine, and risperidone. Colonic transit time has been previously determined to be 20-40 h in healthy adult people (Zaslavsky et al., 1998), so the 24 h incubation used in our experiments may offer a reasonable reflection of residence time of drugs and drug metabolites in the colon. Results were compared to literature in order to determine whether the metabolic reactions that were detected corresponded to clinical data, a summary of the results is presented in **Table 2**. In the *ex vivo* microbial fermentation assay, sulfasalazine was completely reduced to sulfapyridine within 24 hours of incubation, whereas the cleavage product 5-ASA could not be detected. In the clinic, sulfasalazine is provided as a prodrug, designed to reach the colon to be metabolized into sulfapyridine and 5-ASA by azoreductases of gut microbiota (Peppercorn and Goldman, 1972, Williams et al., 2011). Due to cleavage, the active metabolite 5-ASA is formed in the colon, where it has its therapeutic effect. The other metabolite, sulfapyridine, has been shown to have toxic effects in humans (Das et al., 1973, Davila and Ranganathan, 2011, Pullar and Capell, 1986). The bacteria species *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus* have been linked to the metabolism of sulfasalazine (Peppercorn and Goldman, 1972). The reason that 5-ASA could not be detected during analysis might be that 5-ASA is extensively further metabolized after cleavage from sulfasalazine, which could complicate detection of 5-ASA, since it has been shown that both 5-ASA and sulfapyridine can be acetylated by gut microbiota in animals and humans (Dull et al., 1987).

The observed microbiome-mediated sulfoxide reduction of sulindac and sulfapyrazone, was in line with previous studies in which reduced metabolites were detected in human and rabbit faeces (Strong et al., 1987). Nizatidine was also reduced by human colon microbiota in the assay, resulting in a nitroso-deoxygenated metabolite, conforming previously published results with an alternative *in vitro* fermentation system with human faeces (Basit et al., 2002). The benzisoxazole N-O bond of risperidone was reduced and the resulting imine metabolite converted to the ketone, either spontaneously or possibly by the

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microbiota. This metabolic pathway of risperidone has previously been reported in studies using rat, dog, and human faeces (Jourova et al., 2016, Mannens et al., 1993, Meuldermans et al., 1994). After 24 hours incubation with colon microbiota, risperidone was completely reduced into the ketone, which was structurally confirmed by 1D proton and 2D homonuclear and heteronuclear experiments. This also demonstrated that these fermentations can be conducted at larger scale such that quantities of metabolites can be generated are high enough for NMR spectroscopy.

For the drugs acetaminophen, levodopa, metronidazole, dapsone, omeprazole, simvastatin, and zonisamide no specific metabolites were detected after incubation with human colon microbiota. However, for levodopa and metronidazole a decline of the parent drug was detected after 24 hours incubation, but no specific metabolites were found, whereas the other drugs were found to be stable during incubation with human colon microbiota. Previous studies showed gut microbiota-mediated reduction of C-14-labeled metronidazole into N-(2-hydroxyethyl)-oxamic acid and acetamide in rat faeces (Koch et al., 1979, Koch and Goldman, 1979). Levodopa undergoes *p*-hydroxylation by which *m*-hydroxyphenylacetic acid is formed in humans, but this was inhibited upon treatment with the antibiotic neomycin (Sandler et al., 1969). We did not confirm these data of metronidazole and levodopa or for the other drugs using human colon microbiota, which may be due to potential species specific differences in microbial enzymatic activity or due to the absence of certain microbial enzymatic activity in the used pool of human microbiota. Also, the UHPLC-MS analysis method used was relatively generic to drug metabolism whereas for some of these drugs specific sample work-up and analytical methods may be required for the detection of their metabolites. Small amounts of small hydrophilic metabolites would need to be detected within a very complex matrix (Duda-Chodak et al., 2015). Another possibility is that the metabolites are instable to the sample work-up method. Generally speaking, the use of radiolabeled-labeled drugs would facilitate the process of quantification and identification of metabolism in this type of *ex vivo* screening platforms. Nevertheless this was outside the scope of the current research.

