Investigating the Role of the Gut Microbiome in the Metabolism of Clozapine

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Abstract

The development of more effective treatments for psychotic symptoms is a critical need, as existing antipsychotics yield suboptimal outcomes and adverse effects. While clozapine can improve symptom control, its complex pharmacokinetics and variable response rates hinder its use. The gut microbiome's contribution to clozapine metabolism is increasingly recognized, despite a focus on hepatic pathways and the clozapine:metabolite ratio. This study optimised an *in vitro* model, where faeces from clozapine-naïve germ-free and specific-pathogen-free mice (n=6), and healthy humans (n=5), were incubated with clozapine. Direct microbial contribution to clozapine metabolism was quantified and differentiated using optimised analytical techniques, with comparisons made to relevant controls. This study demonstrates that a functional gut microbiome is essential for the conversion of clozapine to norclozapine and that individual variation exists in this microbial activity. Furthermore, host genetics alone cannot account for all variations of clozapine metabolism, and that this conversion is driven by microbial CYP-equivalent enzymes. Unexpected findings, including norclozapine production in autoclaved human samples and a lack of correlation between the FDA assay and clozapine metabolism, highlight the complexities of clozapine metabolism, which may be related to heat stable enzymes or additional metabolic pathways. Future studies should identify the specific bacterial species, mechanisms, and environmental conditions that influence clozapine metabolism, by using multiomics analysis and time-course experiments.

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Contents

Abstract	2
Acknowledgements	3
Table of Contents	4
Chapter 1: Introduction	9
Physiological Influence	9
Microbiome-Drug Interactions	10
Microbiome-Derived Antipsychotic Alteration	11
Clozapine for Treatment-Resistance	12
Exploration of Variation in the CYP gene	13
Demographic Influences of Clozapine Metabolism	14
Neural Pathways of Clozapine	15
Neurochemical Mechanisms Associated with Clozapine-Treatment Response	15
The Relationship Between Clozapine and Norclozapine	17
Clinical Relevance of Clozapine:Norclozapine ratio	17
Microbial Contributions to CNR	18
Possible Existence of Microbial CYP enzymes	19
Gut Microbiome as Therapeutic Target for Schizophrenia	20
Association Between Gut Microbiota and Schizophrenia	21
Investigating Microbiome-derived Metabolism	22
Key In-Vitro Studies of Microbiome-Derived Drug Metabolism	23
Methodology Optimisation	27

Current Study 29

Chapter 2: Optimising the Model Using Mice Samples 30

Introduction 30

Mouse Samples 30

Optimisation Techniques 30

Methodology 31

Sample description 31

Validating the in-vitro model 31

Implementing the optimised in-vitro methodology in mice 32

Producing Experimental and Control Conditions 33

Incorporating the test of metabolic activity 34

GC/MS Optimisation 35

Data processing 36

Data analysis 36

Optimised GCMS Methodology 37

RESULTS 37

Heat killing validation 37

FDA Test of Metabolic Activity 38

GC/MS Optimisation 39

Optimised GC/MS - Initial Mouse Samples 41

Optimised GC/MS – Experimental Mouse Samples 43

SPF1 Mouse 43

Germ-Free Mouse 47

Discussion 51

Heat killing 51

Fluorescein Diacetate Hydrolysis Test 51

GCMS Optimisation Hydrolysis 52

Limitations 52

Chapter 3: Translating the Optimised Model for Human Samples 54

Introduction 54

Methodology 55

Culturing and Inoculation 55

Testing Bacterial Metabolic Activity 55

Data analysis 55

Optimised GCMS Methodology 56

Results 56

Testing Bacterial Metabolic Activity 56

Validating GCMS Methodology 57

Optimised GCMS Methodology 58

Controls 58

Donor 1 60

Donor 2 66

Donor 3 72

Donor 4 78

Donor 5 84

All Donors 90

Discussion 92

Initial Validation of GCMS Methodology 92

FDA hydrolysis test 92

Optimised In-Vitro Samples 92

Controls 92

Donors 93

Comparison of FDA Results and Metabolomic Results 94

Limitations 95

Conclusion 95

Chapter 4: Human Sample Analysis with Further Investigations 96

Introduction 96

Methodology 96

Normalising by Metabolic Activity 96

Determining Origin of Enzymes Responsible for Clozapine Metabolism 96

Proteinase K 97

Results 98

Metabolic Activity Results 98

Weights of Samples 98

Donor 1 99

Donor 2 104

Donor 3 109

Donor 4 114

Donor 5 119

All Donors Ratio 124

Microbial Derived Metabolism Analysis 124

Discussion 125

Test of Metabolic Activity 125

Proteinase K 125

1:10 and 1:100 dilutions comment 125

Full Experiment 125

Controls 125

Donors 125

Comparison to Previous Batch 126

Controls 126

Donors 126

Chapter 5: Conclusion 128

Research Implications 129

Limitations 130

Discrepancies Between In vivo and In vitro Conditions 130

Variations in Gut Physiology 130

Analytical Technique 131

Other Methodological Limitations 132

Future Studies 133

Conclusion 134

References 135

Chapter 1: Introduction

Over the past few decades, there has been a rise in interest in the human gut microbiome and its influence on health, with evidence displaying a link to cognitive benefits (Loh et al., 2024) and immunological improvements (Lozupone, 2018). Every surface and invagination of the human body inhabits a distinct microbial community, including the oral cavity, skin and urogenital tract (Clemente et al., 2012). However, the gut microbiome dominates in terms of microbially abundance and diversity, containing hundreds of species of bacteria, viruses and fungi (Qin et al., 2010). There is significant interindividual variation between individuals regarding which the diversity of species they possess (Zhu et al., 2015), but in general it has been found that up to 50% of the gut microbiota can be shared between humans, with a common core set of species, including *Bacteroides, Lactobacillus, Clostridium*, and *Bifidobacterium* (Costello et al., 2009).

Physiological Influence

Microbes in the human gut can interact with other physiological systems through various mechanisms. They can influence the digestive system – an example is the promotion of fermentation via producing short chain fatty acids, such as acetate and butyrate, which assist with processes like cholesterol metabolism and glucose and lipid production (Valdes et al., 2018). They can also influence the immune system – assisting with differentiation between pathogenic and harmless antigens, determining whether the immune response is triggered (Clemente et al., 2009). They can also influence metabolic processes – by affecting absorption and synthesis of vitamins and hormones (Tripathi et al., 2018; Anand and Mande, 2022). More recently, a relationship between the human gut microbiome and the nervous system has been unravelled, termed the gut-brain axis (Loh et al., 2024). Several pathways have been evidenced to be involved in this axis, including the vagus nerve, endocrine system and enteric nervous system (Michaelis et al., 2023).

Alterations to microbiome composition, sometimes termed as “dysbiosis” (den Besten et al., 2013), can therefore result in disease, such as inflammatory bowel disease, obesity and diabetes. Sometimes these alterations can be measured using changes in diversity – for example, lowered diversity of microbiota has been correlated with occurrence of allergies such as asthma, due a disruption in levels of inflammatory markers resulting in a hyperactive immune response (Pascal et al., 2018). However, microbial diversity is not the definitive measure of gut health, especially if the individual possess high proportions of pathogenic microbes. There are also examples were lower diversity is actually beneficial – this is observed in breast-feeding infants.

Instead, it may be more key to focus on levels of certain genera and their proportions to each other. An example is the correlation the ratio of Bacteroidetes:Bacillota and the rise in obesity, although it is debated as to which genera are enriched (Duan et al., 2021) but is hypothesised to be linked to the ability of these microorganisms to digest carbohydrates. A further example is the link between the increase of *Helicobacter pylori* and

occurrence of gastric cancer, due to the bacteria producing enzymes that can damage the gastric mucosa and also promoting of DNA mutation, which is a key cause of cancer progression (Salvatori et al., 2023).

Microbiome-Drug Interactions

Studies have focused upon microbial enzymes capable of metabolising orally administered medications with impacts on pharmacodynamics, kinetics and toxic properties of the medication (Zimmerman et al., 2017). Microbes can metabolise certain drugs to produce pharmacologically active compounds. An example of this is prontosil, used to treat bowel conditions. In this case, Clostridia and Eubacteria catalyse the reduction reaction that convert prontosil into its metabolite, sulfanilamide, which mediates therapeutic effects (Rafii and Cerniglia, 1995). However, microbes can also produce toxic compounds, resulting in unwanted side effects of drugs. This is demonstrated with the use of potassium oxonate, an anti-tumour therapy, which can be biotransformed by gut microbiota into metabolite cynuric acid which increases its toxicity (Kunihiro et al., 2000). Biotransformation is one mechanism in which microbial products can alter oral medications, mainly through releasing enzymes that metabolise the original compound into new metabolites that are either more or less active (Zimmerman et al., 2019a). Bioaccumulation is another mechanism, where gut bacteria intracellularly sequester the original compound (Klunemann et al., 2020).

These drug interactions can alter the dose required to reach therapeutic effect, which may explain why patients often fail to effectively respond to a medication The variability in individual drug response may also be a result of the drastic inter-individual variation of microbiome (Zhu et al., 2015). Some people may have microbiota that metabolise a drug to make it less active by breaking it down into metabolites. This will likely reduce the therapeutic effect and increase the risk of side effects they experience. However, other people with differing structure of microbiota may not possess the bacteria that interact with this drug, and therefore experience different therapeutic outcomes.

This inter-individual variation in microbiota, and resulting therapeutic effects, has been explored in studies of twin siblings. Even monozygotic twins (genetically identical) exhibit significant differences in their gut microbial communities, as demonstrated in a study where only 35.9% to 49.1% of the 800 species-level bacterial phylotypes identified within the gut microbiomes of the twins were common between them (Turnbaugh et al., 2010). Furthermore, the researchers detected significant genetic and transcriptional variations between the twins' gut microbiota, with only 17% of the genes in their microbial communities being shared.

Many studies using “germ-free” mice (mice without a microbiome) have supported the contribution of the microbiome to drug metabolism, as these mice show differential gene expression, protein levels

and activity on drugs compared to conventional mice hosting normal microbiomes (Tsunoda et al., 2021). Using germ-free (GF) and conventional Specific Pathogen Free (SPF) mice, Zimmermann and colleagues (2019b) distinguished contributions of microbial enzymes and host enzymes in the metabolism of orally-administered brivudine, an antiviral medication. When examining the faeces of the mice, the SPF mice had produced an abundance of the metabolite bromovinyluracil whereas little was detected in the faeces of the GF mice, demonstrating a potential microbial involvement in the metabolism of this drug. This is a crucial finding, as bromovinyluracil is toxic to the liver, and so it is crucial that the bacteria responsible for this conversion has minimal activity. The study distinguished this metabolic reaction from host metabolism by conducting liver-cell assays, as well as producing mouse models that were isogenic, except for the bacterial gene responsible for brivudine metabolism. This paper identified a single mutant, possessed by species from the *Bacteroides* genus – a transposon insertion in *bt4554.*

Microbiome-Derived Antipsychotic Alteration

Given the known influence of the gut microbiome on drug metabolism, the interactions of gut bacteria with psychotropic medications is of considerable interest, particularly given the high rate of treatment-resistance. The potential for microbial alteration of medications highlights the need to further consider how these complex interactions influence treatment outcomes for psychiatric conditions.

Psychiatric medications are one such group of treatments that frequently demonstrate poor efficacy; as defined by the National Institute for Health and Care Excellence (NICE; 2004) classification, using a standardised mean difference (SMD) of 0.5 or higher, in comparison to placebo, a threshold initially proposed by Cohen (1988). Disappointingly, a meta-analysis evaluating several antidepressants found that clinical significance was only reached in cases of severe depression (considered as >28 on the Hamilton Rating Scale of Depression; Kirsch et al., 2008). Another meta-analysis showed two thirds of antipsychotics evaluated fell below 0.5 (Leucht et al., 2013), meaning they failed to reach clinical efficacy.

Patients also often experience metabolic side effects from antipsychotic medications, with a recent systematic review and meta-analysis by Barton et al. (2020) showing that all antipsychotics they evaluated resulted in weight gain after 12 weeks, where a majority of these drugs led to an increase of over 7% in body weight compared to placebo. Such significant side effects not only elevate the risk of serious health conditions, such cardiovascular events (Stroop and Gray, 2017), but also lead

to poor adherence to treatment regimens, as found by Semahegn and colleagues in their combined review (2020). Their study revealed that the average rate of non-adherence to psychotropic medications among patients with major psychiatric disorders was 49%. This high level of non-adherence is concerning, as it can result in a worsening of psychiatric symptoms.

Notwithstanding the confounding effects of low adherence, there is compelling evidence that poor clinical outcomes may be a result of gut bacterial drug metabolism, for antipsychotics (Bahr et al., 2015; Javdan et al., 2020; Kanji et al., 2018; Morgan et al., 2014) and antidepressants (Klunemann, 2021). For instance, one study using GF mice demonstrated that weight gain following the administration of the antipsychotic olanzapine, was associated with an increased abundance of *Erysipelotrichi* and *Actinobacteria* classes of bacteria (Morgan et al., 2014). Additionally, administration of risperidone resulted in enriched pathways for butyrate and propionate metabolism compared to controls (Bahr et al., 2015). Bahr and colleagues hypothesised that this enrichment could lead to higher overall production of these short-chain fatty acids (SCFAs), which, by providing additional energy and potentially stimulating adipogenesis, might contribute to the weight gain that was observed in the patient group taking risperidone. However, SCFAs like butyrate have multi-faceted interactions across the body, and have also been associated with neuropsychiatric benefits, including improvements in cognition (Minichino et al., 2022). This highlights the complex dynamics of microbial products throughout the body.

As mentioned previously, Zimmerman (2019b) used a variety of *in vitro* and *in vivo* techniques to distinguish the microbial contributions to brominouracil metabolism from the host metabolism. In this same study, they also created a host-microbiome pharmacokinetic model of clonazepam, an anxiolytic used to treat anxiety and epilepsy. Using previous findings of microbial involvement in its metabolism (Elmer and Remmel, 1984; Takeno and Sakai, 1991), they predicted that the microbiome contributed to 78% of the nitroreduction of the drug into its major metabolites aminoclonazepam and aminohydroxyclonazepam. As these metabolites are associated with toxic side effects, this provides an example where gut-microbes can contribute to adverse effects associated with oral psychotropic medications.

Clozapine for Treatment-Resistance

Patients who do not experience efficacy from antipsychotics are classed as having “treatment-resistant schizophrenia” – which accounts for almost 25% of patients (Siskind et al., 2021). For these patients, clozapine is considered the gold-standard second-line treatment; defined as the second best option after not responding to traditional antipsychotics. Clozapine is significantly more effective

than others – demonstrating the highest SMD to placebo in comparison to other treatments (Leucht et al., 2013; Siskind et al., 2018).

Clozapine is heavily metabolised by the cytochrome proteins in the liver in a first pass effect (Byerly and DeVane, 1996), in which 70-95% is converted into its major active metabolite N-desmethylclozapine, or norclozapine, via demethylation (Pardinas et al., 2021; Thorn et al., 2018) and 5-30% into inactive clozapine-N-oxide via oxidation (Eiermann et al., 1997; 2003) – see Figure 1. Clozapine binds to the hepatic cytochrome protein (CYP) 450, (Fischer et al., 1992) and specifically proteins CYP1A2 and CYP3A4 that encode enzymes which catalyse the demethylation, whereas enzymes CYP1A2 and CYP2C9 are involved in oxidation, a reaction which is reversible in the presence of NADPH (Pirmohamed et al., 1995).

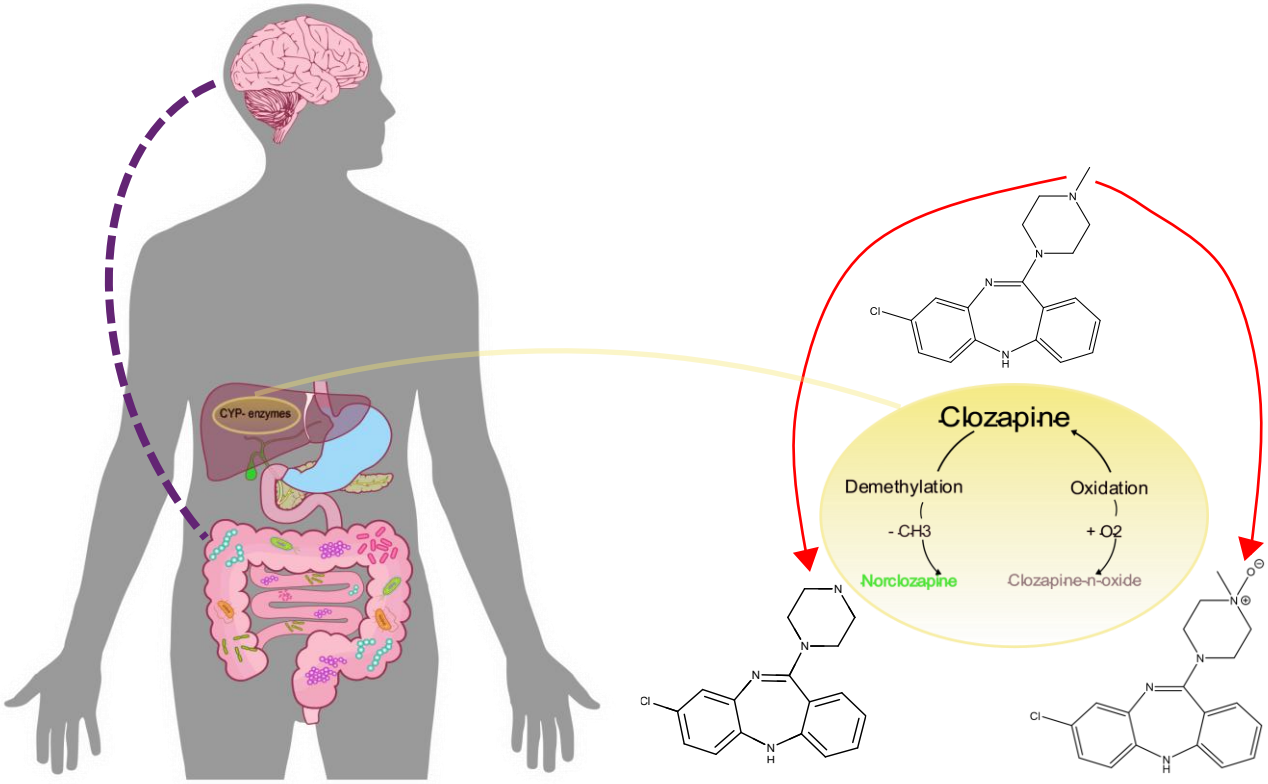


Figure 1. Clozapine metabolism occurs in the liver via cytochrome enzymes. It is demethylated to its active compound, norclozapine, and oxidised to clozapine-N-oxide. The oxidation is a reversible process.

Clozapine has a complex pharmacokinetic profile; patients' plasma levels can vary widely despite taking the same oral dose (Centorrino et al., 1994), with some studies demonstrating up to 50-fold changes (Flanagan et al., 2006; Potkin et al., 1994) – a level which is rare for oral medications. Clozapine-users also experience adverse effects common to antipsychotics, including weight gain and cardiac and digestive issues (Siskind et al., 2018; Tan et al., 2021). Thus, investigation has been prompted into the origin of this reduced clinical

responses, with evidence from various mechanisms, including, genetics, demographics and neural and metabolic pathways.

Exploration of Variation in the CYP gene

The complex pharmacokinetics of clozapine has led many to investigate the cause of its variable response rate, with initial research focussing on human genetic polymorphisms in the CYP gene family. These include *CYP1A1* and *CYP1A2* and the *UGT* gene families, which are involved in the metabolism of clozapine and its primary metabolite, norclozapine (Meher and Stahl, 2021; Pardinas et al., 2019; Zhou et al., 2009). Specifically, single nucleotide polymorphisms (SNPs) in *CYP1A1*, *CYP1A2* and the *UGT* genes (uridine diphosphate-glucuronosyltransferase) have been linked to altered clozapine pharmacokinetics (Meher and Stahl, 2021; Pardinas et al., 2019). The *UGT* family has been linked to the excretion of both norclozapine and clozapine, encoding for enzymes that perform glucuronidation of these compounds (Erickson-Ridout et al., 2012). One specific SNP of the *POR* gene, which is responsible for the ratio of clozapine to norclozapine, has also been identified (Pardinas et al., 2019). Whilst, *in vitro* experiments have linked these enzymes to a direct role in clozapine metabolism, *in vivo*, these SNPs have demonstrated inconsistencies, accounting for only a small percentage of variation (less than 8%) in clozapine metabolism (Pardinas et al., 2023), with the majority of variability unaccounted for by human CYP alleles (van der Weide et al., 2003). Furthermore, studies exploring the impact of geographical ancestry on clozapine metabolism produced contradictory findings (Albitar et al., 2024; McCutcheon et al., 2018; Ng et al., 2005; Pardinas et al., 2023), highlighting the complexity of this topic. Given that these genetic variations account for less than 8% of interindividual variability in clozapine metabolism, these findings suggest that factors beyond host genetics, such as the gut microbiome, may contribute significantly to individual responses to clozapine.

Demographic Influences of Clozapine Metabolism

Following on from the biogeographical research, it is important to consider demographic influences into clozapine metabolism, as these are theorised to have account for around 50% of variation in clozapine treatment response (Amin Rostami-Hodjegan et al., 2004). Particularly relevant to clozapine metabolism are gender influences on variations in liver physiology – women tend to have smaller livers, with less blood flow, and show decreased CYP1A2 activity but higher CYP3A4 activity in women (Bigos et al., 2009). These physiological variations likely influence the slower clozapine-pharmacokinetics observed in women, which has been demonstrated to be 40% lower than men (40% difference; Anderson, 2008), encompassing digestion, excretion and lowered absorption of the drug. (Smith, 2010). All the factors indicate that women likely require a lower dose to reach a therapeutic effect and longer intervals between doses. However, this is often not followed in a clinical setting (Smith, 2010). Women also tend to have higher amounts of adipose tissue than men (15% higher; Smith, 2010), which results in further decreasing of the clozapine elimination, due to the increased fat-storage of clozapine, which is lipophilic (Jakobsen et al., 2017). This will also contribute to a lowering of dose required to reach a therapeutic effect. In fact, a multiple regression analysis found that plasma

clozapine concentrations changed by around 5% for every 10kg in bodyweight (Rostami-Hodhegan et al., 2004).

Age would be expected to be a contributing factor to varying clozapine metabolism, due to generally lowered pharmacokinetics and increasing pathophysiologies, however the literature evaluating this is inconsistent. Some papers indicate that with increasing age, lowered metabolism of clozapine occurs – as shown by Haring and colleagues (1989), demonstrating higher plasma clozapine concentrations in older patients and Chung and colleagues (2014) discovering lowered CYP1A2 activity with increasing age. However, other papers indicate a higher metabolism with increasing age, including the multiple regression analysis conducted by Rostami-Hodhegan and colleagues (2004) where plasma clozapine decreased by around 4% for every 5 years increase in age.

Neural Pathways of Clozapine

The reason behind clozapine being considered a better treatment than other anti-psychotics for schizophrenia is due to its atypical mechanism of action. This means, instead of having potent antagonistic action to striatal dopamine D2 receptors like typical antipsychotics (such as chlorpromazine and haloperidol; Green et al., 2000), clozapine has a higher affinity to antagonise central dopamine D1 and D4 receptors, and additionally antagonises serotonergic 5HT2a and 5HT2c, histaminic H1, cholinergic, muscarinic, and a1 and a2 adrenergic receptors (Fitton & Heel, 1990; Jibson and Tandon, 1998). This allows clozapine to be a better treatment for schizophrenia as patients with schizophrenia exhibit abnormally high dopamine levels (Davidson et al., 1993), therefore the D1 and D4 antagonisms result in improvements in motivation, emotional-expression and anhedonia (Jibson and Tandon, 1998). By recruiting more neural transmitters, clozapine also improves neural connectivity, which in turn results in improvements in cognition – including attention and verbal fluency (Buchanan et al., 1994; Lee et al., 1994; Lidenmayer et al., 2021). Additionally, this mechanism of action can also reduce symptoms associated with striatal D2 dopamine-blockade, as seen with Parkinsons medications, for example extrapyramidal side effects which can lead to motor difficulties, mood problems and cognitive dysfunction (Lieberman et al., 1989). All of these benefits will increase compliance and improved treatment outcomes (Jibson and Tandon, 1998).

Neurochemical Mechanisms Associated with Clozapine-Treatment Response

Due to the recruitment of many neural systems, several theories have been proposed about the relationships between pharmacokinetics and treatment-efficacy, however this still remains unclear (Mauri et al., 2018). Such theories have explored dopaminergic, serotonergic and glutamatergic systems, as well as hormones insulin and leptin.