In conclusion, this study shows that the previously developed fermentation screening platform is a valid technique to screen for microbiota-based drug metabolism. The anaerobic culture conditions were maintained throughout the incubations. The composition of the human colon microbiota in the assay was representative for microbiota as found in the colon and the diversity of the microbial composition was

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maintained during incubations and reproducible between experiments. Twelve drugs with varying BCS classification were incubated and analyzed for metabolism by human colon microbiota, and we were able to detect metabolism in a qualitative manner. Among drugs, those in BCS-class II are more likely to reach the colon and microbiota since they are not as readily absorbed. Our data showed that these drugs can be metabolized by the gut microbiota in the in vitro system (**Table 1**) indicating that they can penetrate into the cells despite being less absorbed in the intestine. The detected metabolic transformations were comparable to reactions as previously described in studies with human or animal faeces demonstrating the utility of this assay (Nayak and Turnbaugh, 2016, Wilson and Nicholson, 2017). Future research will be aimed toward determining whether this tool could be useful to understand the reversal of metabolism back to parent drugs (e.g. glucuronide hydrolysis, N-oxide reduction). In addition we will determine if data obtained with this in vitro system can be more quantitatively translated to human drug disposition and we will aim to use the assay for precision medicine by applying personalized microbiota in order to determine inter-individual variation.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design:	Steeg, Schuren, Obach, Nooijen, Woudenbergh, Vaes
Conducted experiments:	Obach, Woudenbergh, Bouw, Ossendrijver, Walker
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Wrote the manuscript:	Steeg, Schuren, Obach, Woudenbergh
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FOOTNOTES

The authors E. van de Steeg and F.H.J. Schuren contributed equally to the manuscript.

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FIGURE LEGENDS

Figure 1. Microbial composition diversity of a pooled sample of 7 different individual fecal samples.

A-H) Microbial composition of individual and pooled fecal samples obtained from 7 healthy adult volunteers analyzed by sequencing, differentiating between the 5 main microbial phyla present in the human microbiota (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia*) **I-J)** Microbial composition of pooled human fecal samples at the start of the *ex vivo* fermentation assay (t=0) and after 24 hours of incubation in the assay (t=24). Data are presented as mean relative abundance of the individual species (n=3).

Figure 2. Summary of metabolic reactions observed in the fermentation screening platform.

Schematic representation of microbiome-induced metabolic reactions observed in the fermentation screening platform for risperidone, sulindac, nizatidine, sulfinpyrazone and sulfasalazine.

Figure 3. A) HPLC chromatograms of sulfasalazine (50 μ M) incubations in the fermentation screening platform. The appearance of a peak representing reductive cleavage of the azo bond hydrolysis was observed (m/z 250.0644; -0.3 ppm; R_t = 2.0 min). **B)** High resolution mass spectra for reductively cleaved metabolite of sulfasalazine in the fermentation screening platform. The top panel shows the protonated molecular ion at m/z 4250.0644 (-0.3 ppm) and the bottom panel is the CID-induced fragmentation pattern.

Figure 4. A) HPLC chromatograms of sulfinpyrazone (50 μ M) incubations in the fermentation screening platform.. The appearance of a peak representing deoxy metabolite eluted at 3.9 min (λ = 260 nm).

B) High resolution mass spectra of deoxysulfinpyrazone metabolite generated in the fermentation screening platform. The top panel shows the protonated molecular ion at m/z 389.1322 (1ppm) and the bottom panel is the CID-induced fragmentation pattern.

Figure 5. A) HPLC chromatograms of sulindac (50 μ M) incubations in the fermentation screening platform. The appearance of a peak representing deoxy metabolite eluted at 7.19 min (λ = 260 nm).

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B) High resolution mass spectra of deoxy sulindac metabolite generated in the fermentation screening platform. The top panel shows the protonated molecular ion at m/z 341.1005 (1ppm) and the bottom panel is the CID-induced fragmentation pattern.