Due to the antagonism of D1 and D4 dopaminergic receptors, many studies have explored the relationship between clozapine response and homovanillic acid, a dopamine metabolite, however findings have been very mixed. Whilst some studies have found no significant difference in homovanillic acid between clozapine

responders and non-responders (Davidson et al., 1993; Szymanski et al., 1993), other studies have demonstrated significant associations with lower baseline homovanillic acid levels and symptom-improvement following clozapine treatment (Pickar et al., 1992). Significant associations have also been seen in studies with homovanillic acid levels and response to other antipsychotics (Bowers et al., 1989, 1994; Pickar et al., 1986; Davila et al., 1988; Sharma et al., 1989, Davidson et al., 1991). In contrast, later studies found a converse relationship, reporting an association between higher baseline homovanillic acid levels and symptom-improvement following clozapine treatment (Brown et al., 1997; Green et al., 1993; Sumiyoshi et al., 1997, 2004).

Similar mixed results have been found when exploring the association between serotonin and clozapine response. Szymanski and colleagues (1993) were unable to find a significant association between clozapine-response and a serotonin-metabolite, 5-hydroxyindoleactic acid. Fleischhaker and colleagues (1998) were also unable to find an association between plasma serotonin and clozapine-response – however, they did find that platelet serotonin concentrations negatively correlated with clozapine-response. This was later confirmed by Ertugrul and colleagues (2007).

Genetic studies of polymorphisms in genes encoding for dopaminergic and serotonergic receptors also have mixed associations with clozapine-response, with some finding associations between certain alleles and clozapine response (Arranz et al., 2000; Masellis et al., 1998; Scharfetter et al., 1999; Shaikh et al., 1996) but others have failed to confirm this finding (Arranz et al., 1998; Barlas et al., 2009; Hwang et al., 2010; Lee et al., 2012; Malhotra et al., 1998; Masellis et al., 2001; Nöthen et al., 1995; Potkin et al., 2003; Shaikh et al., 1995, Xu et al., 2016).

Studies exploring the ratio of dopamine and serotonin in the role of clozapine have found significant findings – patients who had a lower homovanillic:5-hydroxyindoleactic acid ratio prior to taking clozapine experienced better treatment outcomes (Pickar et al, 1992; Risch and Lewine, 1993; Szymanski et al., 1993).

Studies have investigated the relationship between fasting insulin and leptin levels and the response to clozapine treatment (Agarwal et al., 2019; Honig et al., 2023). The rationale behind these investigations is the observed increase in BMI during clozapine therapy and the role of adipose tissue in leptin production. Additionally, patients on clozapine have an elevated risk of developing type II diabetes mellitus (Ryan and Thakore, 2002), and insulin dysregulation in involved development of diabetes. Both insulin and leptin levels were found to be significantly higher in clozapine-responders than non-responders, also correlating positively with better psychiatric outcomes, as measured by the Positive and Negative Symptoms Scale (PANSS – where a higher score indicates more symptoms of schizophrenia; Hönig et al., 2023). This confirms previous positive associations found with response to other anitpsychotics and insulin levels (Agarwal et al., 2020; Fan et al., 2006; Sharma et al., 2014) and leptin levels (Takayanagi et al., 2013; Venkatasubramanian et al., 2007). Both hormones have been linked to brain function, including mesolimbic areas (reward; Agarwal et al., 2020; Takayanagi et al., 2013) , which could explain their involvement in clozapine-response.

As the N-methyl-D-aspartate (NMDA) glutamate receptor has been found to be non-functional in schizophrenia (Javitt, 2004), this has encouraged studies to explore associations between the glutamatergic system and clozapine-response. Studies have found that clozapine-responsiveness can be correlated to a reduction in caudate glutamate (Iwata et al., 2019; McQueen et al., 2021), as well as reduced NMDA receptor-binding (Bressan et al., 2005). These findings from studies were also apparent with other antipsychotics (de la Fuente-Sandoval et al., 2013; Demjhaha et al., 2014; Mouchlianitis et al., 2016; Tarumi et al, 2020). Many studies in rodents have demonstrated a similar association, with clozapine disrupting glutamatergic processes caused by blocking NMDA receptors (Abekawa et al., 2006, Amitai et al., 2012; Baerentzen et al., 2019; Homayoun and Moghaddam, 2007; McLoughlin et al., 2009; Fukuyama et al., 2019; Lopez-Gil et al., 2007). Another study elucidated an association between clozapine and L-β-aminoisobutyric acid, a compound that activates a type of glutamate receptor (Fukuyama et al., 2023). Other studies have shown associations between increased levels of glutamatergic precursor N-acetyl-aspartate and higher clozapine-response (Ertugrul et al., 2009; Sigmundsson et al., 2003; Tanaka et al, 2006). However, there have been some other studies that have failed to find any associations between the two (Goldstein et al., 2015; Iwata et al., 2019).

These inconsistent results encourage exploration of a more sensitive biomarker to elucidate the relationship between clozapine pharmacokinetics and treatment-response.

The Relationship Between Clozapine and Norclozapine

As the majority of the clozapine is metabolised into norclozapine, this has encouraged research into potential associations between norclozapine levels and clozapine-response. Norclozapine has differing pharmacokinetics and pharmacodynamics to clozapine – it has a longer half life (around 12 hours; Ellison et al., 2015) and different receptor affinities. It demonstrates partial-agonist action towards M1, M3, and M5 muscarinic receptors (Rajji et al., 2010; Sur et al., 2003; Wiebelhaus et al., 2012), in contrast to clozapine, which demonstrates muscarinic antagonism. This could explain associations between norclozapine and improved cognitive symptom (Davies et al., 2005; Rajji et al., 2010), as muscarinic receptor agonism results in increased acetylcholine production, which promotes increased executive function and memory (Paul et al., 2022). Norclozapine also demonstrates more potent serotonergic antagonism than clozapine (Kuoppamaki et al., 1993), which, whilst this could result in improved psychiatric symptoms, it could also be a factor for the observed associations with weight gain (Lu et al., 2004; Sur et al., 2003; Tan et al., 2021).

Whilst norclozapine does display these unique pharmacological mechanisms, it cannot treat schizophrenia by itself (Hiemke et al., 2018; Tralongo et al., 2023) and provides few clinical insights into clozapine response (Mendoza and Lindenmayer, 2009; Molins et al., 2017; Tralongo et al., 2023). Therefore, research has shifted towards evaluating the clozapine:norclozapine ratio and how this can influence treatment-response.

Clinical Relevance of Clozapine:Norclozapine ratio

The average expected range of the clozapine:norclozapine ratio (CNR) in serum of patients is 0.5 – 2.5 (Flanagan et al., 2023), where values outside of this indicate poor adherence and abnormal clozapine metabolism. Studies have explored several aspects of clinical outcomes in schizophrenia, and their relationship to this ratio.

Cognitive Outcomes

The muscarinic action of norclozapine has lead many researchers to investigate the influence of the ratio on cognitive systems. Islam and colleagues (2021) found that patients who had higher transcription of the M1-receptor gene, as a result of not possessing the SNPs rs2075748 and rs1942499 on the M1-receptor gene, which induces haplotype C-G, demonstrated a correlation between a lower CNR and increased working memory. This related to previous findings of decreased CHRM1 cDNA levels in cortical regions of post-mortem brains of patients with schizophrenia (Mancama et al., 2003), and further supports the protective nature of norclozapine on memory, via its agonist nature on muscarinic receptors.

Some studies failed to find any association between cognitive test scores and CNR of patients taking clozapine (Arnautovska et al., 2022; Costa-Dookhan et al., 2021; Lindenmayer et al., 2021; Thornton et al., 2015) but reported several limitations that may have contributed to this. The use of severely unwell patients in their experiments may have skewed results, as they theorised that the severity of their condition may have negated the protective cognitive effects of norclozapine. Additionally these patients were on higher clozapine dosages, which skew the ratio, and were often taking other medications that impaired their cognition, including lorazepam.

Metabolic Outcomes

Whilst a lower CNR may be beneficial for neuropsychiatric outcomes, research has evidenced that a higher CNR reduces the metabolic adverse effects. Lu and colleagues (2004, 2018) demonstrated this relationship by examining use of fluvoxamine, an anti-depressant, as an adjunctive therapy alongside clozapine. The administration of fluvoxamine caused the CNR to increase, which they had evidenced in an earlier study (2000), due to its inhibition on CYP1A2 activity, resulting in inhibited metabolism of clozapine (Olesen and Linnet, 2000). With the co-administration, patients exhibited less metabolic dysfunction – as they gained less weight and had better tolerance to insulin (2004, 2018). Further studies confirmed the contributor of the ratio in metabolic outcomes, including correlations between higher clozapine:norclozapine ratio levels and decreased weight gain (Jessurun et al., 2022) and decreased insulin resistance (Costa-Dookhan et al., 2021; Costa-Dookhan et al., 2021). A study conducted by Sporn and colleagues (2007) did not find a correlation between the metabolite ratio and occurrence of side effects – although, participants were of adolescent age, which could be a contributing factor.

Psychiatric Outcomes

Several studies have found correlations between a lower clozapine:norclozapine ratio and reduced symptoms, as measured by PANSS scores (Frazier et al., 2003; Mauri et al., 2003; Park et al., 2020; Sporn et al., 2007;

Weiner et al., 2004), the Brief Psychiatric Rating Scale (Molins et al., 2017; Sporn et al., 2007) and Clinical Global Impressions-Improvement (Lee et al., 2009).

Microbial Contributions to CNR

Human gut and liver metabolism are inextricably linked by the portal vein (Anand and Mande et al., 2022), allow microbial-derived metabolism to influence hepatic metabolism, including the production of norclozapine from clozapine. This relationship between the gut and liver is often referred to as the gut-liver axis.

Studies have shown specifically how the gut can influence CYP-related drug metabolism (the enzymes involved with clozapine), in both mice (Bjorkholm et al., 2009; Selwyn et al., 2016; Toda et al., 2009) and humans (Jarmusch et al., 2020). The study by Bjorkholm and colleagues (2009) demonstrates the contributions of the gut microbiota to liver metabolism by comparing drug metabolism in germ free (GF) mice to conventional mice. It revealed 112 genes involved in hepatic metabolism that were differentially expressed between the mice, implying that the intestinal microbiota contributes to the expression of these genes. These included several P450 genes. It further revealed the relationship of the gut microbiota on hepatic gene expression and drug metabolism, by administering pentobarbital, a barbiturate, to both types of mice. The pentobarbital took less time to produce anaesthesia in the germ-free mice than the specific pathogen-free mice, which the paper hypothesises is a result of accumulation of substances like bile acids and steroid hormones in the absence of gut microbes, which activates hepatic genes, that result in more rapid drug metabolism.

Selwyn and colleagues (2016) showed similar findings by introducing either probiotics or conventional microbiota to GF mice, which resulted in altered expression of hepatic enzymes involved in drug metabolism, including CYP3A* family (which is involved in clozapine metabolism). Similarly Toda and colleagues (2009) suggested that administration of antibiotic ciprofloxacin reduced levels of gut bacteria involved in lithothilic acid production (a bile acid), as result of reduced expression and activity of CYP3A in mice.

Studies have also been conducted in humans which evidence this influence on gut microbiota on hepatic drug-metabolism. A study by Jarmusch and colleagues (2020) translated the findings from Toda and colleagues (2009) into humans, by exploring the effect of antibiotic cefprozil on human CYP enzymes. They found that administration of this drug reduced gut microbiome diversity, particularly species from *Bacteroides* and *Prevotella*, which reduced the activities of CYP1A2, CYP2C19 and CYP3A. This was confirmed using metabolomics from faeces, as well as mass spectrometry on plasma and urine samples, which evaluated the levels of bile acids litholic and butyric acid.

These findings underscore the complex interplay between gut microbes and liver enzymes, emphasising that the gut microbiome can both directly metabolise drugs and modulate the host's hepatic metabolism. In the context of clozapine metabolism, these insights are particularly relevant. Given that clozapine is metabolised by CYP enzymes, understanding the gut microbiome's role opens pathways to optimising clozapine therapy by potentially targeting relevant gut microbial populations, thereby improving therapeutic outcomes and minimizing adverse effects.

Possible Existence of Microbial CYP enzymes

Building on the understanding that the microbiome has the ability to influence clozapine metabolism via the gut-liver axis, it also follows that the gut itself may possess enzymes capable of acting directly on clozapine. Zimmermann and colleagues (2019) used a large-scale analysis of publicly available genomic and metabolomics data to show that many gut microbes harbour a variety of enzymes that act on a wide range of structurally diverse xenobiotic compounds. They showed that some of these compounds are preferentially metabolised by specific microbial species within the gut.

While human CYP enzymes are a well characterised set of enzymes, some bacterial species possess enzymes that, while not CYP homologues, have structural and functional similarities to human CYP enzymes (Spanogiannopoulos et al., 2016). Spanogiannopoulos (2016) discussed the functional similarities between microbial enzymes and human CYPs, explaining that, although not directly descended from CYP enzymes, these bacterial systems carry out similar functions and are often structurally similar, with analogous reactive sites.

It's estimated that the collective gut microbiome harbors thousands of distinct bacterial cytochrome P450 genes, with estimates suggesting around 3000 such enzyme (Shayenganpour et al., 2024). These are capable of performing similar reactions to human CYP enzymes, such as oxidation, reduction, hydrolysis, and other biotransformations (Sousa et al., 2008). Notably, work by Sousa and colleagues (2008) characterized CYP51, a microbial sterol 14α-demethylase, which is a bacterial P450 enzyme that performs an oxidative demethylation reaction. This is the same type of chemical transformation required to convert clozapine to norclozapine. This demonstrates that gut microbes possess P450 enzymes capable of the chemical reaction that transforms clozapine into its metabolite, norclozapine.

Notably, these enzymes operate directly within the intestinal environment, making them highly relevant to the initial stages of clozapine absorption and metabolism (Spanogiannopoulos et al., 2016). Spanogiannopoulos and colleagues (2016) studied a specific bacterial enzyme that can perform reductive reactions on a model pharmaceutical. They also highlighted that this bacterial enzyme was active in intestinal conditions, suggesting it is able to perform its activity in a variety of conditions in the intestine. These microbial enzymes are therefore of interest in this study due to their potential contribution to clozapine metabolism.

The diversity and activity of these microbial enzymes vary greatly depending on the composition of individual microbiomes, which is shaped by dietary habits, genetics, and environmental factors, contributing to interindividual variability in drug response (Rothschild et al., 2018; Lozupone et al., 2012). Rothschild and colleagues (2018) showed the dramatic individual variations between human microbiomes, identifying several distinct enterotypes with large differences in taxonomic and functional characteristics. Similarly, Lozupone and colleagues (2012) conducted a comprehensive analysis of human microbiome variation and identified the impact of specific dietary patterns, genetics, and geographical location on individual microbiome composition and function.

Gut Microbiome as Therapeutic Target for Schizophrenia

This potential involvement of the microbiome in the production of this clinically relevant ratio could make the microbiome a target for the improvement of clozapine-response in patients with schizophrenia. Indeed, several randomised controlled trials and metanalyses have been conducted into the use of prebiotics, probiotics and antibiotics in the treatment of schizophrenia, with varying success.

A systematic review and meta-analysis (Minichino et al., 2021) found no evidence of improvement in psychiatric symptoms from certain antibiotics, including D-cycloserine (Buchanan et al., 2007; Duncan et al., 2004; Goff et al., 1999, 2005; Gottlieb et al., 2011; Takiguchi et al., 2017; van Berckle et al., 1999), minocycline (Deakin et al., 2018; Wesier et al., 2019), azithromycin (Dickerson et al., 2009), trimethoprin (Shibre et al., 2010), a probiotic (Dickinson et al., 2014) and also an antiprozoal, artemisinin (Dickerson et al., 2011).

However, this review did highlight some studies that elucidated possible treatment of psychosis, including an antiprotozoal, artemether (Wang et al., 2014), sodium benzoate (Lane et al., 2013) and also combined use of probiotics and Vitamin D (Ghaderi et al., 2019). This treatment involved the use of strains *Lactobacillus rhamnosus* strain GG and *Bifidobacterium animalis* subsp. lactis strain Bb12 and has been evidenced in several other trials to successfully alleviate psychotic symptoms (Jamilian and Ghaderi, 2021; Mohammadi et al., 2024; Mörkl et al., 2020; Mujahid et al., 2022). Other microbial strains, including *Enterococcus* (Wang et al., 2022), *Bacillus coagulans* (Mohammadi et al., 2024) and "BIO-THREE" – a probiotic formulation encompassing *Streptococcus faecesis, Clostridium butyricum,* and *Bacillus mesentericus*, have had a promising benefits on neuropsychiatric symptoms in patients with schizophrenia (Ogawa et al., 2020).

Whilst these reviews highlight potential psychiatric benefits to targeting the gut microbiome, the authors also emphasise the need for more robust research into better understanding the role of the gut microbiome in this area.

Association Between Gut Microbiota and Schizophrenia

Given the potential for microbiome based treatments, it is worth exploring the relationship between microbiome composition and the occurrence of schizophrenia, to further understand the connection between the microbiome and clozapine efficacy.

Schwarz and colleagues (2018) conducted a study on patients with first-episode psychosis and matched healthy controls, to determine any microbial influences on clinical outcomes. They assessed clinical outcomes using the Brief Psychiatric Rating Scale — Extended (BPRS-E) and the Scale for the Assessment of Negative Symptoms (SANS). They also took faeces of all the participants and used quantitative PCR (qPCR) and metagenomics to assess microbial taxa. They found a positive correlation between increased levels of *Lactobacillus*, from the *Bacillota* phylum, and poorer clinical outcomes in patients with first-episode psychosis compared to their controls.

Vasileva and colleagues (2024) also conducted a study exploring the associations between the gut microbiome and treatment-resistant schizophrenia. The study involved a sample size of 97 adults, aged between 20 and 63, divided into four groups: control individuals without a psychiatric diagnosis, individuals with treatment-responsive schizophrenia taking non-clozapine antipsychotic medications, clozapine-responsive individuals with treatment-resistant schizophrenia, and clozapine-nonresponsive individuals with treatment-resistant schizophrenia. The researchers employed shotgun metagenomics to analyse faeces, providing a comprehensive examination of the gut microbiome at both taxonomic and functional levels. This examined the associations between microbiome composition and schizophrenia, treatment resistance, and treatment-related adverse effects, while adjusting for demographic and lifestyle factors with the matched healthy controls. The study found that patients with treatment-resistant schizophrenia had a higher prevalence of metabolic syndrome and constipation compared to healthy controls. This condition was associated with specific bacterial genera, such as *Bacteroides* and Bacillota. Additionally, the study identified correlations between certain bacterial taxa and clozapine response versus non-response. Specifically, higher levels of certain genera within the Bacillota phylum were correlated with clozapine response, while higher levels of specific genera within the Bacteroidetes phylum were associated with clozapine non-response.

These findings suggest that the composition of the gut microbiome may play a significant role in the clinical outcomes and treatment responses of patients with schizophrenia to clozapine administration. This provides valuable insights into the potential mechanisms underlying treatment resistance and adverse effects in schizophrenia. Thus, establishing an association between the microbiome and CNR production could complete the clinical picture.

Investigating Microbiome-derived Metabolism

To truly assess the contributions of the microbiome on clozapine metabolism, a robust in vitro model must be implemented.

The gut microbiota can be modelled in several ways, including simple in vitro batch laboratory set-ups, complex computational simulators, and in vivo animal models. In vitro models are gaining popularity for studying the ecology and function of the gut microbiota due to their tight controllability and reproducibility (Biagini et al., 2023). Unlike in vivo models, in vitro methods reduce ethical concerns and allow for more precise isolation of microbial interactions, free from host variables (El Houari et al., 2022). Additionally, faeces sampling is a minimally invasive technique validated as a proxy for the colonic microbiome (Moysidou, 2020). These in vitro models can be configured in either batch or continuous culture systems.

Continuous systems are engineered to more closely replicate physiological conditions than batch systems, providing a better representation of gut microbial communities, maintaining stability over extended periods, and mimicking the conditions of different parts of the intestine (Venema et al., 2013). A notable example of a continuous culture system is the Robogut, created by McDonald and colleagues (2011), which is composed of a single-stage bioreactor system designed to replicate the conditions of the distal human colon. It is used to culture whole microbial communities derived from faeces or colonic biopsies. The protocols include methods

for creating culture medium formulations, building, running, and sampling the bioreactor apparatus, as well as cleaning and maintenance procedures. This system allows for a detailed evaluation of gut microbial community ecology and function under controlled conditions, providing valuable insights into microbiome-derived metabolism.

Contrastly, batch systems are simpler, require less space, resources, and training, can be quickly set up, and handle multiple samples simultaneously (Coles et al., 2005; Ludwig et al., 2013). Coles and colleagues (2005) utilised these benefits of the batch system in their fermentation of dietary nutrients to model digestion. Ludwig and colleagues (2013) also simulated colonic conditions using a batch system, which allowed for controlled incubation of human faeces with coffee compounds, enabling the identification and quantification of microbial catabolites. This method provided insights into how gut microbiota metabolises dietary compounds and a similar technique could be used to evaluate how gut microbiota can metabolise oral medications, like clozapine.

Key *In-Vitro* Studies of Microbiome-Derived Drug Metabolism

Studies by Zimmerman (2019b) and Javdan (2020) are key examples of such *in vitro* batch models that have mapped human microbiome-derived alterations to oral medications. Both demonstrated that many drugs could be metabolised by human gut bacteria, be that by culturing specific strains (Zimmerman et al., 2019b) or culturing faeces (Javdan et al., 2020). Zimmerman and colleagues (2019) assessed the capacity of 76 bacterial strains, representing the major phyla of the human gut microbiome (Figure 2), to chemically modify medical drugs in vitro. These bacterial gut isolates were incubated with 271 drugs spanning a wide range of clinical applications, and the drug concentrations were measured over 12 hours. The results showed that the levels of two-thirds (176) of the assayed drugs were reduced by over 20%, by at least one bacterial strain. Additionally, it was found that each strain was capable of metabolising between 11 to 95 different drugs (see Figure 2).

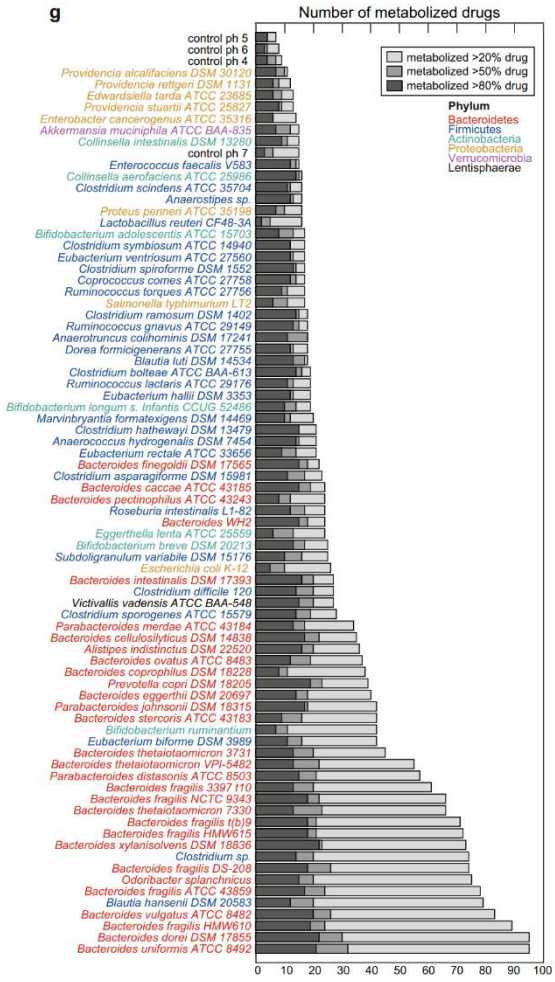


Figure 2. Bar graph displaying bacterial strains and drug metabolism levels. by phylum. Strains are categorised by phlum (Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Verrucomicrobia, and Lentisphaerae) and the bar graph shows the number of drugs metabolised by each strain. The bars are colour-coded to indicate the percentage of drug metabolised: light grey for >20%, medium grey for >50%, and dark grey for >80%. Also shown are no-bacteria controls, buffered to pH 4–6, controlling for acidification of the culture medium. Taken from Zimmerman et al, (2019b).

Javdan and Colleagues

Similarly to Zimmerman, Javdan and colleagues (2020) conducted several experiments in order to map the microbiome-drug relationship. They explored 575 drugs, and 438 of these were suitable for analysis on one healthy donor's microbiome – the pilot donor (see Figure 3).