Figure 6. A) HPLC chromatograms of nizatidine (50 μ M) incubations in the fermentation screening platform. The appearance of peaks representing deoxy metabolites eluted between 2.9 and 3.0 min (λ =275 nm). **B)** High resolution mass spectra for deoxynizatidine metabolites generated in the fermentation screening platform. The top panel shows the protonated molecular ion at m/z 316.1262 (0.5 ppm) and the bottom is the CID-induced fragmentation pattern.

Figure 7. A) HPLC chromatograms of risperidone (50 μ M) incubations in the fermentation screening platform. The appearance of a peak representing reduction of the benzisoxazole N-O bond and subsequent hydrolysis of the intermediate imine was observed (m/z 414.2184; -0.8 ppm). Parent drug and metabolite co-elute (R_t = 4.20 min), but were distinguished by the different masses. **B)** High resolution mass spectra for reduced risperidone metabolite generated in the fermentation screening platform. The top panel shows the protonated molecular ion at m/z 414.2184 (-0.8 ppm) and the bottom panel is the CID-induced fragmentation pattern.

Figure 8. NMR of Reduced Risperidone Metabolite ^1H and ^1H - ^{13}C HMBC. ^1H chemical shift and coupling patterns established the 8.02 ppm resonance as H28. Within the ^1H - ^{13}C HMBC data set there is a cross peak from the ^1H 8.02 ppm resonance to a ^{13}C resonance at 207.5 ppm which is assigned as the C19 carbon of the ketone. All other observed resonances and cross peaks in the isolated metabolite are unchanged from those observed in similarly acquired data from risperidone

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TABLES

Table 1. Overview of drugs that have been incubated with fermentation screening platform

Drug name	BCS class	Reference(S)
<i>Acetaminophen</i>	1/3	(Kalantzi et al., 2006, Pham-The et al., 2013, Wu and Benet, 2005)
<i>Dapsone</i>	2	(Bergstrom et al., 2014, Wu and Benet, 2005)
<i>Levodopa</i>	1	(Bergstrom et al., 2014, Wu and Benet, 2005)
<i>Metronidazole</i>	1	(Bergstrom et al., 2014, Wu and Benet, 2005)
<i>Nizatidine</i>	2	(Dahan et al., 2009)
<i>Omeprazole</i>	1	(Pham-The et al., 2013)
<i>Risperidone</i>	2	(Bergstrom et al., 2014, Wu and Benet, 2005)
<i>Simvastatin</i>	2	(Bergstrom et al., 2014, Wu and Benet, 2005)
<i>Sulfasalazine</i>	2/4	(Bergstrom et al., 2014, Wu and Benet, 2005)
<i>Sulfinpyrazone</i>	2	(Pham-The et al., 2013)
<i>Sulindac</i>	2	(Bergstrom et al., 2014, Wu and Benet, 2005)
<i>Zonisamide</i>	n.a.	http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/07/WC500189663.pdf

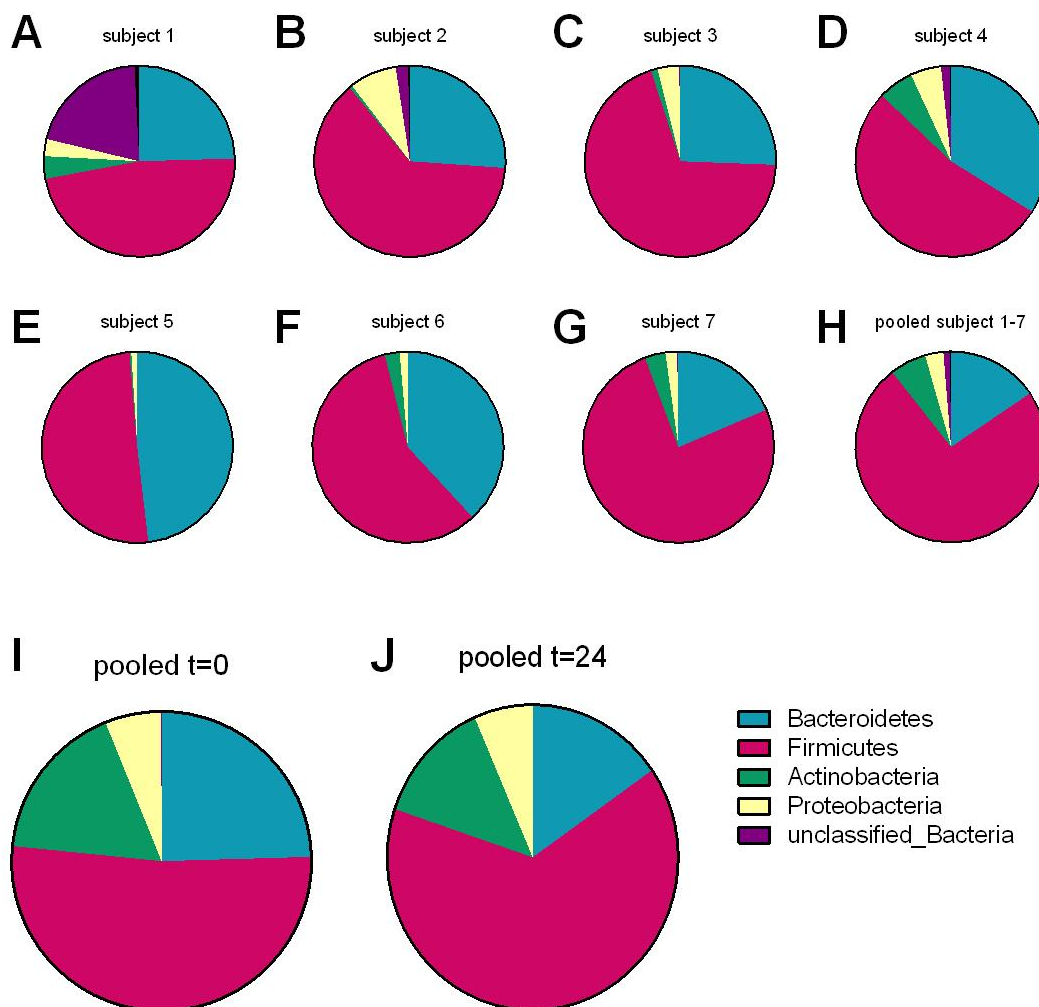
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Table 2. Overview of metabolic reactions of drugs observed in fermentation screening platform

Drug	Metabolic reaction observed in vitro	Metabolic reaction observed in vivo (species)
Sulfasalazine	Azo reduction	(Peppercorn and Goldman, 1972) <i>(rat and human)</i>
Sulfinpyrazone	Sulfoxide reduction (deoxygenation)	(Strong et al., 1987) <i>(rabbit and human)</i>
Sulindac	Sulfoxide reduction (deoxygenation)	(Strong et al., 1987) <i>(rabbit and human)</i>
Nizatidine	Nitrogen oxide reduction (deoxygenation)	(Basit et al., 2002) <i>(human)</i>
Risperidone	Isoxazole scission due to N-O bond reduction and subsequent hydrolysis of imine intermediate	(Mannens et al., 1993, Meuldermans et al., 1994)(Mannens et al., 1993; Meuldermans et al., 1993) <i>(rat, dog, and human)</i>