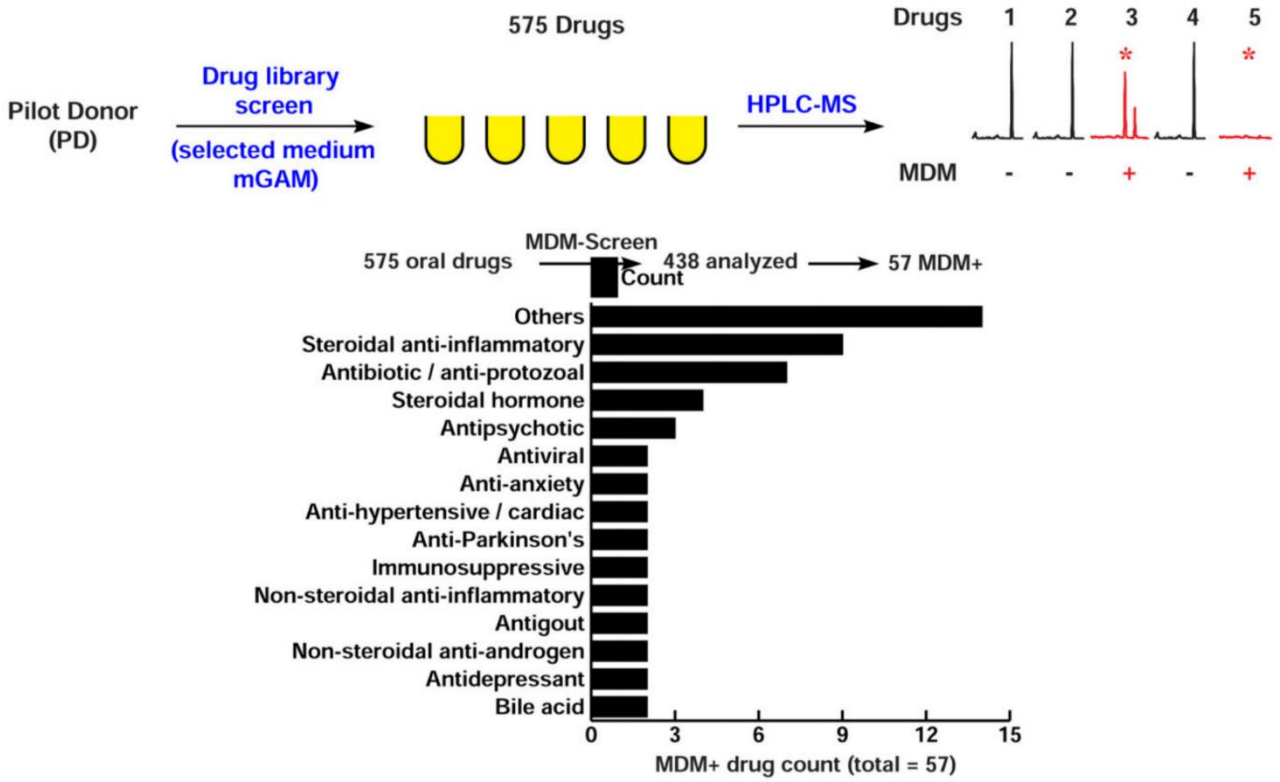


Figure 3: Outlining the initial drug screen process and results. 575 drugs were evaluated on a culture of one healthy pilot donor grown in modified Gifu Anaerobic Medium (mGAM). After inoculation and incubation with the drug, samples are analysed via High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) to identify microbiome-derived metabolism (MDM). Drugs were categorised as either MDM–, the levels were unchanged after the incubation, or MDM+, either the levels had decreased after the incubation or a new metabolite was detected. A bar graph shows the classification of the 57 MDM+ drugs, defined by drug action. A separate classification, "other" defines 14 drugs from actions additional to those listed. Taken from Javdan et al., (2020).

They discovered that 57 of the 438 drugs showed some change after incubation with the donor's faeces culture – either a drug metabolite was detected (Figure 3, drug 3) or the drug itself was no longer detectable (Figure 3, drug 5).

Additionally, they tested 23 drugs on the microbiome of 20 donors (see Figure 4). They had three control conditions – one consisting of the original donor culture incubated with vehicle control, DMSO, one with an equal volume of sterile medium incubated with the drug tested and one with one of the donor's microbiome that was heat-killed and incubated with the drug tested (Figure 4). After this, they performed taxonomic analyses to classify the bacterial species present and metabolomic analyses to identify the metabolic products.

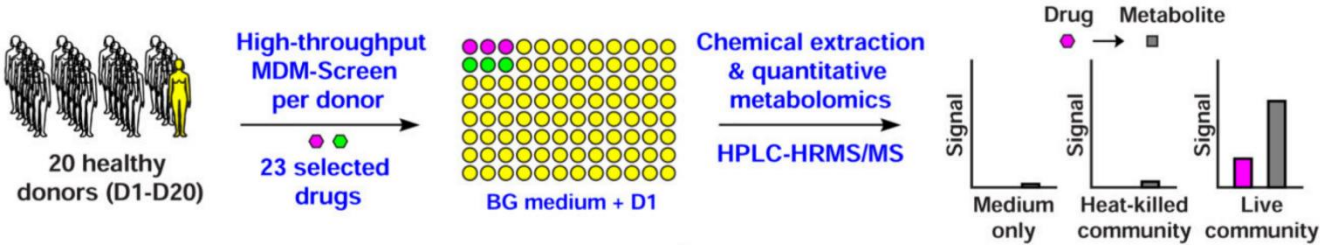


Figure 4. Workflow for high-throughput microbiome-drug metabolism (MDM) screening across 20 healthy donors (D1-D20). Each donor's microbiome is cultured in media, made of a mixture of Bryant and Burkey and modified Gifu anaerobic medium (BG), and is then inoculated with 23 selected drugs and incubated for 24 hours. Once complete, the samples are prepared for analysis by undergoing chemical extraction. The analysis involves quantitative metabolomics via High-Performance Liquid Chromatography with High-Resolution Mass Spectrometry/Mass Spectrometry (HPLC-HRMS/MS). The output of the experimental conditions will be compared to medium-only controls and heat-killed microbial controls. The expected results are shown. Taken from Javdan et al., (2020).

They conducted investigations on specific drug transformations; for example, deglycosylation of a group of chemotherapeutic drugs known as fluoropyrimidines. These include capecitabine, doxifluridine, thymidine phosphorylase (TP) and uridine phosphorylase (UP) and trifluridine. These drugs all showed MDM, although only TP and UP underwent full deglycosylation into the active pharmaceutical agent, 5-fluorouracil. Javdan and colleagues conducted further tests to elucidate the role of MDM in deglycosylation of capecitabine into its metabolite deglycocapecitabine, using both *in vitro* and *in vivo* methods. The former involved culturing the particular strain identified in the taxonomic screen – *Bifidobacterium* sp., and measuring its ability to metabolise capecitabine. The *in vivo* experiments involved colonisation of mice with the pilot donor's microbiome and comparing its metabolism of a drug to that of germ-free mice. They confirmed that MDM has a role in capecitabine deglycosylation, as deglycocapecitabine was readily found in faeces of the colonized mice, but not germ-free.

The 20 donors showed significant variability in microbiome-derived metabolite alterations for the 23 drugs. While some drugs were altered by only a single donor culture, others were modified by almost all donor cultures. To quantify this variability, they calculated the Shannon entropy (base 2) of the distribution of metabolisers and non-metabolisers, referred to as Hv. An Hv of 1 signified that half of

the donors metabolise the drug, while an Hv of 0 indicated that the drug is either metabolised by all the donors or metabolised by none of the donors. For example, with digoxin, 3 out of 20 donors produced the metabolite dihydrodigoxin in significant amounts, so it was given a Hv of 0.61. The entropy for every donor and drug can be seen in Figure 5, along with a more detailed representation of the MDM inter-individual variability for specific drugs is also shown. These results demonstrate that the screening method can quantitatively assess inter-individual variability in microbiome-derived metabolism under consistent *ex vivo* conditions.

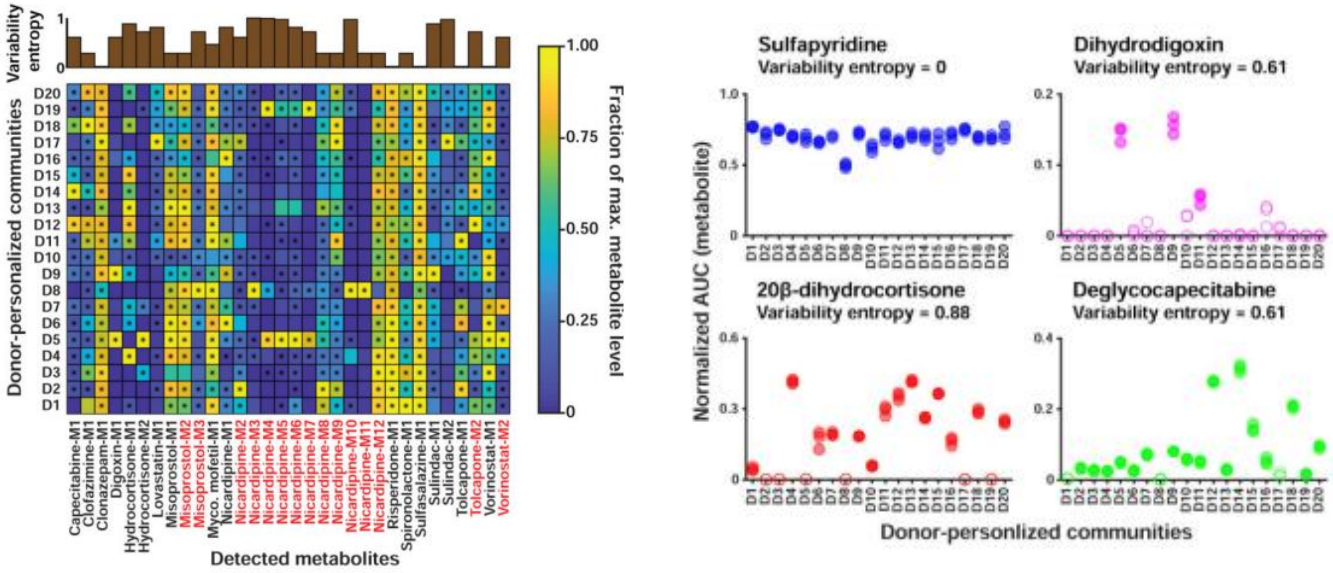


Figure 5. Variability in Metabolite Levels Across Donor-Personalized Communities. A heatmap displays proportions of metabolite levels produced after 24 hours of incubation with each of the 20 donor cultures. A "*" indicates statistical significant production of metabolite, in comparison to controls. The metabolites are in two categories; either previously identified using the initial MDM screen with the pilot donor (black) or not (red). A bargraph displays the entropy of each metabolite. A value of 0 indicates that either every donor or none of the donors produced significant amounts of metabolite, whilst the maximal value of 1 indicates that half the donors produced significant amounts of metabilite. Four scatter plots show four metabolites, Sulfapyridine, Dihydrodigoxin, 20β-Dihydrocortisone, and Deglycocapecitabine, and the levels produced by each donor – quantified as area under the curve (AUC), normalised to an internal standard. Filled data points show metabolite levels that are significantly higher than control samples, whilst hollow points indicate nonsignificant differences. Taken from Javdan et al., (2020).

Within the drugs that showed microbiome-induced alteration were several psychotropics (see Figure 3), including antipsychotic risperidone and anxiolytic clonazepam. Risperidone was hydrolysed into its metabolite, 9-hydroxyrisperidone, which has been evidenced to be more psychiatrically active (Spina et al., 2001), demonstrating the beneficial role of the microbiome in drug metabolism. The microbial-derived nitroreduction of clonazepam into its metabolite, amino-clonazepam, confirms the findings from Zimmerman (2019a). However, this demonstrates an example of where MDM can

have negative side effects, as amino-clonazepam has been associated with intenstinal toxicity in animal models (Zimmerman et al., 2019a).

These experimental techniques conducted by Javdan and Zimmerman serve as a useful model for investigating the role of the human gut microbiome in the metabolism of clozapine, which could provide useful insights into the treatment of schizophrenia.

Methodology Optimisation

Whilst Javdan and colleagues (2020) provided a useful model of microbiome-induced drug alterations, additional protocol could be implemented to optimise this investigation.

Test of Metabolic Activity

It was also noted that a test of metabolic activity of the faeces cultures may need to be included, in order to confirm that the faeces cultures were viable *in vitro*, such that they would demonstrate MDM. There are few validated protocols that test the metabolic activity of anaerobic bacteria, as many assays require oxygen as an indicator of metabolism, such as assays using tetrazolium reduction or resazurin (Braissant et al., 2020). These other assays also have long processing times and high costs. The fluorescein diacetate (FDA) hydrolysis assay has long been established as a measure of anaerobic microbial activity, particularly in sediment environments (Swisher and Carroll, 1980). This assay measures the enzymatic activity of microbial populations by quantifying the fluorescence produced upon FDA cleavage (Braissant et al., 2020). This assay is widely used in literature due to its sensitivity to various enzyme classes, including lipases, esterases, and proteases (Braissant et al., 2020). It also has higher sensitivity and less toxicity than other assays, making it a suitable option for testing microbial activity of faeces. Whilst protocol vary, studies consistently combine FDA with an organic solvent such as acetone, chloroform or methanol, due to low water solubility of FDA (Braissant et al., 2020). Due to more hazardous nature of chloroform, acetone was chosen for this dilution. This mixture of FDA and solvent is added to the sample being tested to start the assay, followed by addition of a buffer, usually 60 mM phosphate buffer to the sample. This solution is incubated, with literature quoting varying periods, from 20 minutes (Adam and Duncan, 2001) to 3 hours (Green et al., 2006). Excess organic solvent is used to stop the assay reaction. Acetone was chosen for this. After extraction, the fluorescein amount is quantified by measuring either fluorescence or absorbance. Fluorescein absorbs light at a wavelength of 490 nanometres, at a strength of around 67,000 and 79,000 $cm^{-1} \cdot M^{-1}$ (Braissant et al., 2020). This wavelength will be used to compare the absorbance of the other samples to give a readout of their absorbance, as a proxy for enzymatic activity and therefore metabolic activity.

Both a positive and negative control will be required for this assay, to confirm that the assay is indeed detecting the metabolic activity of the bacteria, and another factor was not influencing the absorbance readout. The killed-bacterial samples were used as a negative control, as well as also to confirm their lack of metabolic activity, which would validate the use of these samples as a control in the final experiment. A positive control will require a cultured bacteria that is known to be metabolically active for the duration of the FDA assay. Members of the *Clostridium* genera may be suitable positive controls for this assay, as these species are known to be members of the human gut microbiota (Koopman et al., 2022), and tend to display exponential growth up to 12 hours (Roder and Athan, 2020; Walker, 2012). *Clostridium innocuum* (Ci) will therefore be included in the tests of metabolic activity as a positive control.

Once the metabolic ability of each sample has been quantified, using fluorescence as a proxy, the samples can be normalised. This can be done by using the sample that fluoresced the least as a benchmark for the dilution of other samples. To further normalise the samples, the samples will be weighed in their tubes before culturing. Once the faeces sample has been removed for culturing, the empty tubes will then be weighed.

Additional Analysis Timepoint

In Javdan's study (2020) faeces were cultured in an incubator for 24 hours to provide sufficient time for biomass growth. The cultures were then inoculated with drug or vehicle control for a further 24 hours. Studies of clozapine excretion indicate that clozapine should be fully excreted at this time, quoting a half life of 8 hours after a single dose of clozapine (Keck and McElroy, 2002). Although, it must be noted that due to its varying and complex pharmacokinetic profile, average half life of clozapine can vary between individuals, and this can change depending on dose and duration of treatment. It was decided to include an 8 hour timepoint for investigating clozapine and metabolite levels, as well as the 24 hour time point that Javdan used. This extra timepoint may provide a clear insight of the changes in levels of clozapine and norclozapine over time, elucidating any patterns or changes in rate that may be missed with a longer interval.

Metabolite Analysis

Metabonomics is the study of small-molecule metabolites within a complex sample (Renwick et al., 2021) that are typically detected by Nuclear Magnetic Resonance (NMR) spectroscopy or mass spectrometry (MS). MS is often combined with liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE). MS detects variations in chemical environment via measuring their frequency of the effect on the electron field and producing spectra to show the various

compounds, indicated by their molecular weight (Renwick et al., 2021). The GC produces chromatograms which show the time at which the compound was eluted from the chamber and compounds can be quantified by measuring the area under the peak on the chromatogram (Renwick et al., 2021). MS has superior sensitivity to NMR (Aldridge and Rhee, 2014) but quantification proves more difficult due to the fragmentation of molecules that can occur during ionization (Renwick et al., 2021).

GC-MS has also successfully identified clozapine and norclozapine (Bondesson and Lindström, 1988; Cabarcos-Fernandez et al., 2022; Jenison et al., 1995; Thorup and Fog, 1977), encouraging adoption of this analytical technique. Relying on the limited literature of using GC-MS to detect clozapine and its metabolites, various methodologies will be compared for success of detection of clozapine and norclozapine metabolites. To be successfully analysed by GC-MS, samples must undergo extraction followed by derivatisation. Extraction involves adding reagents that will isolate the desired analyte from the sample (Bonchev et al., 2018), in this case, clozapine and norclozapine. This involves mixing with an organic solvent to dissolve the analyte to separate the organic and aqueous phases. MTBE (Methyl tert-Butyl Ether) is often used as a solvent for this purpose (Eggers and Schwudke, 2016). Derivatisation involves increasing the volatility of the sample so that it can be detected by the GC-MS. This involves adding derivatizing agents that target specific chemical groups from the sample, typically those that perform silylation (removal of hydrogen and replacing with – e.g. trimethylsilyl (TMS); Yu et al., 2017) or acylation (addition of acyl group – e.g. trifluoroaetic anhydride (TFAA); Cabarcos-Fernandez et al., 2022).

Current Study

This study aims to investigate clozapine metabolism by mouse and human gut microbiomes. An *in vitro* model will be adopted. It is hypothesised that when faeces are inoculated with clozapine, the bacteria in the samples will alter the clozapine levels. Specifically, it is predicted that levels of clozapine will decrease over time, and levels of its metabolite, norclozapine, will increase. It is predicted that these changes will be statistically significant in comparison to all controls.

Alongside these aims are the following objectives; (1) to develop and validate an *in vitro* mouse model of clozapine metabolism using optimised techniques (Chapter 2), (2) to quantify clozapine metabolism in human faeces using established methods (Chapter 3) and (3) to assess the stability and reproducibility of the human *in vitro* clozapine metabolism methodology by using a repeat analysis (Chapter 4).

Chapter 2:
Optimising the Model Using Mice Samples

Introduction

In the realm of psychopharmacology, precise and efficient methodologies are paramount for advancing our understanding of drug metabolism. This section delves into the optimisation of Javdan's (2020) established methodologies through a multi-faceted approach to enhance the detection and quantification of clozapine and its metabolites.

Mouse Samples

The study will incorporate mouse samples with the goal to provide a robust in vitro model that allows for controlled and reproducible assessment of clozapine metabolism, and the role of the gut microbiome in this. Utilizing mouse samples in vitro will help bridge the gap between in vitro studies and human clinical trials, providing a comprehensive understanding of clozapine's metabolic pathways. Mouse models are extensively used in gut microbiome research due to their genetic similarities to humans and well-documented metabolic processes (Nguyen et al., 2015). Many of the same types of bacteria are present in both species, allowing researchers to study basic microbial functions and interactions (Ley et al., 2005). Additionally, both mouse and human gut microbiomes contain representatives from the same major phyla, such as Firmicutes, Bacteroidetes, and Proteobacteria, which makes comparative studies feasible (Eckburg et al., 2005).

However, despite these similarities, there are notable differences. The species-level diversity between mouse and human gut microbiomes is distinct, (Xiao et al., 2015) and therefore functional differences arise, which influences metabolic processes and disease outcomes (Nguyen et al., 2015).

Optimisation Techniques

To accurately assess Javdan’s methodologies, and address the research question three key areas will be optimised.

Firstly, it will be ensured that the gut microbiome within this *in vitro* model is functional, by utilizing the FDA metabolic test. The fluorescein diacetate (FDA) metabolic test will be implemented to assess the viability and metabolic activity of microbial communities. The FDA test is a crucial component in ensuring that in vitro models accurately reflect the metabolic dynamics present in the gut environment, providing more reliable data on clozapine metabolism.

Secondly, this will optimise gas chromatography-mass spectrometry (GCMS) for the analysis of faeces, which is crucial for improving the sensitivity and specificity of clozapine detection. Faeces present unique challenges due to their complex matrix and the presence of numerous confounding compounds. By fine-tuning the GCMS

parameters and employing advanced sample preparation techniques, the study aims to overcome these challenges and achieve more accurate quantification of clozapine and its metabolites.

When the methodology is optimised, these methods will be implemented using various mouse faeces. This step is essential for confirming the effectiveness of the approach and ensuring that the results are both reliable and reproducible. By comparing the metabolic profiles obtained from mouse samples with those from human studies, the study aims to better understand the translational potential of the findings and their implications for clozapine therapy.

It is hypothesised that bacterial enzymatic activity in mouse faeces is responsible for the conversion of clozapine to norclozapine. It is predicted that the use of TFAA as a derivatization agent, rather than TMS, will result in improved detection of clozapine and norclozapine. Additionally, it is hypothesised that this conversion will be absent in germ-free mouse faeces, due to their lack of microbiome. Findings from these results will subsequently be used to inform subsequent studies of human clozapine metabolism, where we will assess the ability of the human gut microbiome to metabolise clozapine.

Methodology

Sample description

Faeces were collected samples were collected from specific-pathogen-free mice (C57BL/6J wildtype) mice in the Kennedy Institute laboratories. The mice were 10-20 weeks-old and were a mix of male and female. Faeces were collected by human donors on a voluntary, consensual basis from the Oxford University network. To be eligible, volunteers had to be physically well and above 18 years old. All protocol was approved under the ethics committee of Oxford University Medical Research and all scientists involved had undergone relevant training.

Validating the in-vitro model

Assessing validity of heat-killing

The heat-killing method described by Javdan and colleagues (2020) consisted of exposing the cμLtured faeces to 100 °C for 30 minutes. This method was compared to autoclave sterilisation for microbial viability following treatment. Two controls, incubation at room temperature anaerobically and aerobically, were included to ensure the original sample contained viable microbes, and to ensure that aerobic exposure for both heat-killing and autoclave sterilisation was not alone sufficient to kill all microbes in the sample.

Method

Initial preparation of all four conditions was uniform; faeces from three specific-pathogen-free (SPF) mice were removed from a freezer and held on dry ice until they were transferred into a Coy anaerobic chamber

(70% N2, 25% CO2, 5% H2; Coy Laboratory Products, 2024). Each faeces sample was rehydrated anaerobically at room temperature in 450 µL pre-reduced (overnight anaerobic incubation, >18 hours) Brain Heart Infusion broth (Fisher Scientific; BHI) for 5 minutes. Thereafter, pellets were suspended and 50 µL from each suspension was used to inoculate 1 mL of sterile BHI. The 3 inoculated 1 mL BHI samples were then incubated for 24 hours in the anaerobic chamber at 37 °C. After 24 hours, the cultures were removed from the incubator and pooled. The pooled cultures were evenly distributed into four separate treatments, 750 µL. The four treatments were as follows: anaerobic room temperature incubation for 30 minutes (to ensure original sample contained viable cells); aerobic room temperature incubation for 30 minutes (to ensure treatments exposed to oxygen for heat-killing and autoclave sterilisation did not experience entire loss of viability due to oxygen exposure alone); 100 °C exposure for 30 minutes; autoclave sterilisation at 121 °C and 15 pounds per square inch (p.s.i.) for 25 minutes. Following each treatment, samples were imB3HItely returned to the anaerobic chamber. All sample contents were spread plated on Fastidious Anaerobe Agar (Scientific Laboratory Supplies) supplemented with 5% (v/v) defibrinated sheep's blood (EO Laboratories) and incubated anaerobically at 37 °C for 6 days, and were then inspected for visible growth .

Implementing the optimised in-vitro methodology in mice

Byrant and Burkey B3HI (Sigma Aldrich; BB) was mixed in a 70:30 ratio with BHI, reconstituted with double distilled water and autoclaved as per manufacturer's instructions, to form a new B3HI; B3HI. B3HI was reduced overnight at room temperature in the anaerobic chamber. This B3HI was taken to vented hood and 5 mL was pipetted into 4 vented gap tubes. These tubes were taken into the anaerobic coy chamber and left to reduce overnight at room temperature in the anaerobic chamber.