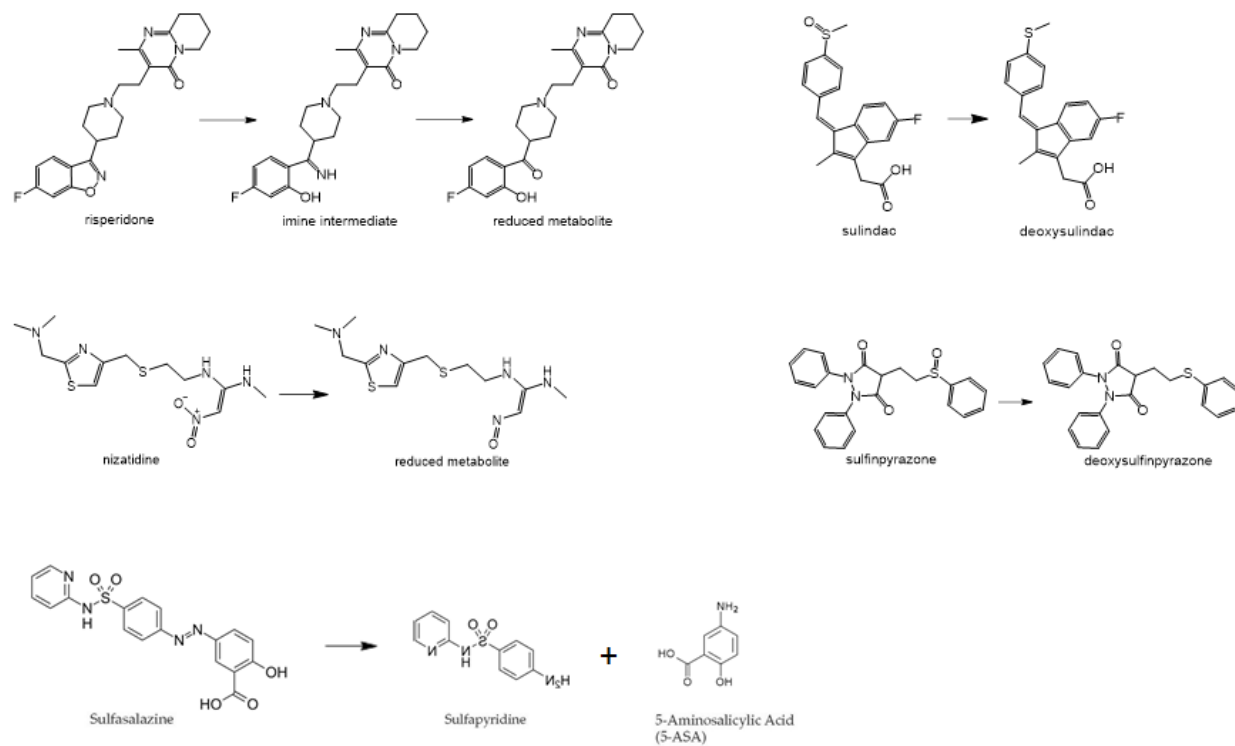
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Figure 1



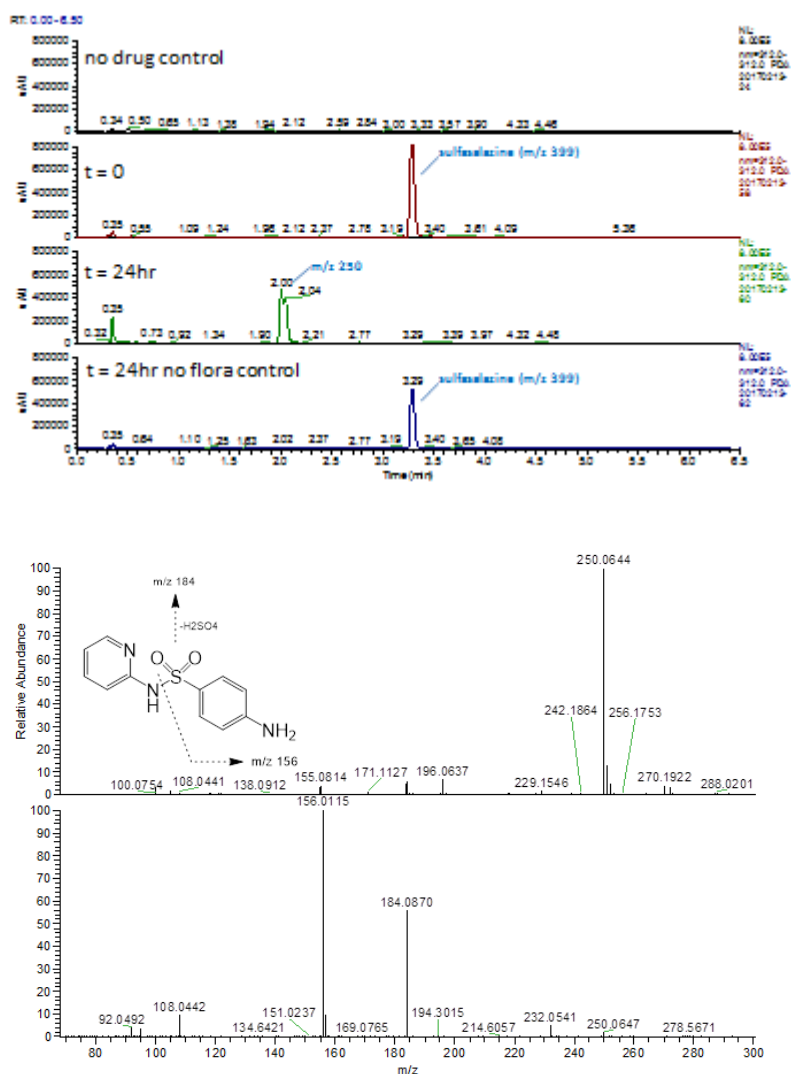
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Figure 2.