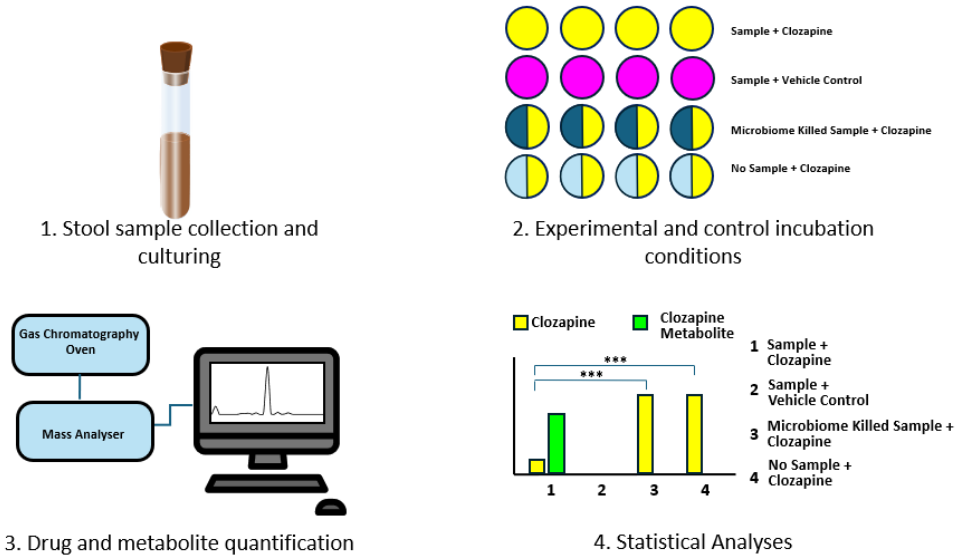


Figure 6. Schematic representation of the *in vitro* experimental protocol used to investigate clozapine metabolism by the gut microbiome. The four-panel schematic illustrates: (1) the initial step of faeces sample collection and anaerobic culturing; (2) the different experimental and control conditions, including: samples incubated with clozapine; samples with a vehicle control, a heat killed microbiome sample incubated with clozapine and a clozapine only sample; (3) the quantification of clozapine and its primary metabolite using

Gas Chromatography-Mass Spectrometry (GC-MS); and (4) the final step of statistically analysing the obtained data using relevant t-tests. The coloured circles in panel 2 depict different components used for each condition, including: sample (blue), clozapine (yellow) and a vehicle control (pink), where the split circles denote heat killed microbiome samples.

Faeces from three SPF mice and three germ-free (GF) mice were retrieved from a freezer on dry ice to the anaerobic chamber. To each faeces sample, 1 mL of medium was added to rehydrate the faeces sample suspended in broth. Each solution was then transferred to a vented cap tube containing 5 mL of media to inoculate. These six inoculated samples were then incubated for 24 hours in the anaerobic chamber at 37 °C. After 24 hours, the cultured samples were removed from the incubator.

Producing Experimental and Control Conditions

To produce the heat-killed-microbiome condition, 67 µL of each cultured sample was taken and pooled into one 1.5 mL microfuge tube and this was repeated in quadruplicates. These four 1.5 mL microfuge tube tubes were removed from the Coy anaerobic chamber and autoclaved. Once completed, these were returned to the anaerobic chamber.

The clozapine and DMSO stock were produced by mixing in a 1:100 (v/v) ratio with B3HI medium. Into each microfuge tube, 400 µL of culture and 15 µL of either clozapine stock or DMSO stock. This was completed in quadruplicates for every mouse culture (see Figure 7). Another set of quadruplicates from each donor was incubated with clozapine for a further 24 hours to quantify the degradation of clozapine over 48 hours.

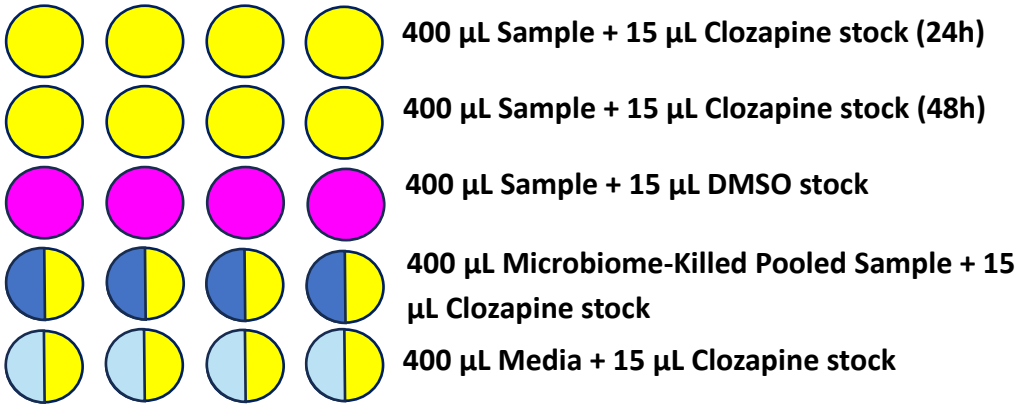


Figure 7. Experimental Setup for Clozapine and DMSO Stock Incubation with Mouse Cultures. This figure shows the setup for incubating mouse cell cultures with 10 µM clozapine and DMSO. Three groups were used: clozapine-treated, DMSO-treated (vehicle control), and untreated controls. Samples were collected at 0, 24, 48, and 72 hours, with additional experiments exploring clozapine concentrations from 1 µM to 100 µM.

Once incubation time was complete, cultures and controls were taken out of the Coy chamber and centrifuged at 16,000 g for 10 minutes, to separate supernatant from pellet. All samples were transferred to a freezer at -

70 °C in the anaerobic chamber at 37 °C to await extraction and analysis via Gas Chromatography-Mass Spectrometry.

Incorporating the test of metabolic activity

To ensure that the bacteria from the faeces were still metabolically active *in vitro* after 24 hours of incubation and after a futher 24 hr incubation with vehicle control or drug, their ability to hydrolyse fluorescein diacetate (FDA) was measured.

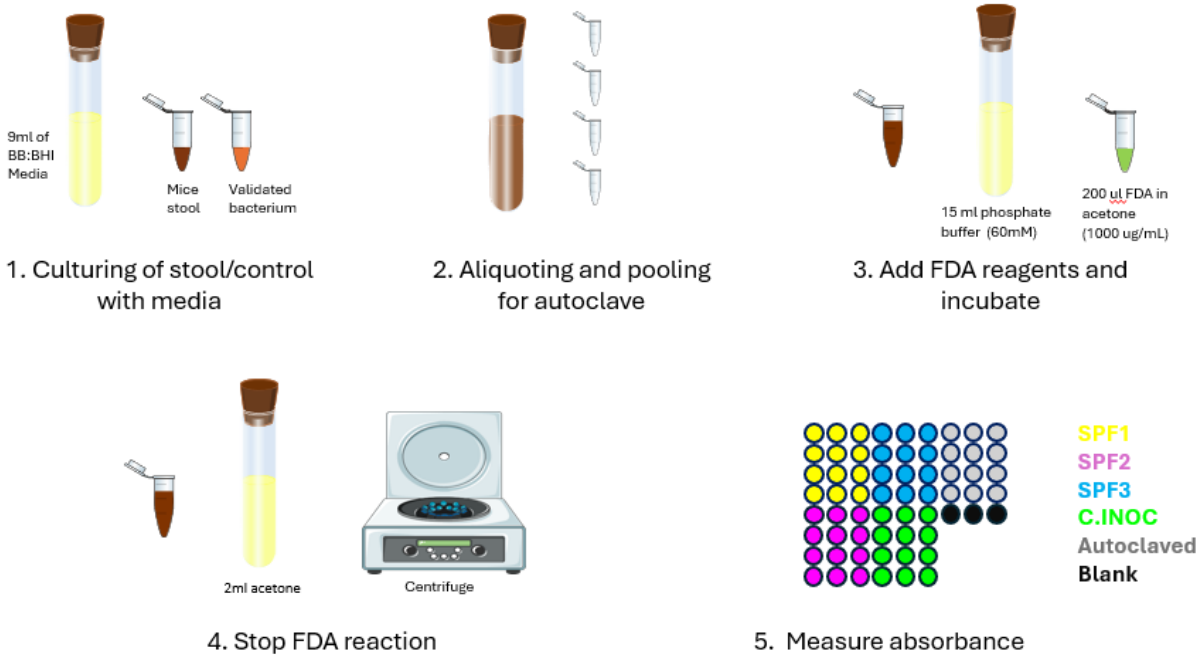


Figure 8. Schematic representation of the experimental protocol for measuring bacterial metabolic activity using Fluorescein Diacetate (FDA).

Initially, *the Clostridium inoculum* (Ci) sample was cultured on a plate of BHI for four hours at 37 °C in the anaerobic chamber. Just before the end of this incubation, three mouse faeces, all SPF Wildtype, were retrieved from the -70 °C freezer and degassed into the chamber. The Ci growth was transferred into a 1.5 mL microfuge tube. To each faeces sample and the Ci sample, 1 mL of previously degassed medium was added to rehydrate the faeces. Each solution was then transferred to a vented cap tube containing 5 mL of media to inoculate. These four vented caps containing inoculated samples were then incubated for 24 hours in the anaerobic chamber at 37 °C.

Approximately 10 mg FDA was weighed and mixed with acetone (100%) in 1:1000 v/v to create a 1000ug / mL solution of FDA in acetone. Phosphate buffer (60 mM, pH 7.6) was prepared according to manufacturer instructions. Briefly, 11.9956 g Na_2HPO_4 and 1.8596 g NaH_2PO_4 were added to 1.767 L distilled water, stirred, and filter sterilised with a Stericup.

Once the 24 hours of incubation was complete, the cultured samples were removed from the incubator. The mouse faecess were pooled into four 50 mL conical tubes, where 167 µL of each of the three vented cap tubes containing a mouse faeces sample was put into each conical tube, and these conical tubes were removed from the chamber to be autoclaved. Whilst these samples were autoclaving, all four vented caps were aliquoted into conical tubes, in quadruplicates of 400 µL. Each vented cap was returned to the incubator for a further 24 hours. Once the four conical tubes had been autoclaved, they were returned to the anaerobic chamber. To every conical tube, 15 mL of 60 mM phosphate buffer and 200 µL of a 1000 µg/mL solution of FDA in acetone was added. They were then put into the incubator for an hour at 37 °C. Subsequently, 2 mL of acetone to each sample. The conical tubes were then centrifuged and were aliquoted into a 96 well plate with triplicate technical repeats, with each well containing 200 µL of each conical tube, as shown in Figure 2. This plate was measured for absorbance via a microplate Reader. This FDA hydrolysis test was repeated on the three mouse samples after the further 24 hours of incubation.

GC/MS Optimisation

We compared reagents for extraction and derivitization by observing their ability to detect our chosen metabolites.

Metabolite Extraction

Samples for GC/MS were retrieved from the -70 °C freezer on dry ice. Once thawed, 200 µL of each sample were pipetted into a 2 mL ube and pure methanol was added (1:1, 200 µL). The samples were homogenized using a bead beater (Precellys 24, Bertin Technologies) for three cycles (6000 g, 30 seconds). Following this, 1 mL of tert-butyl ether (MTBE) was added to each sample and the samples were shaken once more for 30 seconds to extract metabolites. The samples were centrifuged for 30 minutes at 15,000 g and 4 °C. The organic phase from each sample was transferred into a glass vial. The samples were dried using Speed Vac centrifugation (miVac, GeneVac). To the remaining aqueous phase of each sample, 1 mL of methanol was added and then samples were homogenized for one cycle (5000 g, 20 seconds). These samples were put on dry ice for an hour and centrifuged for 30 minutes at 15,000 g and 4 °C to precipitate any proteins. After this, the supernatant was added to the dried organic phase in the glass vials and these were subsequently dried in the Speed Vac.

Chemical derivatization

An internal standard diaminoheptane was spiked into the samples (2.5 µL, 10 mM in acetonitrile; ACN) before resuspending in trifluoroacetic acid (300 µL/ sample) and shaken for 90 minutes at 30 °C and a speed of 1200 g (Bioshake IQ). After this, the lids of the samples were removed and the contents allowed to evaporate in the fume cupboard. To ensure sufficient drying of the samples, they

were put in the Speed Vac. Once dried, 60 µL of ACN) was added to each sample. Samples were vortexed at the highest speed to mix this. The samples were then injected directly for GC/MS.

GC-MS analysis

Samples were analyzed using a GCxGC-MS system comprising of a gas chromatograph coupled to a quadrupole mass spectrometer (Shimadzu GCMS QP2010 ultra) and Shimadzu AOC-20i/s auto sampler. The first dimension separation was carried out on a SHM5MS capillary column (30 m×0.25 mm i.d.×0.25 µm film thickness, Shimadzu) while the second dimension separation was on a BPX-50 capillary column (5 m×0.15 mm i.d.×0.15 µm film thickness, SGE). Helium gas was used as a carrier gas at a 73 psi constant inlet head pressure. The modulation period was set as 4 seconds. The samples were injected at 300 °C in three different split ratios – 1:10 total ion monitoring, 1:10 single ion monitoring (SIM), and one further injection splitless SIM (m/z: 70, 71, 83, 126, 127, 196, 227, 253, 352, 504). The oven temperature was programmed from 80 °C to 320 °C at 40 degrees/min and held at 320 °C for 6 minutes. The interface temperature to the mass spectrometer was set at 330 °C and ion source was heated at 230 °C. The mass spectrometer was operated at scan speed 20,000 amu covering a range of m/z 45-600. Electron Ionization spectra was recorded at 70 eV.

Data processing

Raw GCxGC MS data were processed using GCMSsolution software (v2.72/4.20 Shimadzu. NIST 11/s, OA_TMS, FA_ME and YUTDI in-house libraries were used for data analysis. Differences in metabolite production were measured using area under the graph. For peak picking and peak quantitation using the GCMS Solution software (v4.2), the following parameters were used: Slope: 1000/min, width: 0.04 s, with 0 min areas, drift 0/min and T. DBL: 1000 min without any smoothing methods. Analytes were quantified at the base peaks of m/z 70, 71 and 83 for clozapine, m/z 227, 352, 504 m/z for norclozapine.

Data analysis

Levels of clozapine and norclozapine in all samples will be normalized to the internal standard, d-aminoheptane in order to correct for differences in extraction efficiency.

For assessing the significance of difference in compound levels in media samples to autoclaved samples, donor samples to autoclaved samples and donor samples to media samples, analysis of variance of samples needs to be assessed. For this the F-test is conducted to asses whether they are equal. If significant, the variances in samples are unequal, and thus a two-sample of unequal variance t-test can be performed. When comparing within donor samples, a paired t-test will be used. Significance for these tests is set at 5%.

Optimised GCMS Methodology

Once it was discovered which method was the most sensitive for detecting our compounds of interest, this method was chosen and all the samples (N=72) were analysed by the GC-MS equipment.

RESULTS

Heat killing validation

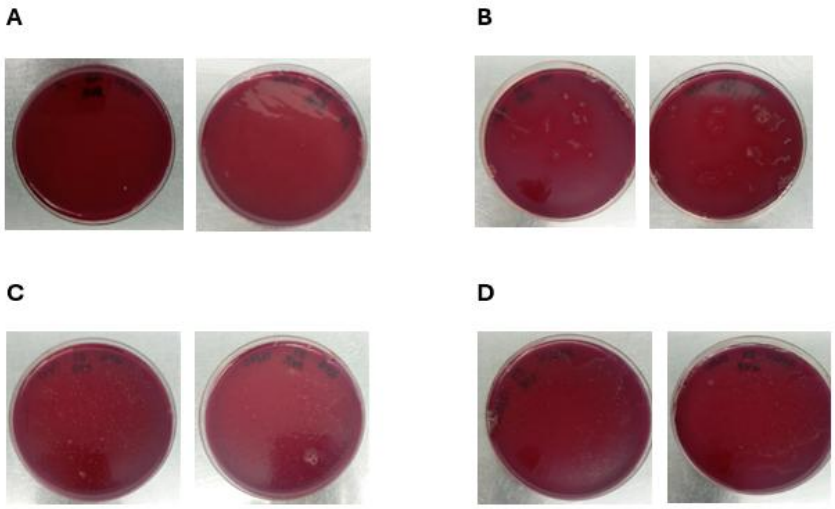


Figure 9. Growth of pooled SPF mouse faeces after treatment of four different conditions and incubation at 37 °C for 6 days. (A) Plates inoculated with culture that was oxygen-exposed during autoclave sterilisation prior to incubation. B) Plates inoculated with culture that was exposed to aerobic conditions and heated to 100 °C for 30 min prior to incubation. C) Plates inoculated with culture that was exposed to aerobic conditions at room temperature for 30 min prior to incubation. D) Plates inoculated with culture that had been in anaerobic conditions at room temperature for 30 min prior to incubation.

Figure 9 shows the growth of SPF mouse faeces after different methods of killing. The plates in Figure 9B and 9C, treated with Javdan's heat-killing method, display abundant growth. The plates in Figure 9D, that were treated anaerobically at room temperature, show no growth. The plates in 9A, that were autoclaved, show one colony formed but overall minimal growth.

FDA Test of Metabolic Activity

This figure shows the results of the FDA test of metabolic activity of mouse faeces compared with controls.

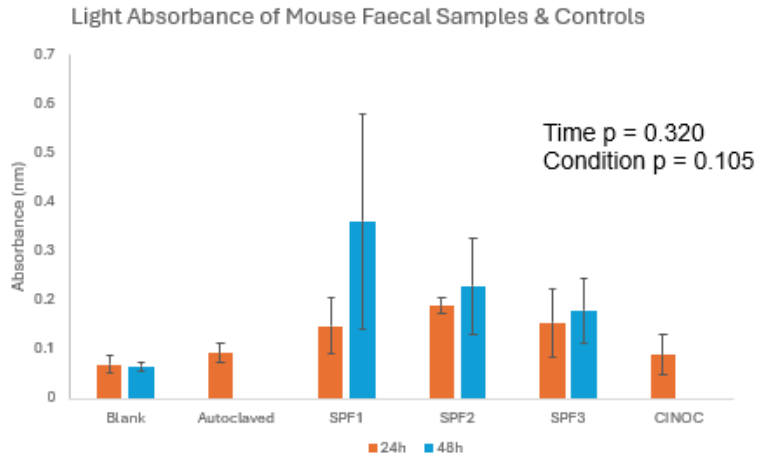


Figure 10. Test of FDA Metabolic Activity. Average absorbance values (nanometres (nm)) of samples from varying conditions, including autoclaved faeces, samples from SPF mouse 1, SPF mouse 2 and SPF mouse 3 and a sample of cultured *Clostridium inoculum*. There is also a blank sample (containing FDA reagents, media, and no faeces). Two timepoints of incubation length are shown for each condition; 24 hours (h) shown in orange, and 48 hours (h) shown in blue – except for autoclaved, which just has a 24 hour incubation. Error bars show the variation of the triplicate samples within each condition, calculated by standard error of the mean. The p values from t tests comparing means of both timepoints (time) and also comparing means of control samples to means of experimental samples (condition) are shown.

The control samples appear lower than the experimental samples, but the t test did not find this difference to be significant ($p = 0.105$). Similarly, time did not seem to affect metabolic activity, with a t test comparing activity at 24 to 48 hours producing a non-significant p value ($p = 0.320$). The sample from Ci was also not significantly different.

GC/MS Optimisation

Figure 11 demonstrates the output of some test samples, prepared using the differing reagents.

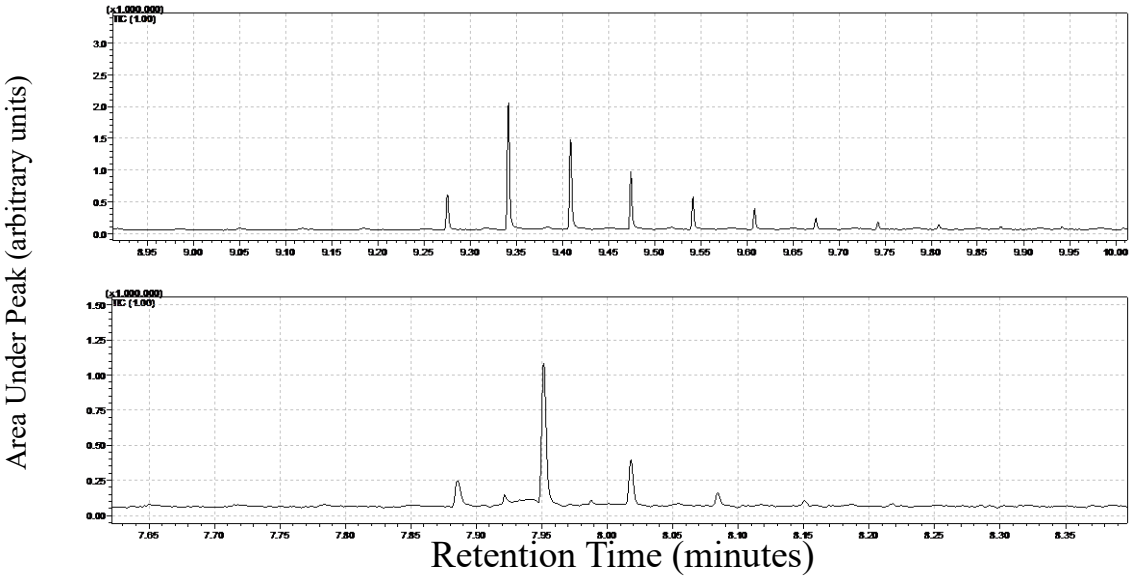


Figure 11. Chromatograms of faeces with clozapine, prepared using (a) TFAA (b) TMS.

From this output, it is evident that TFAA produces a more sensitive detection of clozapine, with a highest peak of 2.0 (absolute intensity), compared to TMS, which produced a highest peak of 1.10.

Figure 12 provides a more quantitative output of the metabolomic result of the two varying reagents.

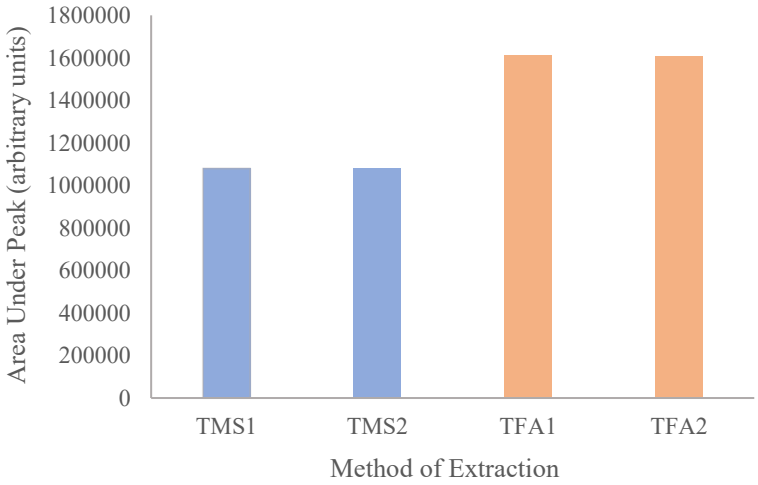


Figure 12. Comparing the detection of metabolite clozapine (A) using TMS and TFAA. Shows area under the graphs in Figure 11.

This bar chart in Figure 12 illustrates the area under the curve (AUC) for different extraction methods (TMS1, TMS2, TFA1, TFA2) used in the detection of clozapine and its metabolites. The AUC values for the TFAA

methods (TMS1 and TMS2) are higher compared to the TMS methods (TFA1 and TFA2), indicating better detection and quantification of clozapine.

Similar to clozapine, the chromatogram for norclozapine shows distinct peaks (Figure 13), and the samples with TFAA added also yielded higher AUCs than the TMS methods (Figure 14).

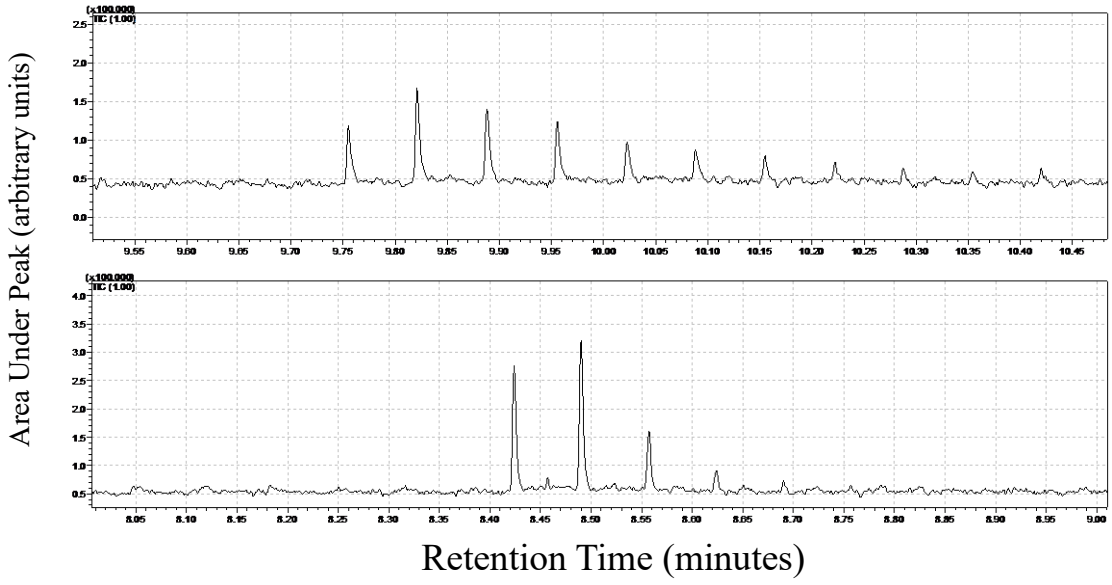


Figure 13. Norclozapine (A) TMS (B) TFAA

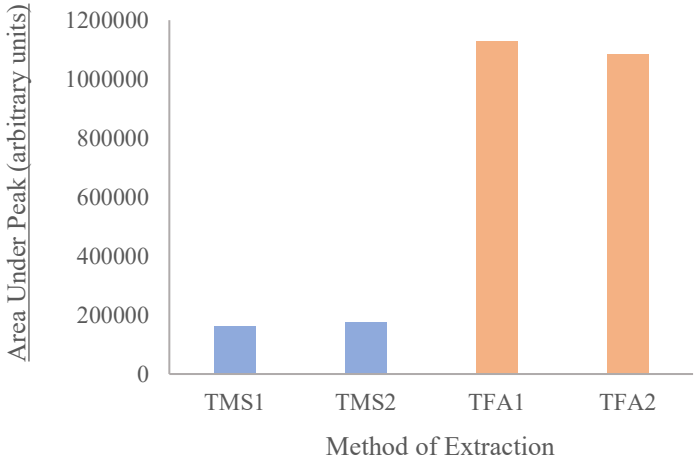


Figure 14. Comparing the detection of metabolite norclozapine (B) using TMS and TFA. Shows area under the graphs in Figure 13.

The bar graph in Figure 15 compares the effectiveness of different extraction methods for clozapine using three solvents: Trifluoroacetic anhydride (TFAA) (A), Methyl tert-butyl ether (MTBE) (B), and Methyl tert-butyl ether with Potassium carbonate (MTBE with K_2CO_3) (C). The methods are categorized as A1, A2, B1, B2, C1, and C2 on the x-axis. The y-axis represents the area under the curve (AUC), with values ranging from 0 to 400,000.

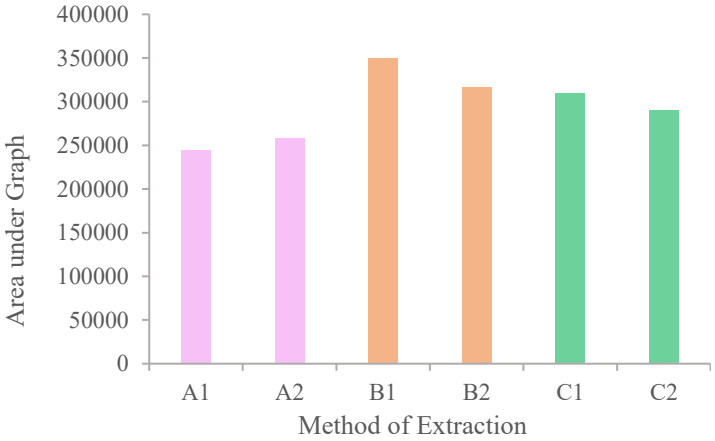


Figure 15. Comparing extraction methods of clozapine along with methanol, using Trifluoroacetic anhydride (TFAA) (A), Methyl tert-butyl ether (B) and MTBE and K2CO3 (C)

The results indicate that both TFAA (B) extraction methods show lower AUC values compared to MTBE (A) and MTBE with K_2CO_3 (C) methods, suggesting that the MTBE and MTBE with K_2CO_3 solvents are more effective at extracting clozapine.

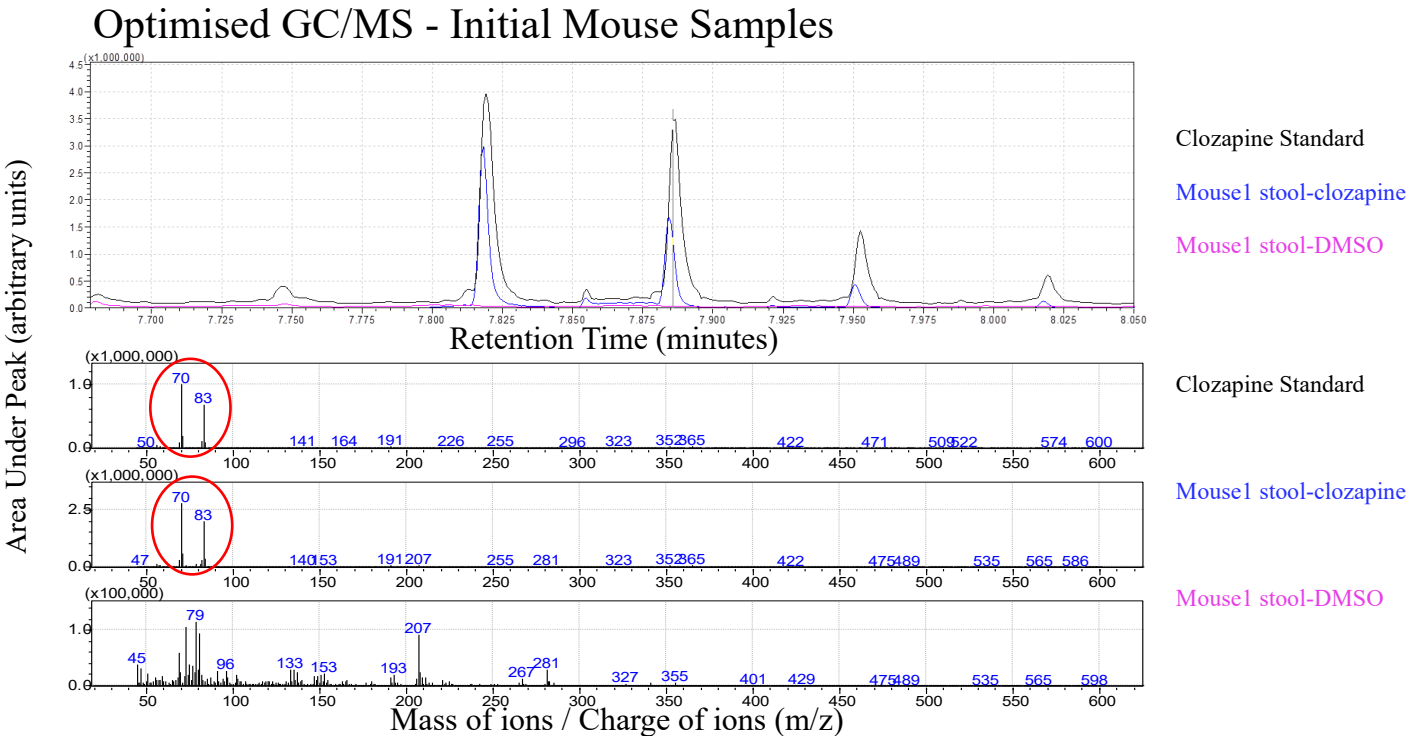


Figure 16. Chromatogram and mass spectra of the clozapine standard, mouse1 faeces with clozapine and mouse1 faeces-DMSO at 7.885 minutes. Red circles denote qualifying ions for clozapine. DMSO = dimethyl sulfoxide.

Figure 16 illustrates the results of Gas Chromatography-Mass Spectrometry (GC/MS) analysis for clozapine, which includes three samples: “Clozapine Standard," in black, "Mouse1 faeces-clozapine,", in blue, and "Mouse 1 faeces-DMSO", in pink. The chromatogram, displays the separation and detection of clozapine and its related compounds. The y-axis represents "Absolute Intensity," and the x-axis denotes "Retention Time (minutes)." The mass spectra of the detected compounds are shown, with both graphs having the y-axis labeled "Absolute Intensity" and the x-axis marked "Mass of ions (m/z)." Both the chromatogram and mass spectra show that the Mouse1 faeces-DMSO sample does not contain clozapine – with no peak being shown in the chromatogram or the mass spectra, using 70, 71 and 83 m/z as quantifying ions, indicated by red circles.

Figure 17 illustrates the results of Gas Chromatography-Mass Spectrometry (GC/MS) analysis for norclozapine, which includes three samples: "Norclozapine Standard," in black, "Mouse1 faeces-clozapine,", in pink, and "Mouse 1 faeces-DMSO", in blue.

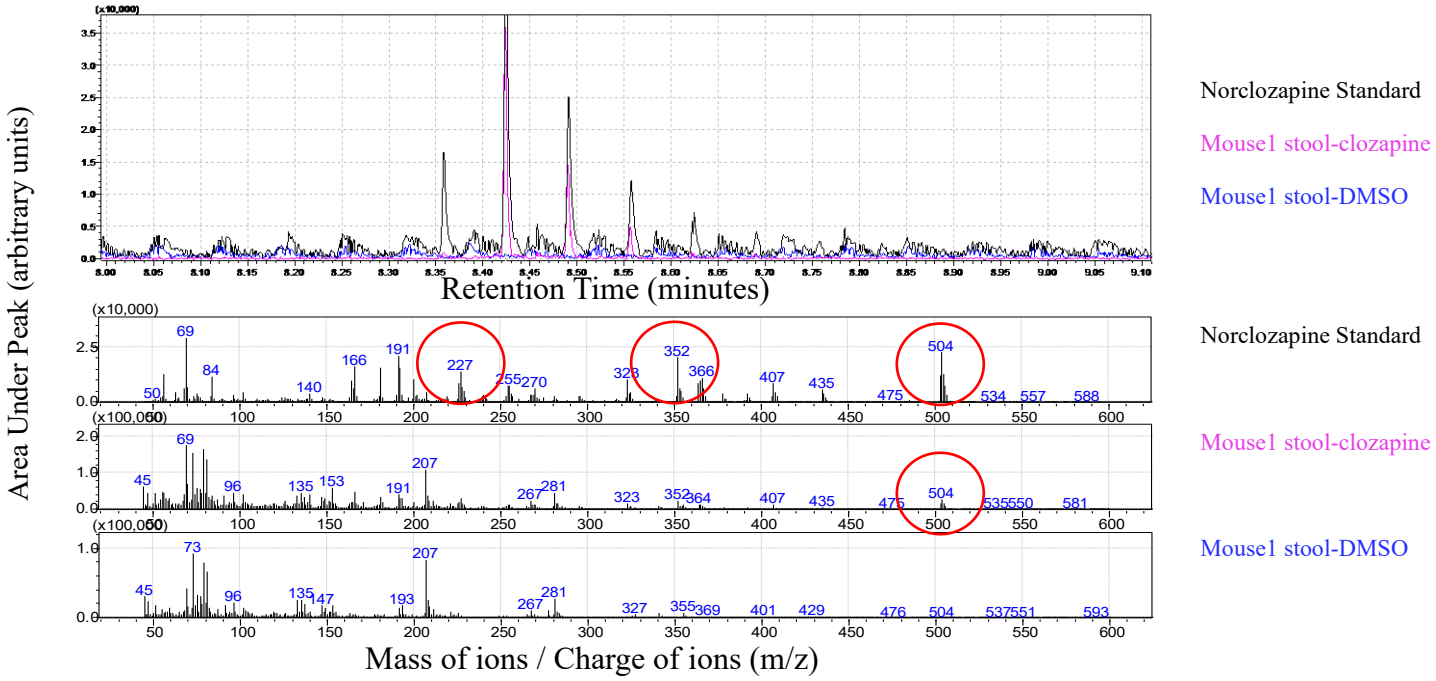


Figure 17. Chromatogram and mass spectra of the norclozapine standard, mouse1 faeces with clozapine and mouse1 faeces-DMSO at 7.885 minutes. Red circles denote qualifying ions for norclozapine. DMSO = dimethyl sulfoxide.

Similarly to Figure 16, both the chromatogram and mass spectra show that the Mouse1 faeces-DMSO sample does not contain norclozapine – with no peak being shown in the chromatogram or the mass spectra, using 227, 352 and 504 m/z as quantifying ions, indicated by red circles.

Optimised GC/MS – Experimental Mouse Samples

SPF1 Mouse

Clozapine

Figure 18 illustrates the results of Gas Chromatography-Mass Spectrometry (GC/MS) analysis for clozapine, which includes three samples: autoclaved mouse faeces with clozapine in black, SPF mouse faeces with clozapine in blue, and SPF mouse faeces with DMSO in pink.

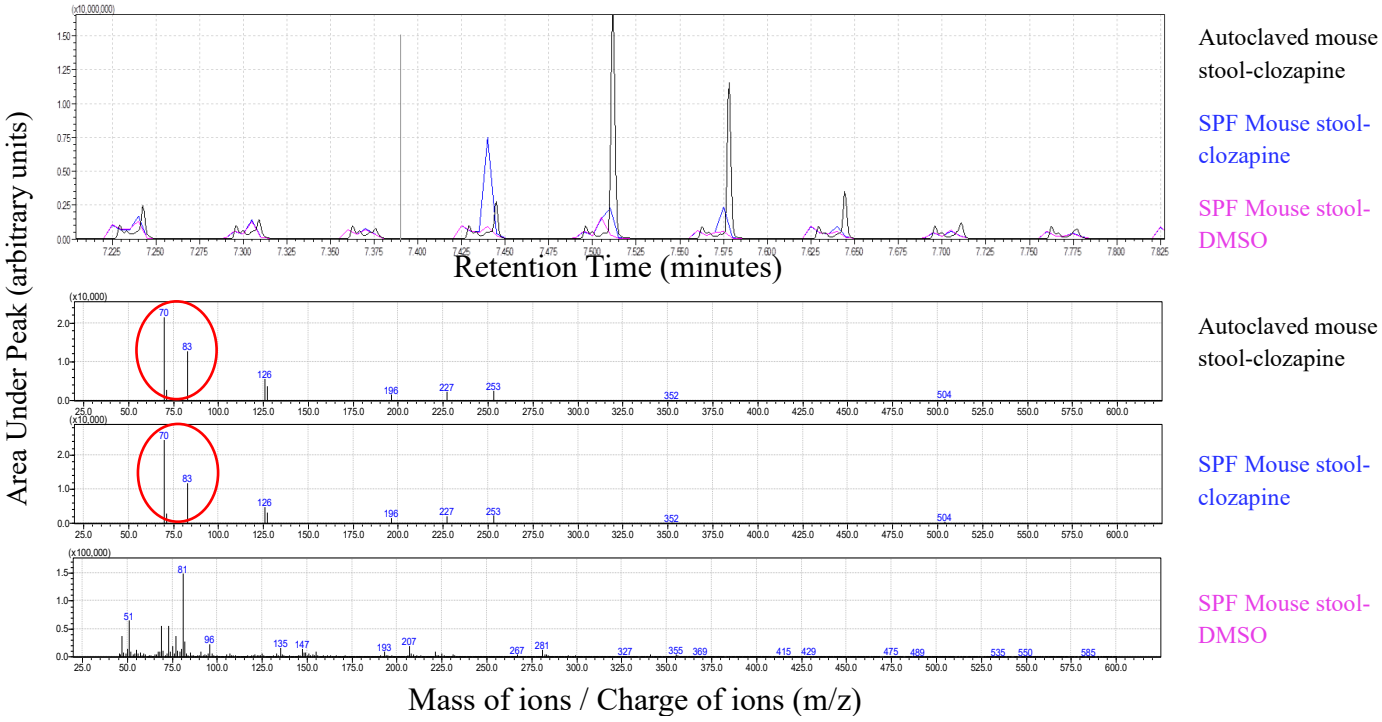


Figure 18. Chromatogram and mass spectra of the clozapine standard, SPF mouse faeces with clozapine and mouse1 faeces-DMSO at 7.515 minutes. Red circles denote qualifying ions for clozapine. SPF = specific pathogen free, DMSO = dimethyl sulfoxide.

Both the chromatogram and mass spectra suggest that the SPF mouse faeces with DMSO sample does not contain clozapine – with no peak being shown in the chromatogram or the mass spectra, using 70, 71 and 83 m/z as quantifying ions, indicated by red circles.

The clozapine levels in the SPF mouse samples incubated with clozapine for were found to have an equal variance to the clozapine levels in the autoclaved mouse faeces sample, incubated with clozapine ($p = 0.0870$) and the two-sample t test of equal variance did not meet significance ($p = 0.31$).

Table 1. Analysis of Equal Variance F-tests and T-tests Reported of Clozapine Levels in SPF Mouse Samples. Results reported as (f test p value, t test p value).

	SPF Mouse - Clozapine	SPF Mouse-DMSO
Autoclave-Clozapine	0.0870, 0.310	x
SPF Mouse-Clozapine	x	0.0167, 0.103

A paired t test could not be conducted on the samples with mouse faeces due to varying group size. Instead, the variance was assessed and the two groups deemed to have unequal variance ($p = 0.0167$). This is also evident from the error bars in the bar graph (Figure 19). The two-sample t test of unequal variance did not meet significance ($p = 0.103$).

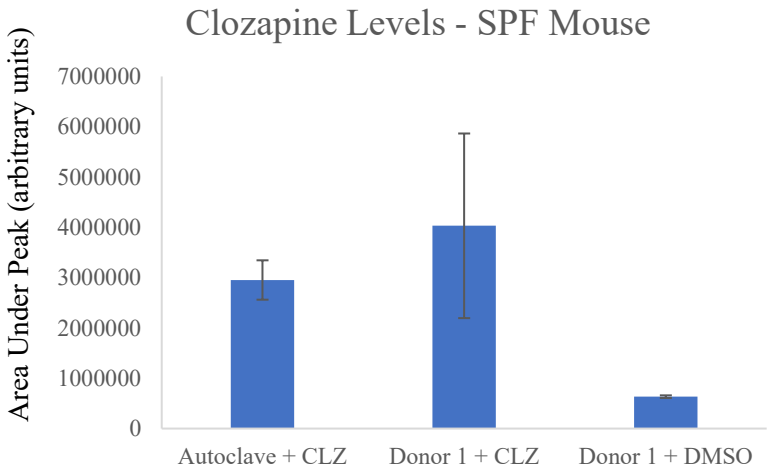


Figure 19. Bar graph displaying the clozapine levels in samples using SPF mouse faeces, including samples with autoclaved mouse faeces incubated with clozapine, mouse faeces incubated with clozapine and mouse faeces incubated with DMSO. Error bars shows standard error of the mean of the variation of triplicate repeats. SPF = specific pathogen free, CLZ = clozapine, DMSO = dimethyl sulfoxide.

Norclozapine

Figure 20 illustrates the results of Gas Chromatography-Mass Spectrometry (GC/MS) analysis for clozapine, which includes three samples: autoclaved mouse faeces with clozapine in black, SPF mouse faeces with clozapine in blue, and SPF mouse faeces with DMSO in pink.

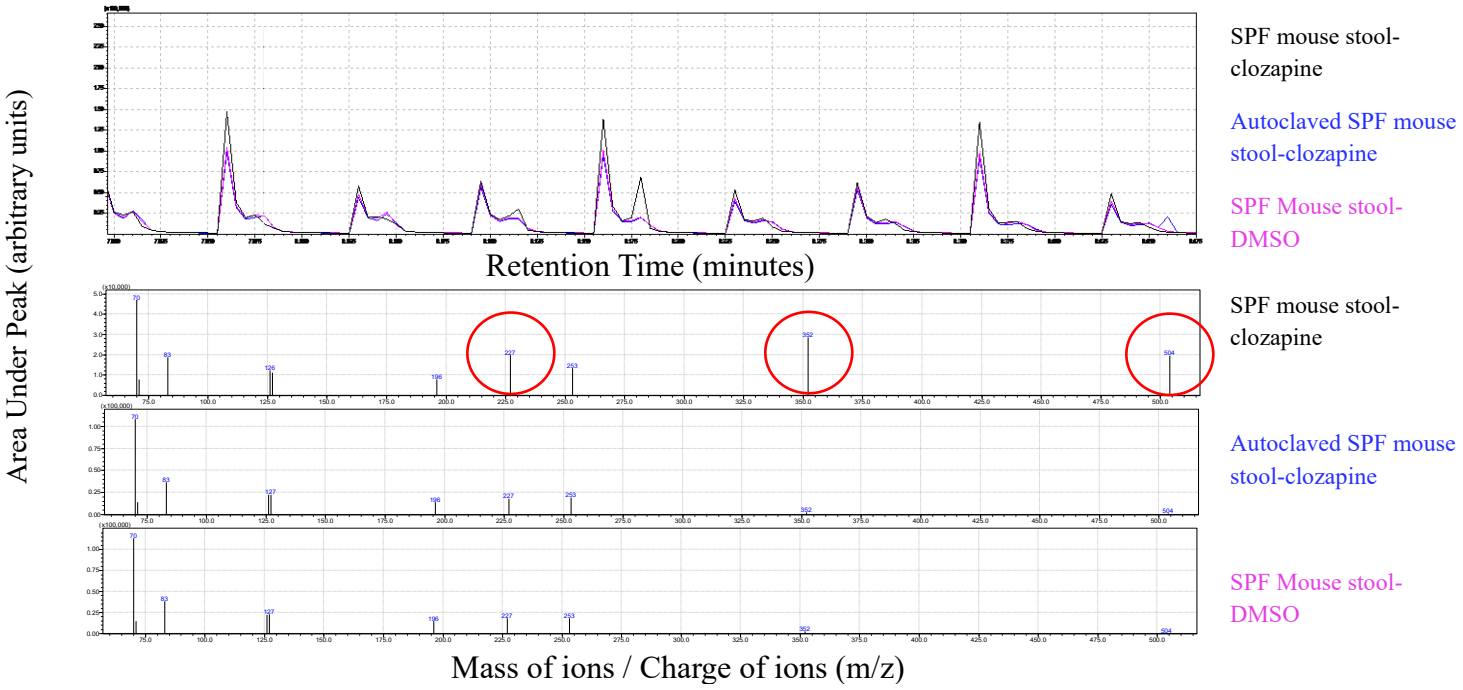


Figure 20. Chromatogram and mass spectra of the norclozapine standard, mouse1 faeces with clozapine and mouse1 faeces-DMSO at 8.180 minutes. Red circles denote qualifying ions for norclozapine. SPF = specific pathogen free, DMSO = dimethyl sulfoxide.

Both the chromatogram and mass spectra suggest that the experimental sample does contain norclozapine, but the autoclaved sample and the SPF mouse faeces with DMSO sample do not, using 227, 352 and 504 m/z as quantifying ions, indicated by red circles.

The norclozapine levels in the SPF mouse samples incubated with clozapine for were found to have an unequal variance to the clozapine levels in the autoclaved mouse faeces sample, incubated with clozapine ($p = 0.00420$). This is also evident from the error bars in the bar graph (Figure 21). The two-sample t test of unequal variance did not meet significance ($p = 0.254$).

Table 2. Analysis of Equal Variance F-tests and T-tests Reported of Norclozapine Levels in SPF Mouse Samples. SPF = specific pathogen free, DMSO = dimethyl sulfoxide. Results reported as (f test p value, t test p value).

	SPF Mouse - Clozapine	**SPF Mouse-DMSO**
Autoclave-Clozapine	0.00420, 0.254	x
SPF Mouse-Clozapine	x	0.0842, 0.276

A paired t test could not be conducted on the samples with mouse faeces due to varying group size. Instead, the variance was assessed and the two groups deemed to have equal variance (p = 0.0842). The two-sample t test of equal variance did not meet significance (p = 0.276).

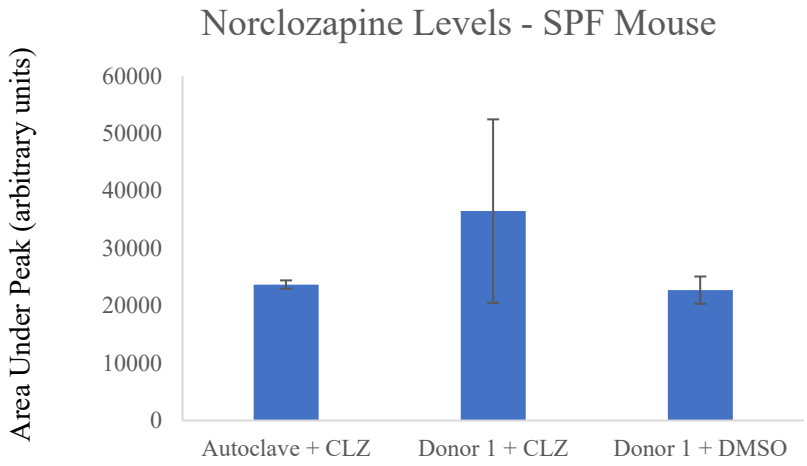


Figure 21. Bar graph displaying the norclozapine levels in samples using SPF mouse faeces, including samples with autoclaved mouse faeces incubated with clozapine, mouse faeces incubated with clozapine and mouse faeces incubated with DMSO. Error bars shows standard error of the mean of the variation of triplicate repeats. SPF = specific pathogen free, CLZ = clozapine, DMSO = dimethyl sulfoxide.