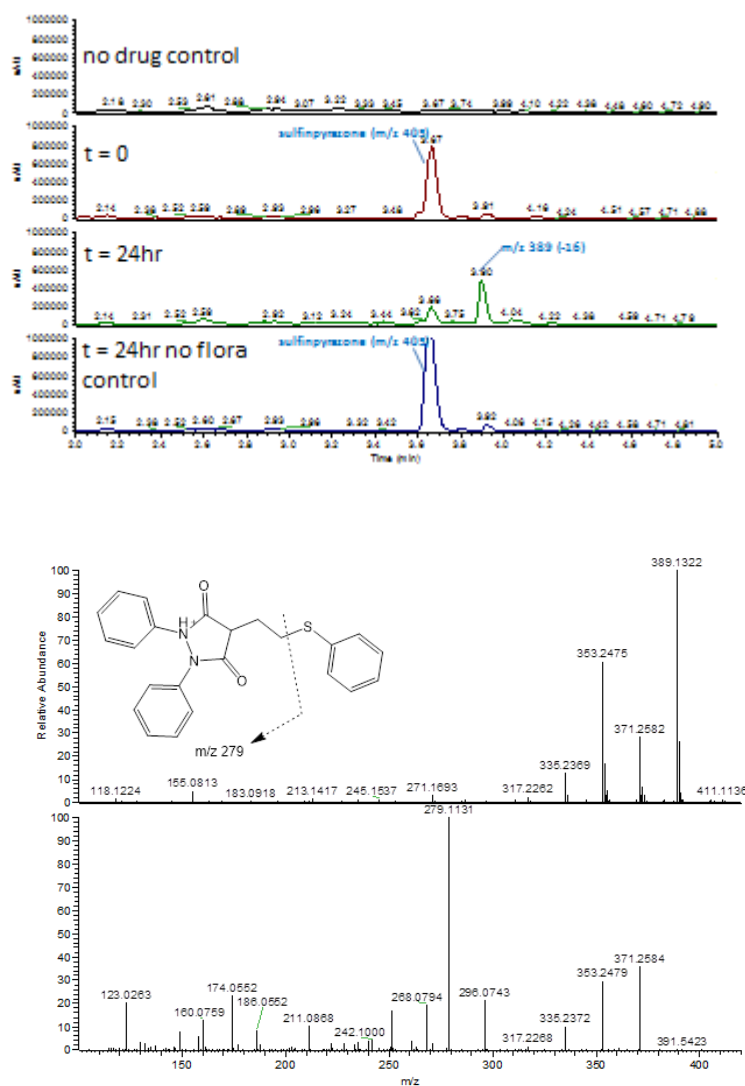
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Figure 3.