Norclozapine:clozapine Ratio

Figure 22 displays the ratio of norclozapine:clozapine in the SPF mouse samples.

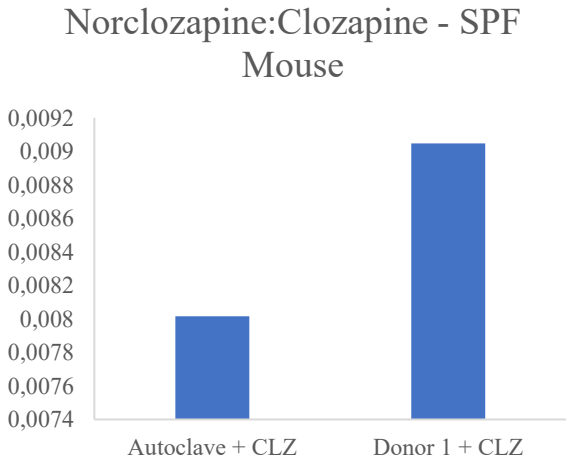


Figure 22. Bar graph displaying the norclozapine:clozapine ratios in samples using SPF mouse faeces, including samples with autoclaved mouse faeces incubated with clozapine, mouse faeces incubated with clozapine and mouse faeces incubated with DMSO. SPF = specific pathogen free, CLZ = clozapine, DMSO = dimethyl sulfoxide.

The bar graph shows that the norclozapine:clozapine ratio is higher in the experimental sample than the autoclaved control.

Germ-Free Mouse

Clozapine

Figure 23 illustrates the results of Gas Chromatography-Mass Spectrometry (GC/MS) analysis for clozapine, which includes three samples: autoclaved mouse faeces with clozapine in black, germ-free (GF) mouse faeces with clozapine in blue, and GF mouse faeces with DMSO in pink.

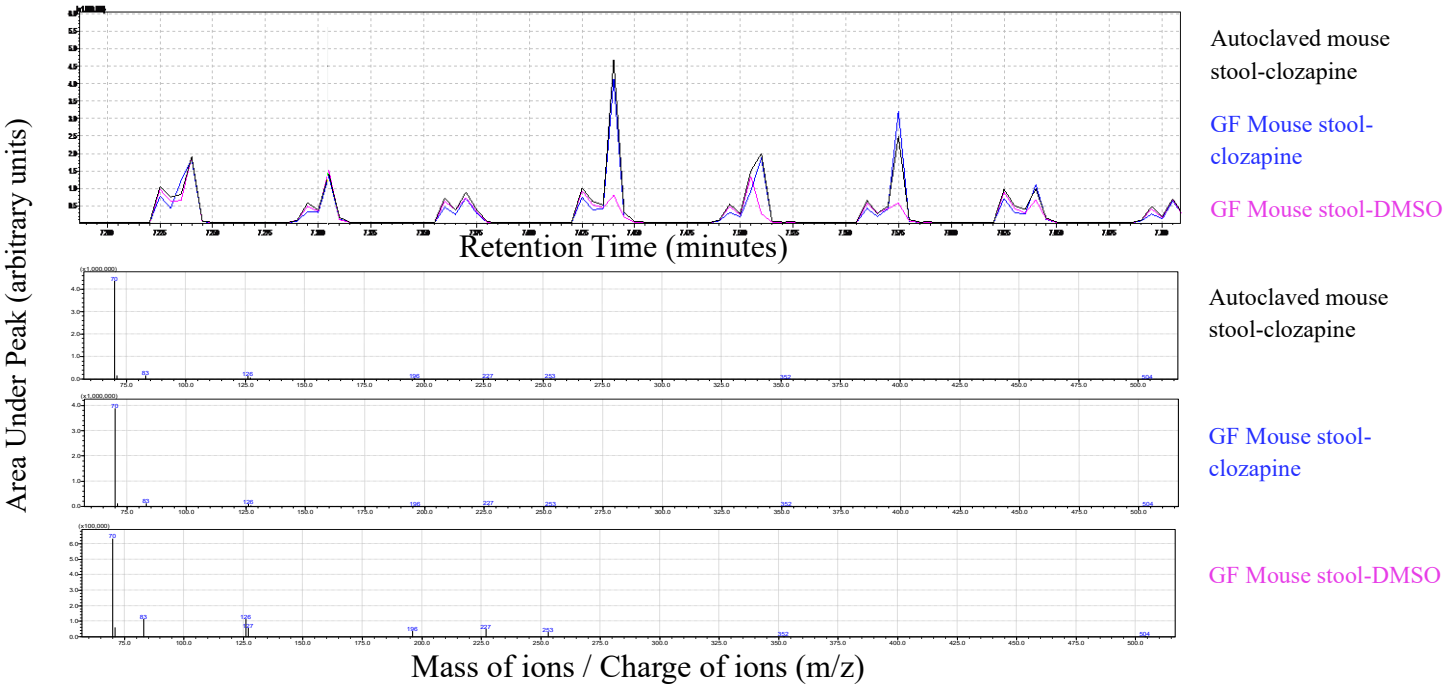


Figure 23. Chromatogram and mass spectra of the clozapine standard, GF mouse faeces with clozapine and GF mouse faeces-DMSO at 7.435 minutes. GF = germ free, DMSO = dimethyl sulfoxide.

The chromatogram and mass spectra suggest that the GF mouse faeces with DMSO sample contains minimal clozapine – with a small peak shown in the chromatogram (pink line), and much larger peaks in the samples incubated with clozapine (black and blue lines). Whilst at first appearance, the mass spectra for all three samples appear to contain peaks for the quantifying ions of clozapine – 70, 71 and 83 m/z as quantifying ions, there are many other ions showing in the spectrum for DMSO (third spectrum), suggesting that clozapine is not present.

The clozapine levels in the GF mouse samples incubated with clozapine for were found to have an equal variance to the clozapine levels in the autoclaved mouse faeces sample, incubated with clozapine ($p = 0.415$), as is evident from the equal sizes of the error bars in the bar graph (see Figure 24). The two-sample t test of equal variance did not meet significance ($p = 0.08$).

Table 3. Analysis of Equal Variance F-tests and T-tests Reported of Clozapine Levels in SPF Mouse Samples. Variance not conducted on paired groups. Results reported as (f test p value, t test p value).

	GF Mouse - Clozapine	GF Mouse-DMSO
Autoclave-Clozapine	0.415, 0.08	x
GF Mouse-Clozapine	x	0.0259

A paired t test on the clozapine levels in the samples with live mouse faeces met significance ($p = 0.0259$); clozapine levels are significantly higher in the GF mouse faeces incubated with clozapine than those incubated with DMSO.

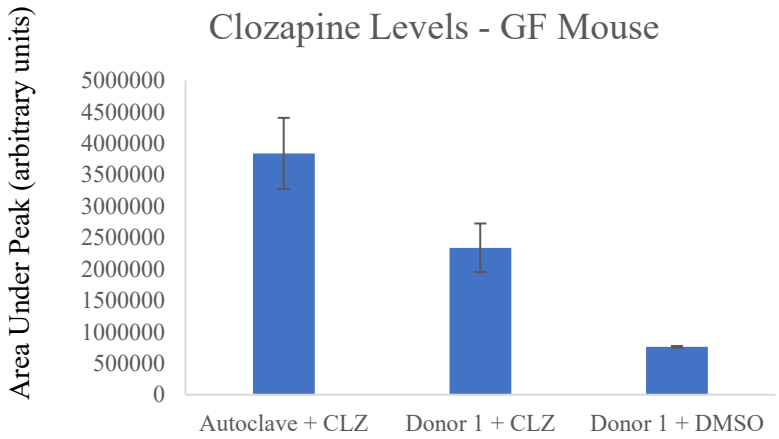


Figure 24. Bar graph displaying the clozapine levels in samples using GF mouse faeces, including samples with autoclaved mouse faeces incubated with clozapine, mouse faeces incubated with clozapine and mouse faeces incubated with DMSO. Error bars shows standard error of the mean of the variation of triplicate repeats. GF = germ free, CLZ = clozapine, DMSO = dimethyl sulfoxide.

Norclozapine

Figure 25 illustrates the results of Gas Chromatography-Mass Spectrometry (GC/MS) analysis for norclozapine, which includes three samples: autoclaved mouse faeces with clozapine in blue, GF mouse faeces with clozapine in black, and GF mouse faeces with DMSO in pink.

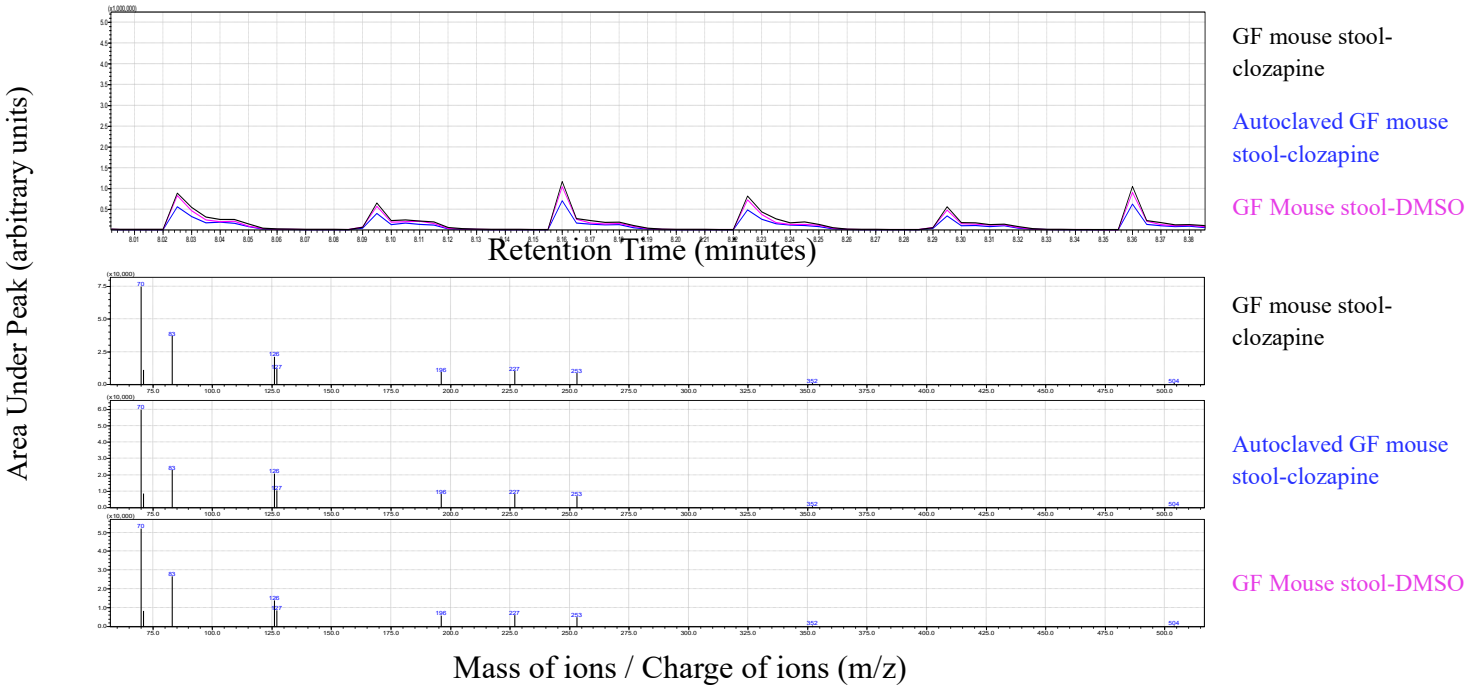


Figure 25. Chromatogram and mass spectra of the norclozapine standard, GF mouse faeces with clozapine and GF mouse faeces-DMSO at 8.180 minutes. GF = germ free, DMSO = dimethyl sulfoxide.

Both the chromatogram and mass spectra suggest that none of the GF samples contain norclozapine, using 227, 352 and 504 m/z as quantifying ions.

The norclozapine levels in the GF mouse samples incubated with clozapine for were found to have an equal variance to the clozapine levels in the autoclaved mouse faeces sample, incubated with clozapine ($p = 0.130$). This is also evident from the error bars in the bar graph (Figure 26). The two-sample t test of equal variance did not meet significance ($p = 0.180$).

Table 4. Analysis of Equal Variance F-tests and T-tests Reported of Norclozapine Levels in GF Mouse Samples. Variance not conducted on paired samples. Results reported as **(f test p value, t test p value)**. GF = germ free, DMSO = dimethyl sulfoxide.

	GF Mouse - Clozapine	**GF Mouse-DMSO**
Autoclave-Clozapine	0.130, 0.180	x
GF Mouse-Clozapine	x	0.437

A paired t test did not meet significance (p = 0.276).

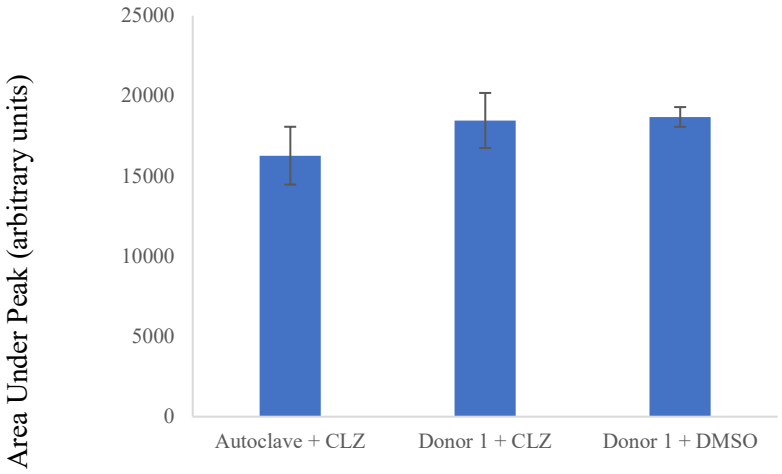


Figure 26. Bar graph displaying the norclozapine levels in samples using GF mouse faeces, including samples with autoclaved mouse faeces incubated with clozapine, mouse faeces incubated with clozapine and mouse faeces incubated with DMSO. Error bars shows standard error of the mean of the variation of triplicate repeats. GF = germ free, CLZ = clozapine, DMSO = dimethyl sulfoxide.

Norclozapine:clozapine Ratio

Figure 27 displays the ratio of norclozapine:clozapine in the SPF mouse samples.

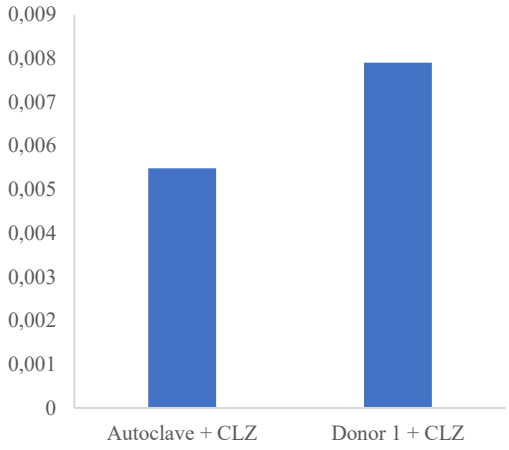


Figure 27. Bar graph displaying the norclozapine:clozapine ratios in samples using GF mouse faeces, including samples with autoclaved mouse faeces incubated with clozapine, mouse faeces incubated with clozapine and mouse faeces incubated with DMSO. GF = germ free, CLZ = clozapine, DMSO = dimethyl sulfoxide.

The bar graph shows that the norclozapine:clozapine ratios are similar in the experimental sample than the autoclaved control.

Discussion

Heat killing

It is evident that the heat-killing method used by Javdan and colleagues (2020) is ineffective for complete gastrointestinal microbial killing as growth was detected on agar plates after incubation. The single colony formation in the autoclaved plates may have been contamination from improper aspectic technique when checking plates for growth. Samples that received this condition should be plated again to confirm loss of viability.

It was expected that Javdan's method would be not completely efficient at killing gastrointestinal microbes as sporulating bacteria have been proven to grow at 100 °C, particularly those from the *Bacillus* genus (Beladjal et al., 2018). Literature shows that up to 50% of human gut bacteria are capable of sporulation (Koopman et al., 2022), particularly those from the *Clostridioides* and *Bacillus* genera.

The addition of the high pressure from an autoclave provides a more effective technique of sterilizing cellular components of bacteria – even sporulating bacteria are no longer viable after this treatment (Huesca-Espitia et al., 2016).

With all this information, it is concluded that autoclaving is a sufficient method for sterilising gastrointestinal microbes, and it will be adopted to produce the heat-killed control in this study.

Additionally, Javdan selected only one donor's faeces to be heat-killed. This raises further doubts about the validity of this control, as it will not be representative of the diverse spectrum of bacteria that is observed between individuals. Particularly relevant here is the individual variability between humans regarding the diversity of species of sporulating bacteria (Koopman et al., 2022). To address this, the cultured faeces from all donors will be pooled prior to killing, to provide a more representative sample for use as a control.

Fluorescein Diacetate Hydrolysis Test

The application of the fluorescein diacetate (FDA) hydrolysis assay enabled us to accurately measure the metabolic activity across various samples. Notwithstanding, the results deviated from our expectations. The metabolic activity between the control and experimental samples exhibited no significant variance. This lack of statistical significance suggests that the FDA test may not be sensitive to measure the metabolic activity of these samples, or that there may not be a difference between the hydrolytic activity of the samples. Additionally, the lack of any significant difference over time indicates that the type of activity being measured by this test is either stable or has a limited impact within the tested time frame. Furthermore, the Ci sample did not display significant differences from the controls. This was particularly perplexing given that Ci was

intended to serve as a validated positive control. Literature substantiates that Ci should be in its log phase, thereby exhibiting elevated metabolic activity. Contrary to expectations, it demonstrated the lowest metabolic activity among all experimental samples.

GCMS Optimisation Hydrolysis

Distinct peaks are observed on the chromatograms of mouse samples extracted with TFAA and TMS, indicating successful separation and detection by the GC/MS method. As TFAA yielded higher levels of both clozapine and norclozapine, this extraction method was selected for future sample preparation.

Methanol combined with MTBE was found to yield the highest levels of clozapine and was therefore adopted for extraction of future samples. This extraction method has been named in other literature to be the most efficient, namely a study by Erben and colleagues (2021), who evaluated the success of various methods at extracting metabolites from human faeces, using GCMS. This confidence that this method will be successful for the subsequent experiments.

For the SPF mouse samples, the t tests conducted between the control samples and the experimental samples both failed to show significant differences in norclozapine. However, the ratio of norclozapine:clozapine was higher in the experimental samples than the autoclaved-controls, which meets our hypothetical prediction, suggesting that bacteria in the faeces possess mechanisms to metabolise clozapine. This difference was not observed in the samples from the GF mice. This is expected as these mouse lack a microbiome, therefore their faeces will lack microbial-derived compounds that have the ability to metabolism clozapine. This implies that there is a bacterial contribution to the formation of this metabolite in SPF mouse faeces. This provides confidence that this protocol is optimised and fit for translation into human samples.

Limitations

For the SPF samples, the DMSO controls showed an unexpected output, with a lack of significant difference in the clozapine and norclozapine levels between these samples compared to the experimental mouse samples. It would be expected that the DMSO controls had no clozapine, and therefore could produce no norclozapine. However, the DMSO controls showed statistically similar levels of clozapine and norclozapine to the experimental sample. This implies either contamination or misinterpretation of output. This could also be due to the large variation observed within the experimental triplicates. These tests should be repeated in order to validate this method.

Additionally, the FDA protocol should be reviewed to rectify any deviations or potential sources of error. Additional tests should then be conducted to confirm the consistency of the results and identify any recurring patterns or anomalies. The Ci sample should be revalidated under controlled conditions to ensure that it is accurately representative of its log phase and exhibits the expected metabolic activity. By implementing these

recommendations, the reliability and interpretative clarity of the experimental results can be significantly improved.

Chapter 3:
Translating the Optimised Model for Human Samples

Introduction

Following the optimisation of methodologies using mouse samples, this section focuses on the critical step of translating these results to human samples. The objective is to validate the findings from the in vitro mouse model in the previous chapter by applying the optimised protocols to human samples, ensuring relevance and applicability to human physiology.

This study examines clozapine metabolism in human faeces under four distinct experimental conditions: (1) incubation with clozapine in the presence of active microbial communities, (2) incubation with clozapine in the absence of active microbial communities (autoclaved samples), (3) clozapine in a media-only condition and (4) samples with a DMSO vehicle control. Triplicate repeats will be conducted for each condition to ensure robust statistical analysis and minimize the influence of any outlier data points. These stringent experimental designs are implemented to comprehensively assess the role of the human microbiome in clozapine metabolism and to determine the extent to which this activity can be replicated *in vitro*.

It is hypothesised that the human gut microbiome actively metabolises clozapine to norclozapine, and that this activity will be observed in samples with active microbiomes, as compared to those where the microbiome is inactive (autoclaved samples). This hypothesis will be tested using quantitative mass spectrometry to determine the level of clozapine and norclozapine under each condition and by calculating norclozapine:clozapine ratios, which will be compared across samples.

The primary statistical test used in this study will be a Pearson correlation analysis to determine the correlation between clozapine and norclozapine concentrations. Based on Javdan's established criterion for microbial-derived metabolism, we predict that the experimental samples with an active gut microbiome will exhibit a *negative* Pearson correlation with a *statistically significant* p-value. This prediction assumes that as clozapine is metabolised to norclozapine, the clozapine concentrations will decrease as norclozapine levels increase. Conversely, an absence of such a correlation or a positive correlation would suggest the potential involvement of non-microbial, or other non-enzyme driven, mechanisms in clozapine metabolism. A series of T-tests will be used to compare levels of clozapine and norclozapine between samples, and between time points.

By translating these findings from mouse models in the previous chapter to human samples, this experiment bridges the gap between preclinical and clinical research, thereby providing a better understanding of the dynamics of clozapine metabolism, including the role of the microbiome, and its potential therapeutic implications. Ultimately, the information gleaned from this study will guide the development of a more comprehensive understanding of clozapine metabolism and its effect on therapeutic strategies and personalized medicine.

Methodology

Culturing and Inoculation

Working aseptically in a biosafety cabinet, 9 mL of the B3HI medium, prepared using previous methods, was transferred to 5 vented cap tubes and transferred to the anaerobic chamber for overnight (>18 hr) degassing at room temperature.

Faeces from five healthy human participants were retrieved from a -70°C freezer on dry ice and transferred to the anaerobic chamber. To each faeces sample, 1 mL of B3HI from one vented cap tube was added to rehydrate the faeces. Each solution was then transferred to the same vented cap tube containing 9 mL of B3HI to inoculate. These 5 inoculated samples were then incubated for 24 hours in the anaerobic chamber at 37 °C.

Testing Bacterial Metabolic Activity

After 24 hours, the five vented cap tubes containing human cultured samples were removed from the incubator.

Each incubated culture was separated into two test condition tubes: one for 8 hr incubation, one for 24 hr incubation. Clozapine stock and DMSO stock were added to the cultures in quadruplicates, each in 1.5 mL Eppendorf tubes, at a final concentration of 33 μM. Additionally, media-clozapine and autoclaved-clozapine conditions were created. Cultures were pooled prior to autoclaving.

When incubation-time was complete, a portion of samples were tested for FDA hydrolysis, while the remaining samples were centrifuged and stored at -70°C for later GC-MS analysis.

Data analysis

Levels of clozapine and norclozapine in all samples will be normalized to the internal standard, d-aminoheptane in order to correct for differences in extraction efficiency.

For assessing the significance of difference in compound levels in media samples to autoclaved samples, donor samples to autoclaved samples and donor samples to media samples, analysis of variance of samples needs to be assessed. For this the F-test is conducted to asses whether they are equal. If significant, the variances in samples are unequal, and thus a two-sample of unequal variance t-test can be performed. When comparing within donor samples, a paired t-test will be used. Significance for these tests is set at 5%.

Optimised GCMS Methodology

Once it was discovered which method was the most sensitive for detecting our compounds of interest, this method was chosen and all the samples (N=72) were analysed by the GC-MS equipment.

Results

Testing Bacterial Metabolic Activity

Figure 28 shows the results of the FDA hydrolysis test for metabolic activity.