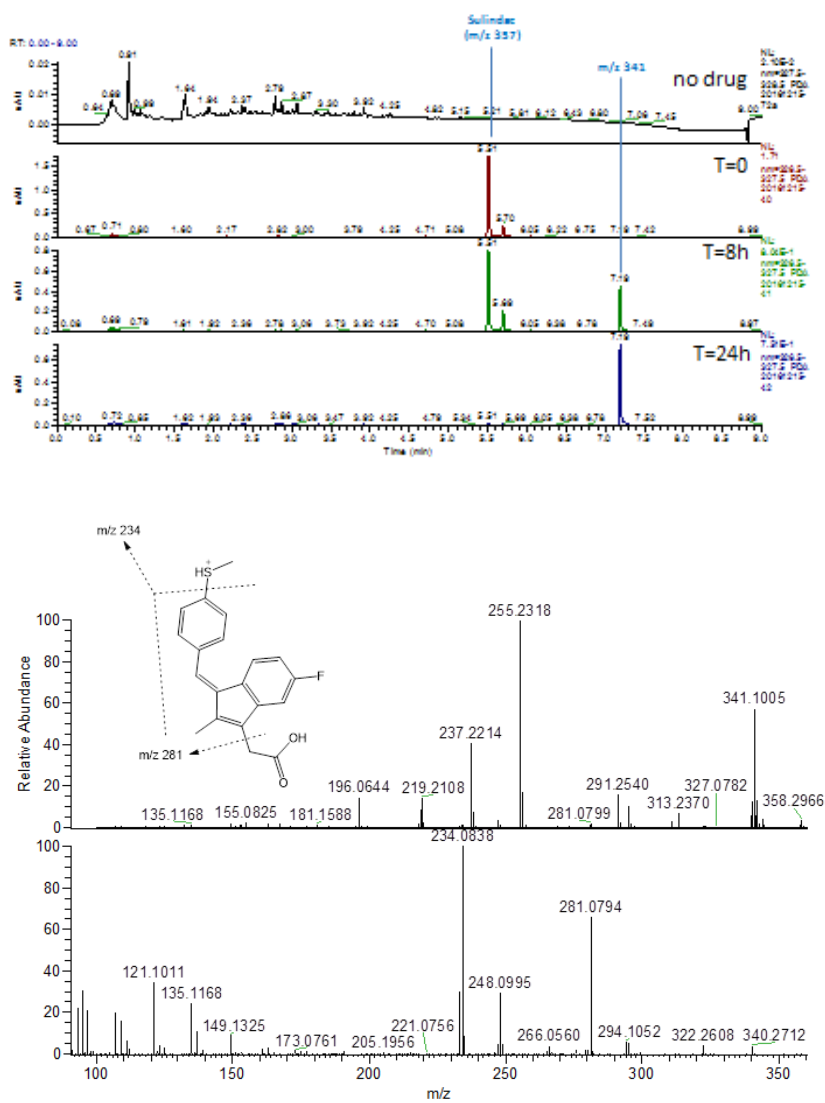
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Figure 4.