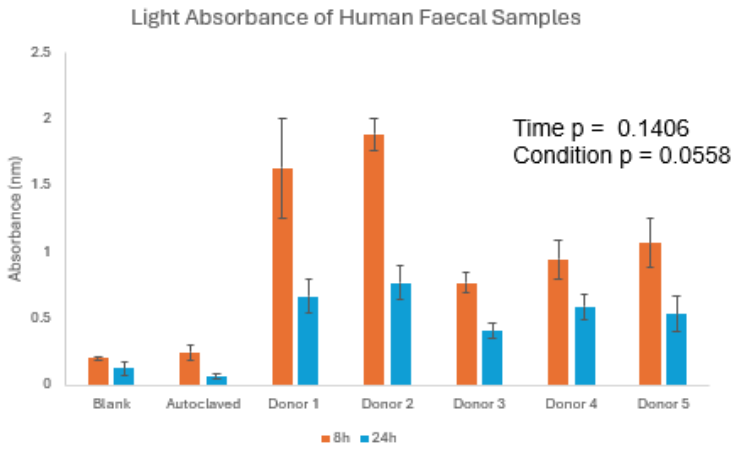


Figure 28. Test of FDA Metabolic Activity. Average absorbance values (nanometres (nm)) of samples from varying conditions, including autoclaved human faeces, samples from human donor 1, human donor 2, human donor 3, human donor 4 and human donor 5. There is also a blank sample (containing FDA reagents, media, and no faeces). Two timepoints of incubation length are shown for each condition; 8 hours (h) shown in orange, and 24 hours (h) shown in blue. Error bars show the variation of the triplicate samples within each condition, calculated by standard error of the mean. The p values from t tests comparing means of both timepoints (time) and also comparing means of control samples to means of experimental samples (condition) are shown.

The control samples appear to exhibit much lower metabolic activity than the experimental samples but this difference was found to lack significance by the t test ($p = 0.0558$). Whilst time did appear to have an effect on metabolic activity, particularly for donors 1 and 2, the t test did not find this difference to be significant ($p = 0.1406$).

Validating GCMS Methodology

Figure 29 shows the results of the initial human samples used to ensure that the previous GCMS sample preparation was efficient in detecting metabolites in human samples.

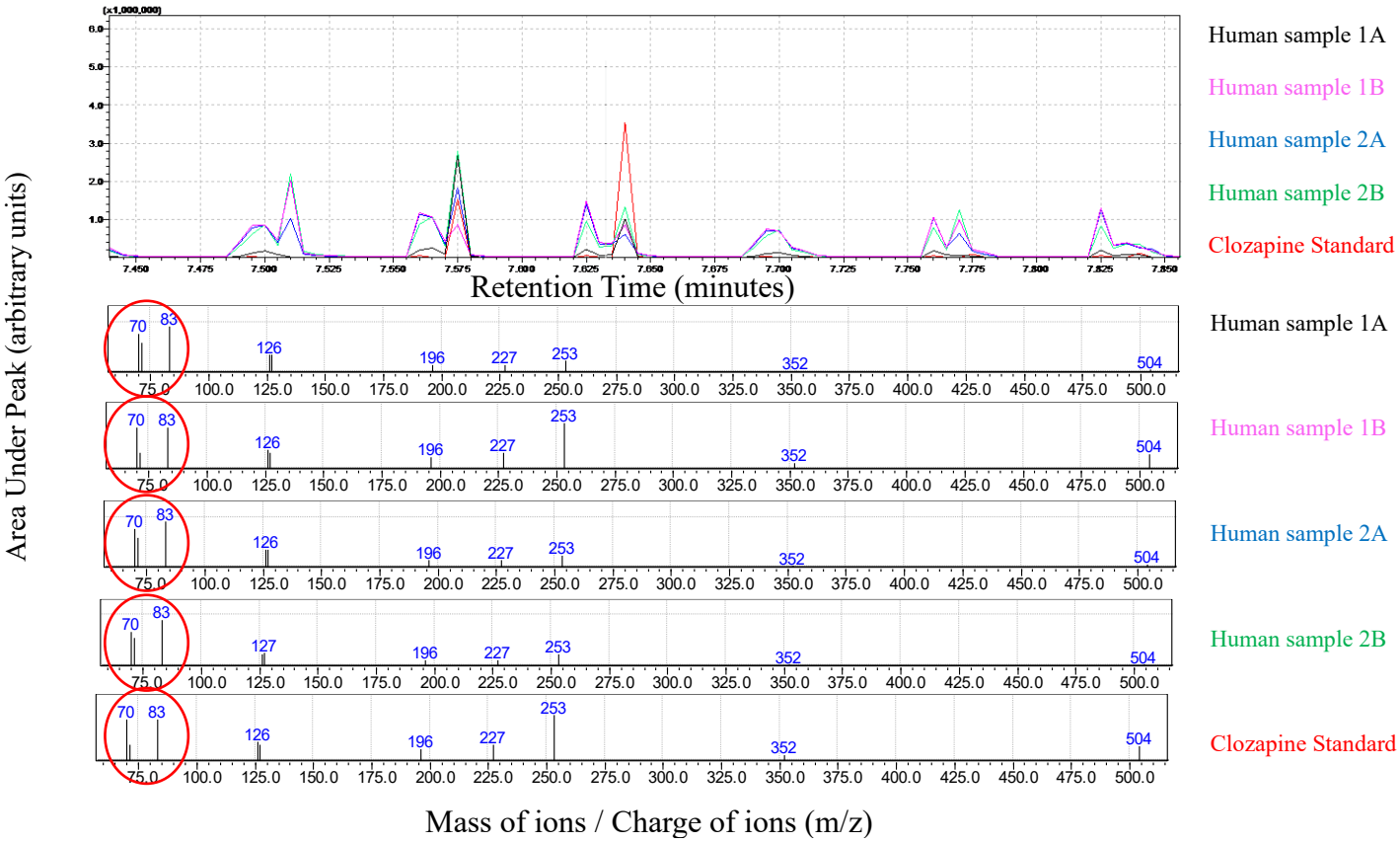


Figure 29. Chromatogram and mass spectra at 7.635 minutes of human faeces prepared using optimised methods including the clozapine standard, two samples from human donor 1 (1A and 1B) and two samples from human donor 2 (2A and 2B).

All samples appear to show a similar output to the clozapine standard (red line in chromatogram, 5th spectrum), both in the chromatogram and mass spectra, suggesting that clozapine can be detected in these samples.

Figure 30 shows output of the initial human samples at 7.975 minutes.

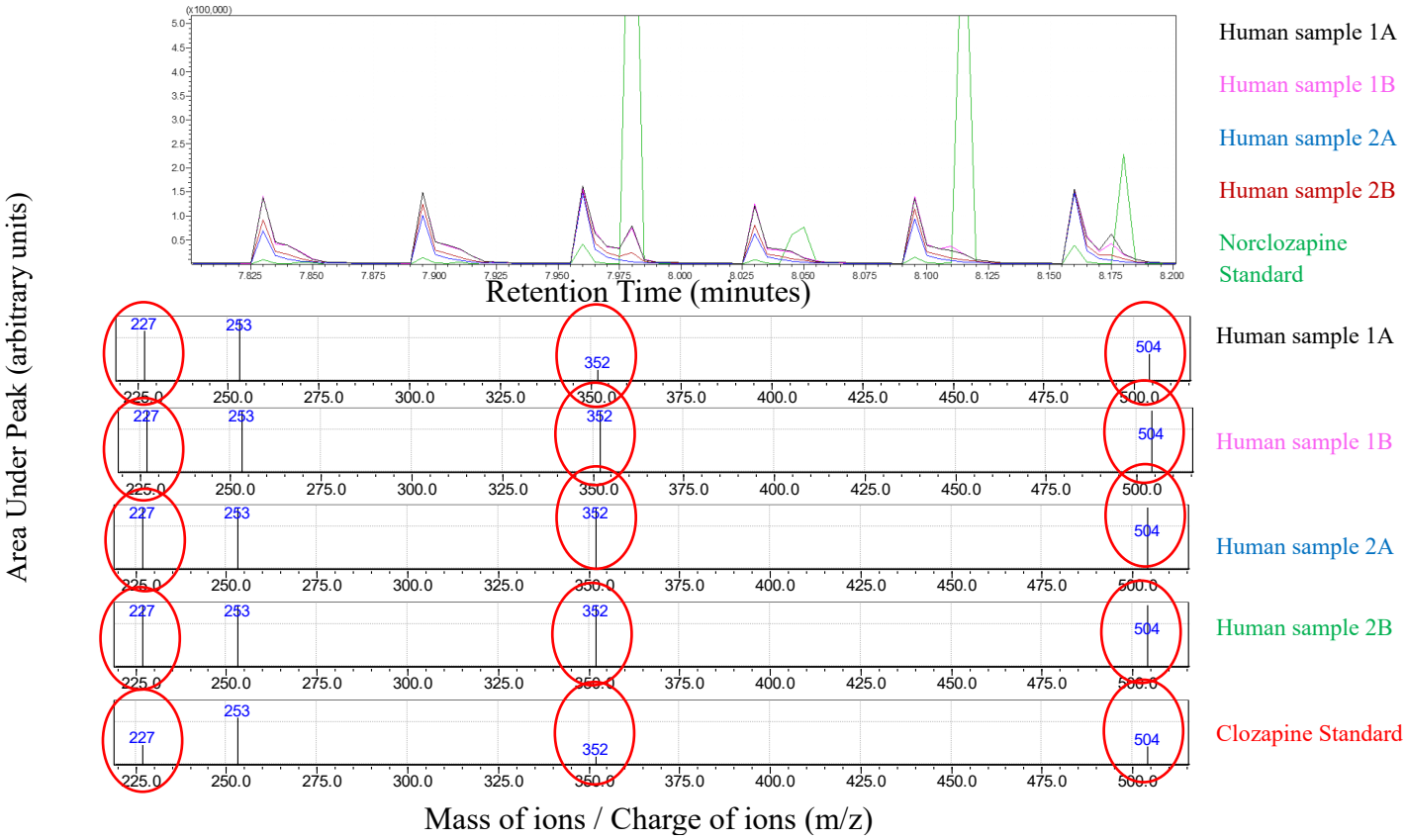


Figure 30. Chromatogram and mass spectra at 7.975 minutes of human faeces prepared using optimised methods including the norclozapine standard, two samples from human donor 1 (1A and 1B) and two samples from human donor 2 (2A and 2B).

All samples appear to show a similar output to the norclozapine standard (green line in chromatogram, 5th spectrum), both in the chromatogram and mass spectra, albeit fair smaller peaks in the chromatogram. This suggests that norclozapine can be detected in these samples.

As clozapine and norclozapine can be detected in human faeces using the established GCMS method, this was selected as the method for the full 72 samples (5 donors, 4 conditions, triplicate repeats).

Optimised GCMS Methodology

Controls

Clozapine Levels

The clozapine levels in the samples with media and clozapine were found to have an unequal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.03$) and

the two-sample t test of unequal variance did not meet significance (p = 0.34). The clozapine levels in the samples with media and clozapine were found to have an unequal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 24 hours (p = 0.004) and the two-sample t test of unequal variance did not meet significance (p = 0.18). A paired t test was conducted on the clozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours and the clozapine levels in the samples that were autoclaved and incubated with clozapine for 24 hours and was not significant (p = 0.30).

Table 5. Analysis of Equal Variance F-tests and T-tests Reported of Clozapine Levels Control Samples. Variance not conducted on paired samples. Results reported as (f test p value, t test p value).

	Media	**Autoclaved-Clozapine 8h**	**Autoclave-Clozapine 24h**
Media-Clozapine	x	0.03, 0.34	0.004, 0.18
Autoclaved-Clozapine 8h	x	x	x, 0.30
Autoclave-Clozapine 24h	x	x	x

Norclozapine Levels

The norclozapine levels in the samples with media and clozapine were found to have an unequal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours (p = 0.0002) and the two-sample t test of unequal variance met significance (p = 0.03). The norclozapine levels in the samples with media and clozapine were found to have an equal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 24 hours (p = 0.06) and the two-sample t test of equal variance met significance (p = 0.02). A paired t test was conducted on the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours and the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 24 hours and was not significant (p = 0.11).

Table 6. Analysis of Equal Variance F-tests and T-tests Reported of Norclozapine Levels in Control Samples. Variance not conducted on paired samples. Results reported as (f test p value, t test p value).

	Media	**Autoclaved-Clozapine 8h**	**Autoclave-Clozapine 24h**
Media-Clozapine	x	0.0002, 0.03	0.06, 0.02
Autoclaved-Clozapine 8h	x	x	x, 0.11
Autoclave-Clozapine 24h	x	x	x

Donor 1

Clozapine Levels

Figure 31 illustrates the GCMS output of Donor 1 samples and controls.

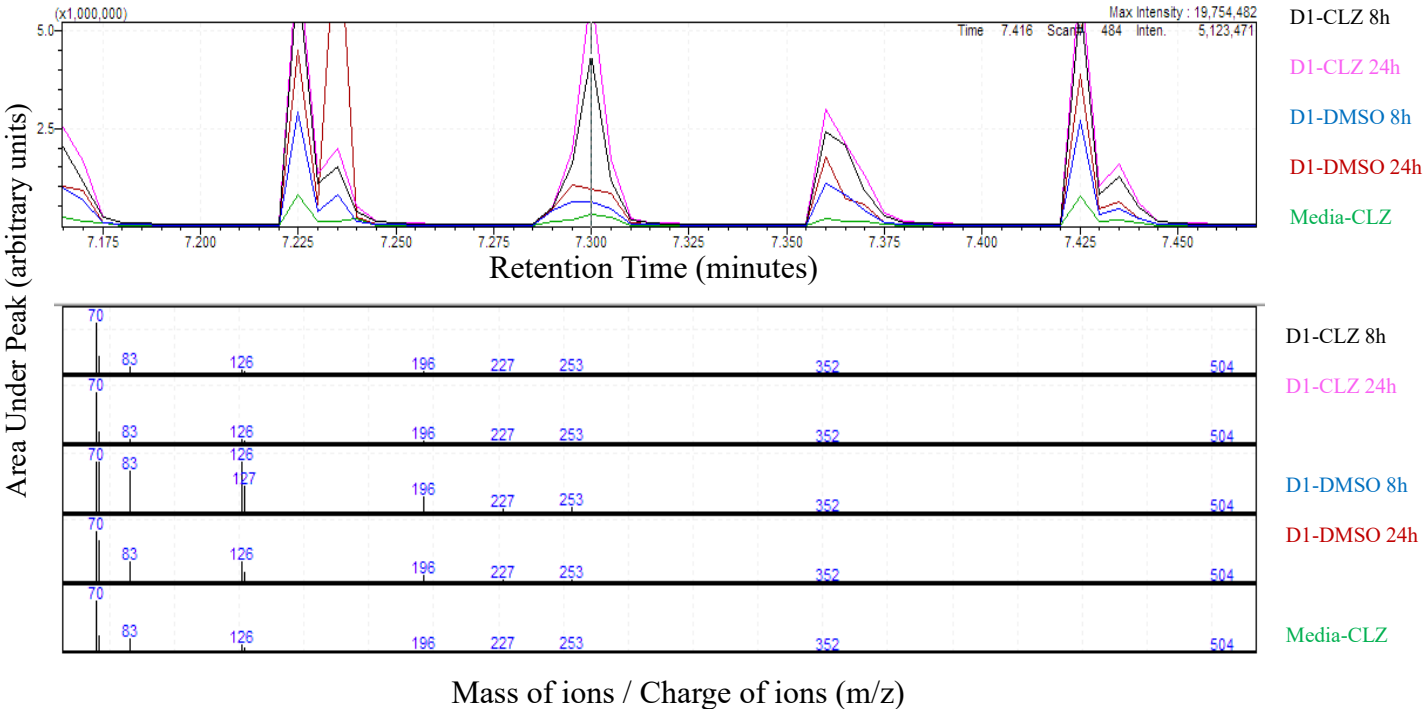


Figure 31. Chromatogram and mass spectra of human samples including donor 1 faeces incubated with clozapine for 8 hours (D1-CLZ 8h), and for 24 hours (D1-CLZ 24h), donor 1 faeces incubated with DMSO for 8 hours (D1-DMSO 8h) and for 24 hours (D1-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.300 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine.

On the chromatogram, there appears to be minimal clozapine in the Donor1-DMSO control conditions at both 8 hour and 24 hour incubation time points (blue and red lines). The donor 1 samples incubated with clozapine show much higher peaks on the chromatogram (pink and black). The sample containing media incubated with clozapine also appears to contain minimal clozapine. Further investigation into the samples in the media-clozapine condition revealed that two of the triplicates appeared to be incorrectly prepared, with only one containing the expected arbitrary concentration of clozapine.

This is also clear from the mass-spectra of the five samples, in which the samples incubated with clozapine only show peaks at 70, 71 and 83 m/z – the qualifying ions for clozapine. The DMSO samples show these peak, but also so other peaks at 126, 127 and 196 m/z, indicating that this spectra is not clozapine. The media-clozapine has the peaks for clozapine, but also 126 m/z.

Quantitative

The clozapine levels in the donor 1 samples incubated with clozapine for 8 hours were found to have an equal variance to the clozapine levels in the samples with media and clozapine ($p = 0.57$). This is evident from the bar graph, with the error bars a similar size (Figure 32). The two-sample t test of equal variance did not meet significance ($p = 0.49$).

The clozapine levels in the donor 1 samples incubated with clozapine for 8 hours were found to have an equal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.08$) and the two-sample t test of equal variance did not meet significance ($p = 0.24$)

A paired t test was conducted on the clozapine levels in the donor 1 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 1 samples incubated with DMSO for 8 hours met significance ($p = 0.15$).

A paired t test was conducted on the clozapine levels in the donor 1 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 1 samples incubated with clozapine for 24 hours was not significant ($p = 0.40$).

The clozapine levels in the donor 1 samples incubated with clozapine for 24 hours were found to have an equal variance to the clozapine levels in the samples with media and clozapine ($p < 0.0001$), likely due to the high variance between the media samples. The two-sample t test of equal variance was not significant ($p = 0.44$).

The clozapine levels in the donor 1 samples incubated with clozapine for 24 hours were found to have an unequal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 24h ($p = 0.01$) and the two-sample t test of unequal variance was not significant ($p = 0.22$).

Table 7. Analysis of Equal Variance F tests and T tests of Clozapine levels in Donor 1 Samples and Controls. Variance not conducted on paired samples. Results reported as (f test p value, t test p value).

	Donor1-Clozapine 8h	**Donor1-Clozapine 24h**	**Donor1-DMSO 8h**	**Donor1-DMSO 24h**
Media-Clozapine	0.57, 0.49	<0.0001, 0.44	x	x
Autoclaved-Clozapine 8h	0.08, 0.24	x	x	x
Autoclave-Clozapine 24h	x	0.01, 0.22	x	x
Donor1-Clozapine 8h	x	x	0.15	x
Donor1-Clozapine 24h	0.4	x	x	0.005
Donor1-DMSO 8h	x	x	x	0.35

A paired t test was conducted on the clozapine levels in the donor 1 samples incubated with clozapine for 24 hours and the clozapine levels in the donor 1 samples incubated with DMSO for 24 hours was significant ($p = 0.005$).

A paired t test was conducted on the clozapine levels in the donor 1 samples incubated with DMSO for 8 hours and the clozapine levels in the donor 1 samples incubated with DMSO for 24 hours was not significant ($p = 0.35$).

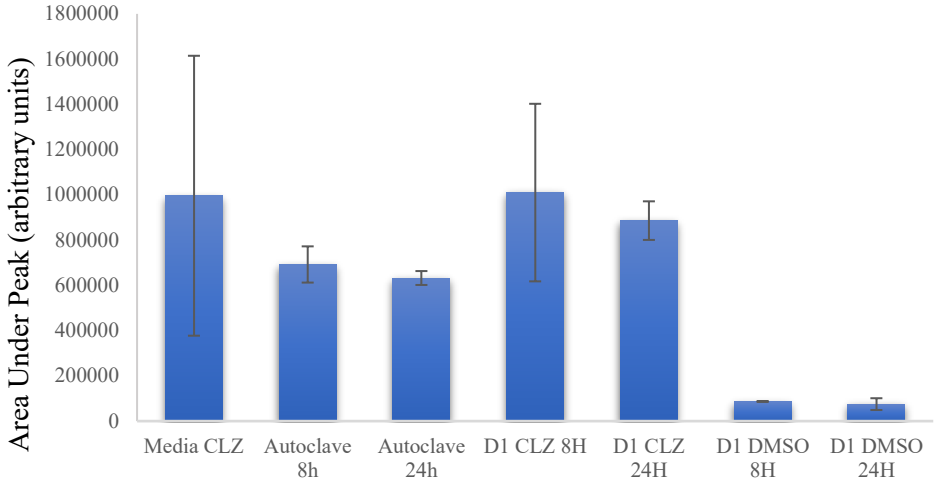


Figure 32. Bar graph displaying the clozapine levels in samples using human donor 1 (D1) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 1 faeces incubated with clozapine for 8 hours (D1-CLZ 8h), and for 24 hours (D1-CLZ 24h), donor 1 faeces incubated with DMSO for 8 hours (D1-DMSO 8h) and for 24 hours (D1-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.300 minutes. DMSO = dimethyl sulfoxide.

Norclozapine Levels

Figure 33 illustrates the GCMS output of Donor 1 samples and controls.

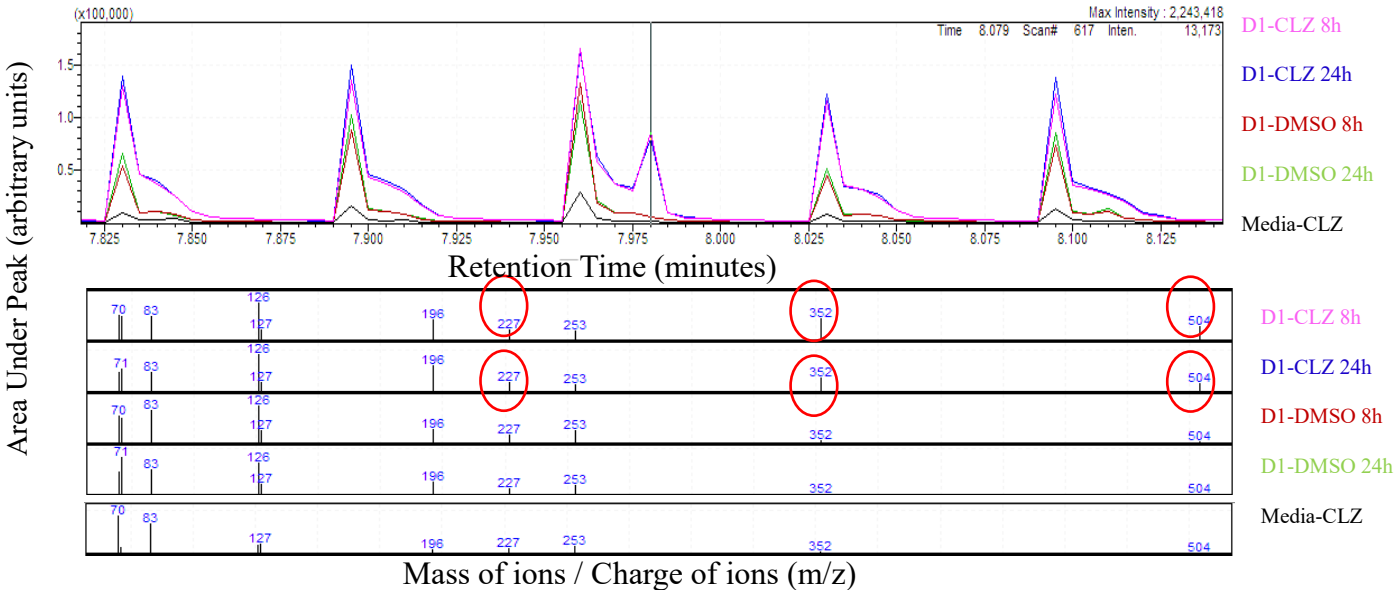


Figure 33. Chromatogram and mass spectra of human samples including donor 1 incubated with clozapine for 8 hours (D1-CLZ 8h), and for 24 hours (D1-CLZ 24h), donor 1 incubated with DMSO for 8 hours (D1-DMSO 8h) and for 24 hours (D1-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.98 minutes. DMSO = dimethyl sulfoxide. Presence of ions 227, 352, and 504 m/z on spectra 1 and 2, denoted by red circles.

Both the chromatogram and the spectra reveal that norclozapine is only present in the samples with donor 1's faeces incubated with clozapine – seen in the pink and blue lines on the chromatogram and the presence of ions 227, 352, and 504 m/z on spectra 1 and 2, denoted by red circles.

All the other lines of the chromatogram show minimal/no levels of norclazpine - the red, green and black lines signifying the donor 1 faeces incubated with DMSO and the sample with media incubated with clozapine. Their spectra (3-5) also do not contain the qualifying ions for norclozapine.

Quantitative

The norclozapine levels in the donor 1 samples incubated with clozapine for 8 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p < 0.0001$). This can be seen in the error bars in the bar graph in Figure 34, with the media samples showing almost no variation. The two-sample t test of unequal variance did not meet significance ($p = 0.09$).

The norclozapine levels in the donor 1 samples incubated with clozapine for 8 hours were found to have an equal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.28$) and the two-sample t test of unequal variance did not meet significance ($p = 0.35$)

A paired t test was conducted on the norclozapine levels in the donor 1 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 1 samples incubated with DMSO for 8 hours and this did not meet significance ($p = 0.10$).