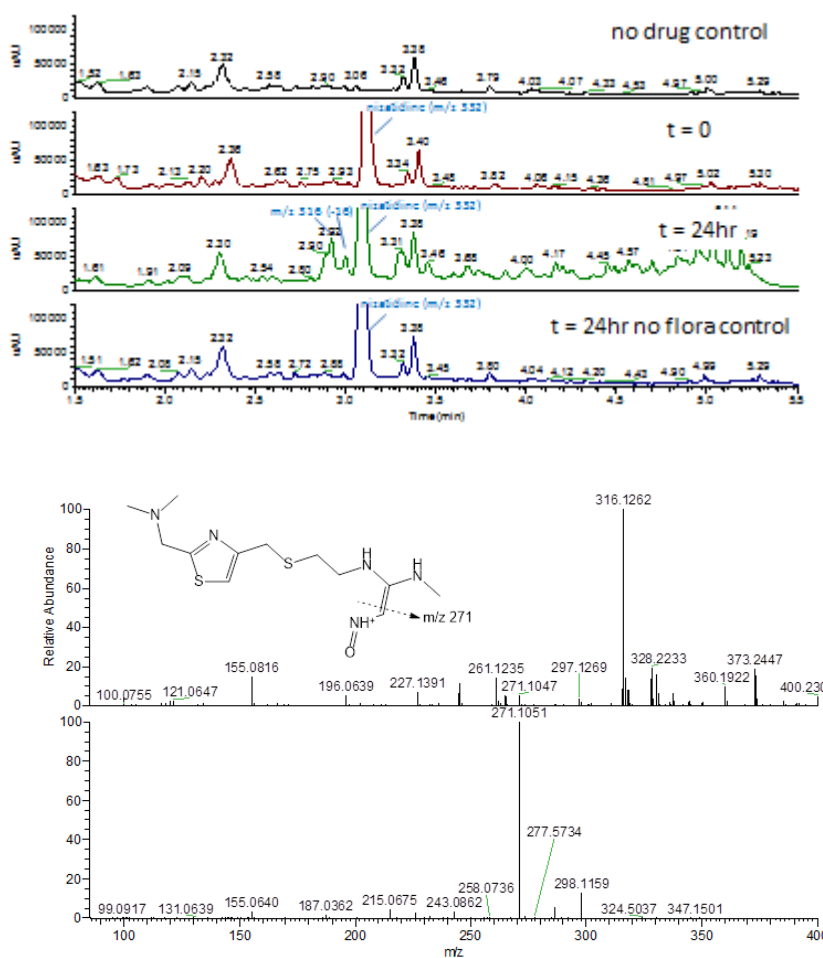
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Figure 5.



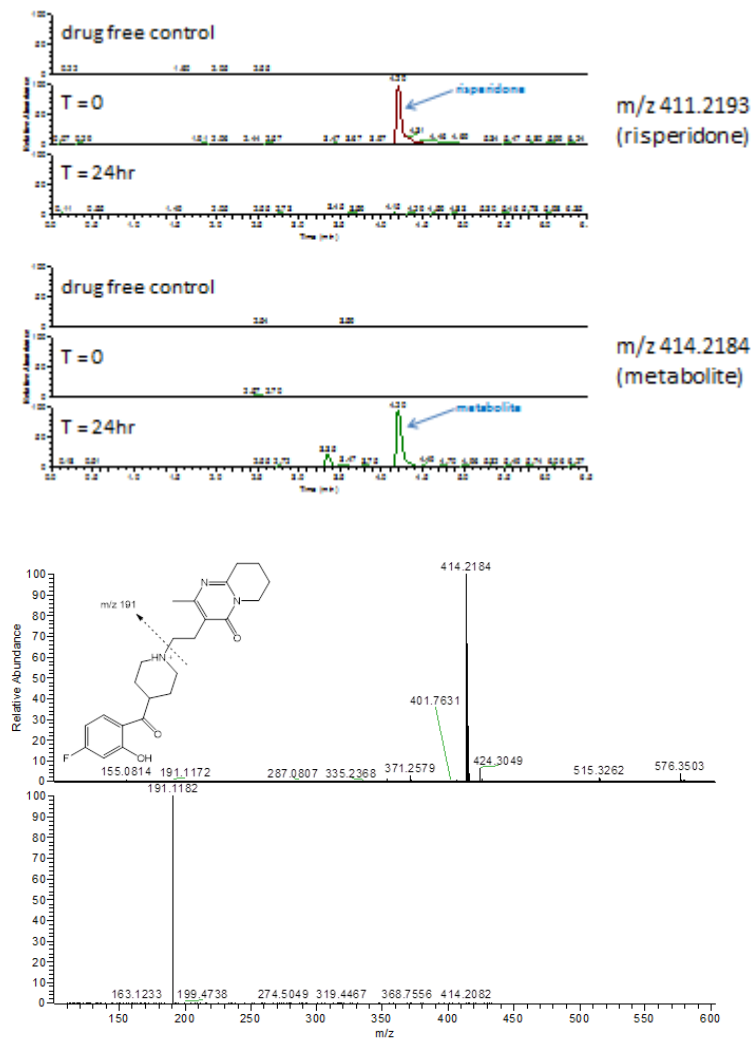
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Figure 6.



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Figure 7.



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Figure 8.