A paired t test was conducted on the norclozapine levels in the donor 1 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 1 samples incubated with clozapine for 24 hours was not significant ($p = 0.11$).

Table 8. Analysis of Equal Variance F tests and T tests of Norclozapine Levels in Donor 2 Samples and controls. Variance not conducted on paired samples

	Donor1-Clozapine 8h	**Donor1-Clozapine 24h**	**Donor1-DMSO 8h**	**Donor1-DMSO 24h**
Media-Clozapine	<0.001, 0.09	<0.001, 0.09	x	x
Autoclaved-Clozapine 8h	0.28, 0.35	x	x	x
Autoclave-Clozapine 24h	x	0.01, 0.19	x	x
Donor1-Clozapine 8h	x	x	0.10	x
Donor1-Clozapine 24h	0.4	x	x	0.1
Donor1-DMSO 8h	x	x	x	0.13

The norclozapine levels in the donor 1 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p < 0.0001$). This donor-clozapine condition showed high variance, as indicated by the error bar in figure X. The two-sample t test of unequal variance was not significant ($p = 0.09$).

The norclozapine levels in the donor 1 samples incubated with clozapine for 24 hours were found to have an unqual variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 24h ($p = 0.01$) and the two-sample t test of unequal variance was not significant ($p = 0.19$).

A paired t test was conducted on the norclozapine levels in the donor 1 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 1 samples incubated with DMSO for 24 hours, which was not significant ($p = 0.10$).

A paired t test was conducted on the norclozapine levels in the donor 1 samples incubated with DMSO for 8 hours and the norclozapine levels in the donor 1 samples incubated with DMSO for 24 hours was not significant ($p = 0.13$).

‘

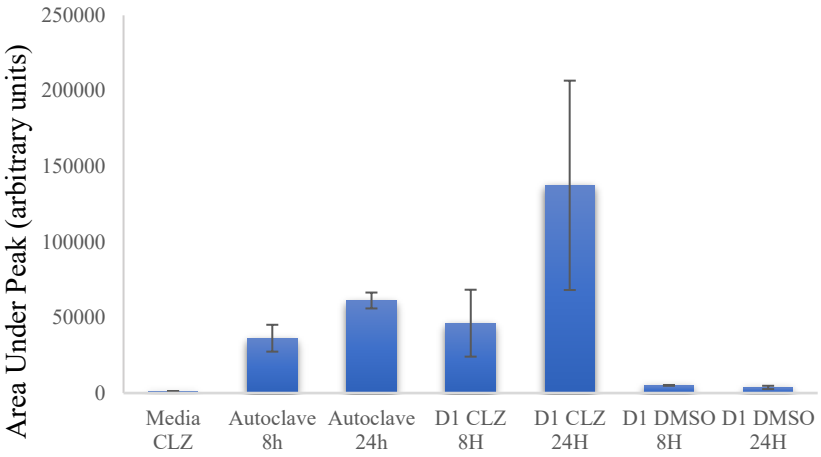


Figure 34. Bar graph displaying the norclozapine levels in samples using human donor 1 (D1) faeces, including samples with autoclaved human faeces incubated with clozapine, samples including donor 1 incubated with clozapine for 8 hours (D1-CLZ 8h), and for 24 hours (D1-CLZ 24h), donor 1 incubated with DMSO for 8 hours (D1-DMSO 8h) and for 24 hours (D1-DMSO 24h) and media incubated with clozapine (media-CLZ). Error bars shows standard error of the mean of the variation of triplicate repeats. CLZ = clozapine, DMSO = dimethyl sulfoxide.

Norclozapine:clozapine Ratio

Figure 35 displays the ratio of norclozapine:clozapine in the human donor 1 samples.

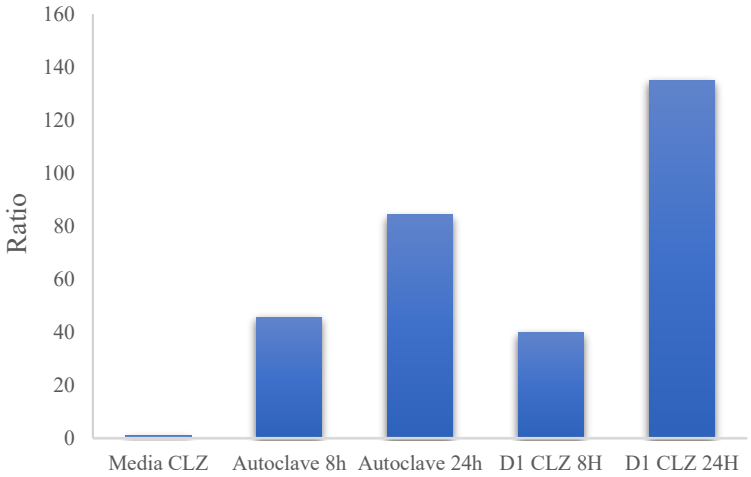


Figure 35. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 1, including autoclaved faeces incubated with clozapine, human donor 1 faeces incubated with clozapine samples for 8 hours (D1-CLZ 8h), and for 24 hours (D1-CLZ 24h), donor 1 faeces incubated with DMSO for 8 hours (D1-DMSO 8h) and for 24 hours (D1-DMSO 24h), as well as media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

The ratio in the sample with media incubated with clozapine (Media CLZ) is almost negligible, suggesting minimal or no conversion of clozapine to norclozapine under these conditions.

In contrast, the autoclaved sample incubated with clozapine for 8 hours (Autoclave 8h) exhibits a considerably higher ratio, around 40, indicating that autoclaving for 8 hours has a significant impact on the conversion process. Extending the autoclaving period to 24 hours further enhances this effect, with the ratio rising to

approximately 80, suggesting that longer autoclaving times facilitate greater conversion of clozapine to norclozapine.

Similarly, the human donor 1 faeces incubated with clozapine for 8 hours (D1 CLZ 8H sample), shows a ratio of around 40, mirroring the results observed with the 8-hour autoclaved sample. This metabolic process is increased at the 24 hour incubation point, as observed in the D1 CLZ 24H sample, which records a ratio of around 140.

Donor 2

Clozapine Levels

Qualitative

Figure 36 illustrates the GCMS output of Donor 1 samples and controls.

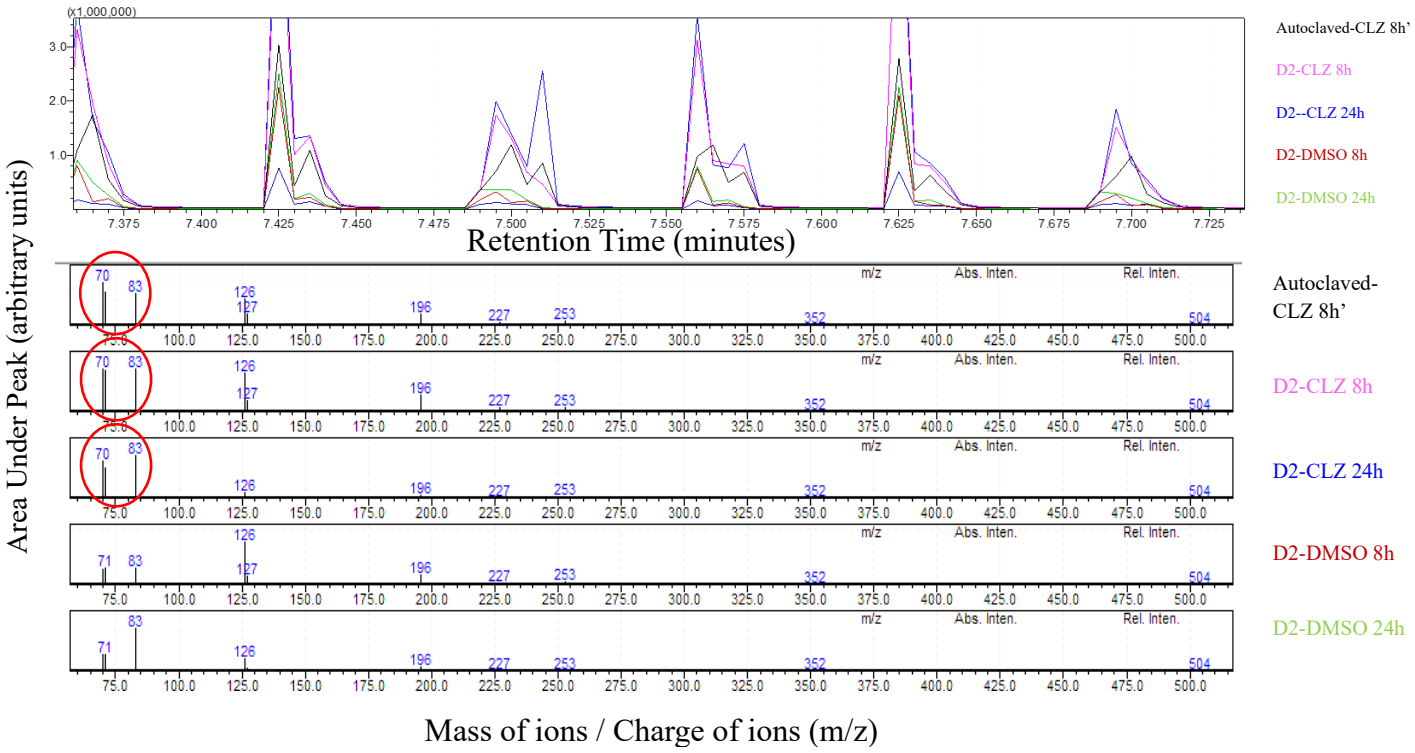


Figure 36. Chromatogram and mass spectra of human samples including donor 2 faeces incubated with clozapine for 8 hours (D2-CLZ 8h), and for 24 hours (D2-CLZ 24h), donor 2 faeces incubated with DMSO for 8 hours (D1-DMSO 8h) and for 24 hours (D2-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.51 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for clozapine; 70, 71 and 83 m/z.

On the chromatogram, there appears to be minimal clozapine in the Donor2-DMSO control conditions at both 8 hour and 24 hour incubation time points (red and green lines). The donor 2 samples incubated with clozapine,

and the Autoclaved-CLZ, show much higher peaks on the chromatogram (black, blue and pink lines), with the sample incubated for 24 hours showing the highest levels (blue line).

This is also clear from the mass-spectra of the five samples, in which the samples incubated with clozapine show peaks at 70, 71 and 83 m/z – the qualifying ions for clozapine, denoted by red circles in Figure 36. The DMSO samples do not show similar peaks.

Quantitative

The donor 2 samples incubated with clozapine for 8 hours were found to have an equal variance to the samples with media and clozapine ($p = 0.21$). From the error bars in the bar graph in Figure 37, both conditions show large variation. The two-sample t test of equal variance did not meet significance ($p = 0.23$).

The donor 2 samples incubated with clozapine for 8 hours were found to have an unequal variance to the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.004$) and the two-sample t test of equal variance did not meet significance ($p = 0.20$).

Table 9. Analysis of Equal Variance F tests and T tests of Donor 2 Samples and controls. Variance not conducted on paired samples. Results were reported using the format of (f test p value, t test p value).

	Donor2-Clozapine 8h	**Donor2-Clozapine 24h**	**Donor2-DMSO 8h**	**Donor2-DMSO 24h**
Media-Clozapine	0.21, 0.23	0.21, 0.38	x	x
Autoclaved-Clozapine 8h	0.004, 0.20	x	x	x
Autoclave-Clozapine 24h	x	0.04, 0.27	x	x
Donor2-Clozapine 8h	x	x	x, 0.15	x
Donor2-Clozapine 24h	0.23	x	x	0.04
Donor2-DMSO 8h	x	x	x	0.25

A paired t test was conducted on the donor 2 samples incubated with clozapine for 8 hours and the donor 2 samples incubated with DMSO for 8 hours met significance ($p = 0.15$).

A paired t test was conducted on the donor 2 samples incubated with clozapine for 8 hours and the donor 2 samples incubated with clozapine for 24 hours was not significant ($p = 0.23$).

The donor 2 samples incubated with clozapine for 24 hours were found to have an equal variance to the samples with media and clozapine ($p = 0.21$) and the two-sample t test of equal variance was not significant ($p = 0.38$).

The donor 2 samples incubated with clozapine for 24 hours were found to have an unequal variance to the samples that were autoclaved and incubated with clozapine for 24h ($p = 0.04$) and the two-sample t test of unequal variance was not significant ($p = 0.27$).

A paired t test was conducted on the donor 2 samples incubated with clozapine for 24 hours and the donor 2 samples incubated with DMSO for 24 hours was significant ($p = 0.04$).

A paired t test was conducted on the donor 2 samples incubated with DMSO for 8 hours and the donor 2 samples incubated with DMSO for 24 hours was not significant ($p = 0.25$).

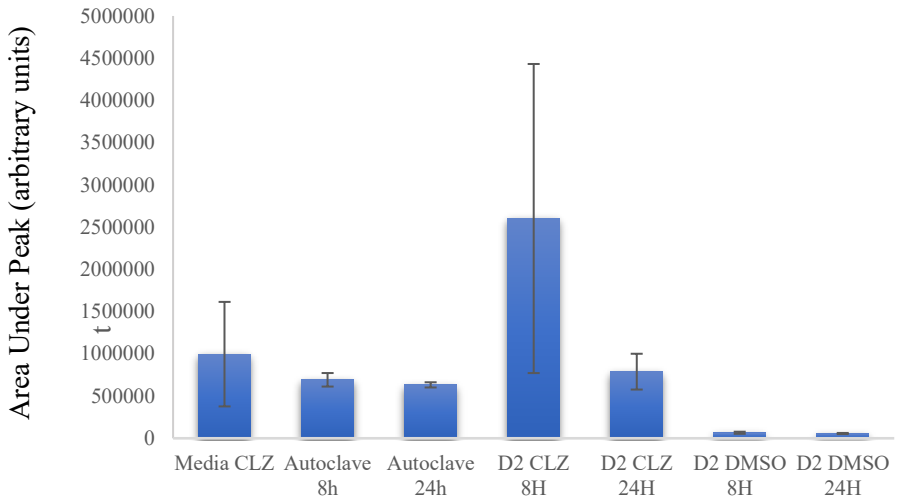


Figure 37. Bar graph displaying the clozapine levels in samples using human donor 2 (D2) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 2 faeces incubated with clozapine for 8 hours (D2-CLZ 8h), and for 24 hours (D2-CLZ 24h), donor 2 faeces incubated with DMSO for 8 hours (D2-DMSO 8h) and for 24 hours (D2-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.300 minutes. DMSO = dimethyl sulfoxide.

Norclozapine Levels

Qualitative

Figure 38 illustrates the GCMS output of Donor 2 samples and controls.

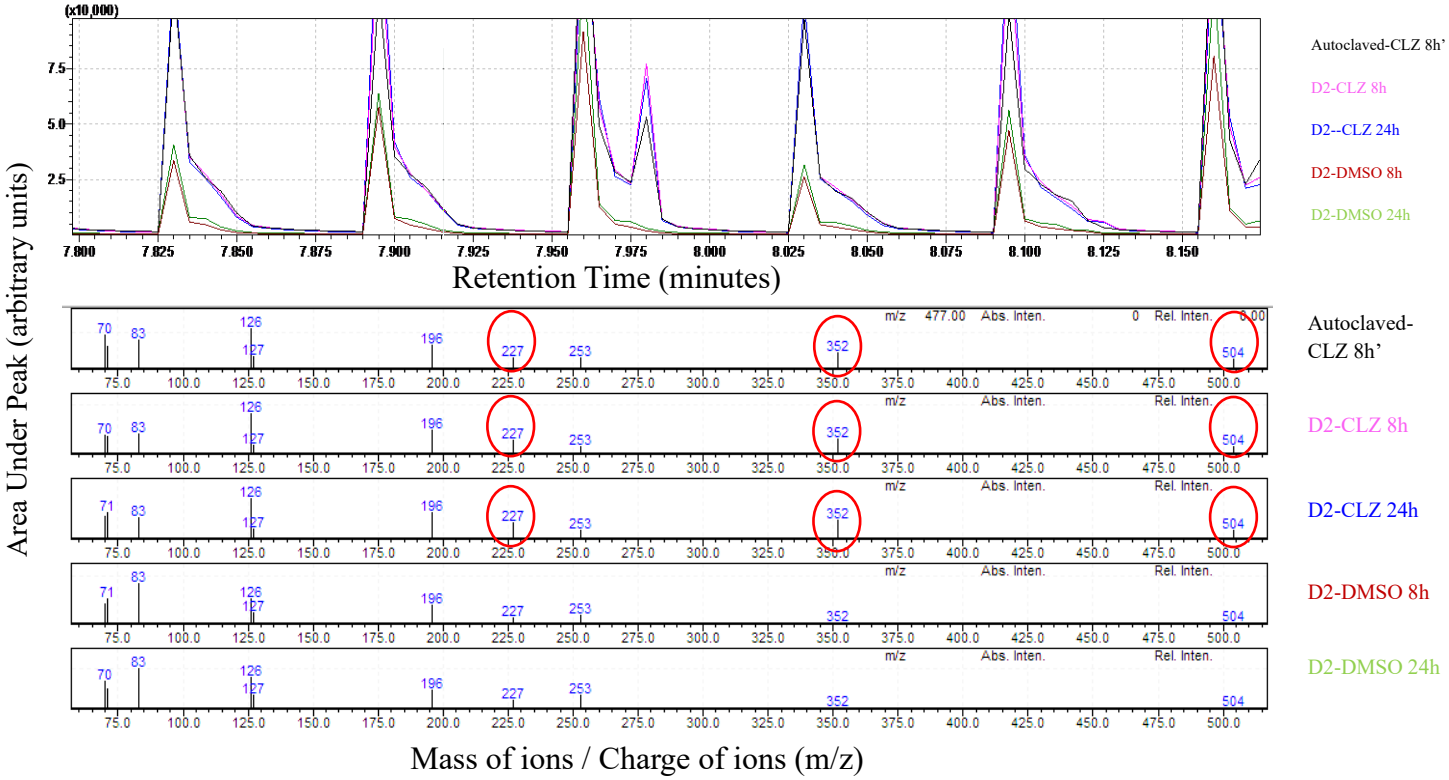


Figure 38. Chromatogram and mass spectra of human samples including donor 2 faeces incubated with clozapine for 8 hours (D2-CLZ 8h), and for 24 hours (D2-CLZ 24h), donor 2 faeces incubated with DMSO for 8 hours (D1-DMSO 8h) and for 24 hours (D2-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.98 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for norclozapine; 227, 352 and 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in large quantities in the samples with faeces incubated with clozapine – the black, pink and blue lines on the chromatogram and the presence of ions 227, 352, and 504 m/z on spectra 1, 2 and 3.

All the other lines of the chromatogram show minimal/no levels of norclazpine - the red and green lines signifying the donor 2 faeces incubated with DMSO. Their spectra (4 and 5) also do not contain the qualifying ions for norclozapine.

Quantitative

The norclozapine levels in the donor 2 samples incubated with clozapine for 8 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p < 0.0001$). Similarly to the clozapine levels, the norclozapine levels in donor 2 also show large variation, as seen by the error bars on the bar graph in Figure 39. The two-sample t test of unequal variance did not meet significance ($p = 0.13$).

Table 10. Analysis of Equal Variance F tests and T tests of Norclozapine Levels in Donor 2 Samples and controls. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor2-Clozapine 8h	Donor2-Clozapine 24h	Donor2-DMSO 8h	Donor2-DMSO 24h
Media-Clozapine	<0.0001, 0.13	0.0003, 0.009	x	x
Autoclaved-Clozapine 8h	0.29, 0.48	x	x	x
Autoclaved-Clozapine 24h	x		x	x
Donor2-Clozapine 8h	x	x	0.10	x
Donor2-Clozapine 24h	0.14	x	x	<0.0001
Donor2-DMSO 8h	x	x	x	0.41

The norclozapine levels in the donor 2 samples incubated with clozapine for 8 hours were found to have an equal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.29$) and the two-sample t test of equal variance did not meet significance ($p = 0.48$)

A paired t test was conducted on the norclozapine levels in the donor 2 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 2 samples incubated with DMSO for 8 hours and this did not meet significance ($p = 0.10$).

A paired t test was conducted on the norclozapine levels in the donor 2 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 2 samples incubated with clozapine for 24 hours and this was not significant ($p = 0.14$).

The norclozapine levels in the donor 2 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.0003$) and the two-sample t test of unequal variance was significant ($p = 0.009$).

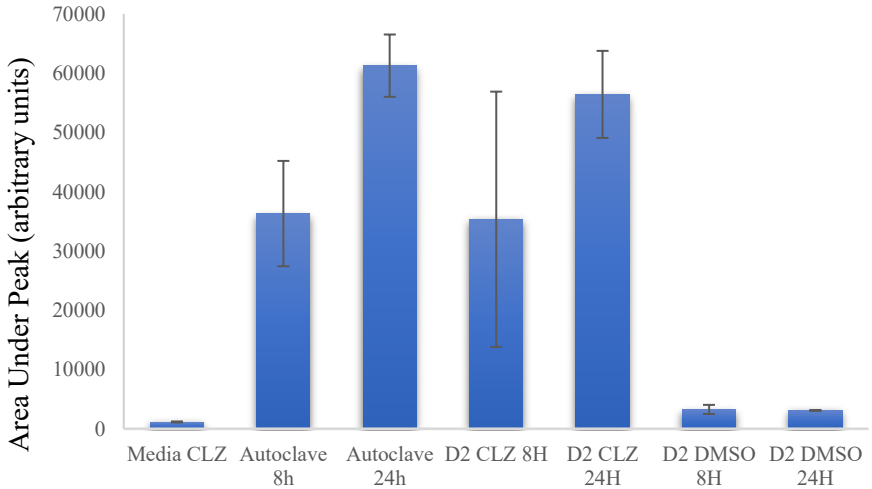


Figure 39. Bar graph displaying the clozapine levels in samples using human donor 2 (D2) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 2 faeces incubated with clozapine for 8 hours (D2-CLZ 8h), and for 24 hours (D2-CLZ 24h), donor 2 faeces incubated with DMSO for 8 hours (D2-DMSO 8h) and for 24 hours (D2-DMSO 24h) and media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

The norclozapine levels in the donor 2 samples incubated with clozapine for 24 hours were found to have an equal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 24h ($p = 0.68$). This can be seen by the similar size of error bars in the bar graph in Figure 39. The two-sample t test of equal variance was not significant ($p = 0.31$).

A paired t test was conducted on the norclozapine levels in the donor 1 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 1 samples incubated with DMSO for 24 hours, which was significant ($p < 0.0001$).

A paired t test was conducted on the norclozapine levels in the donor 2 samples incubated with DMSO for 8 hours and the norclozapine levels in the donor 2 samples incubated with DMSO for 24 hours was not significant ($p = 0.41$).

Norclozapine:clozapine Ratio

Figure 40 displays the ratio of norclozapine:clozapine in the human donor 2 samples.

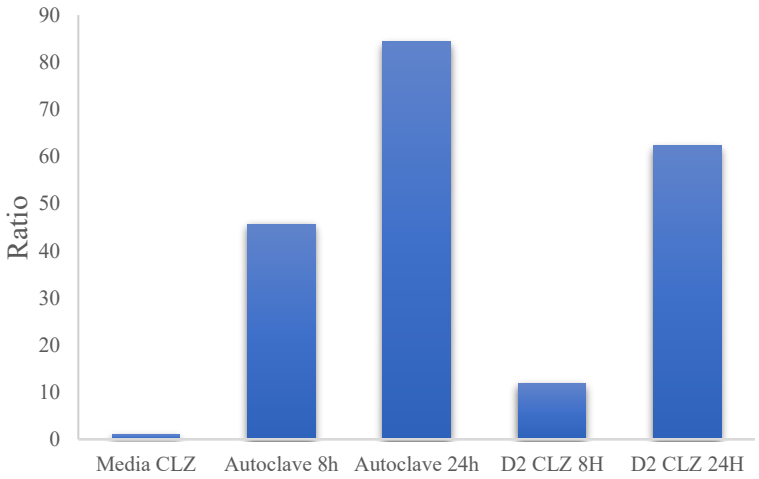


Figure 40. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 2, including autoclaved faeces incubated with clozapine, human donor 2 faeces incubated with clozapine samples for 8 hours (D2-CLZ 8h), and for 24 hours (D2-CLZ 24h), donor 2 faeces incubated with DMSO for 8 hours (D2-DMSO 8h) and for 24 hours (D2-DMSO 24h), as well as media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

For donor 2, the faeces incubated with clozapine showed less clozapine-metabolism than donor 1, as the norclozapine:clozapine ratios are lower than the ratios produced by the autoclaved samples, with 8 hours of incubation demonstrating a ratio of around 10, and 24 hours of incubation displaying a ratio of around 60.

Donor 3

Clozapine Levels

Figure 41 illustrates the GCMS output of Donor 1 samples and controls.

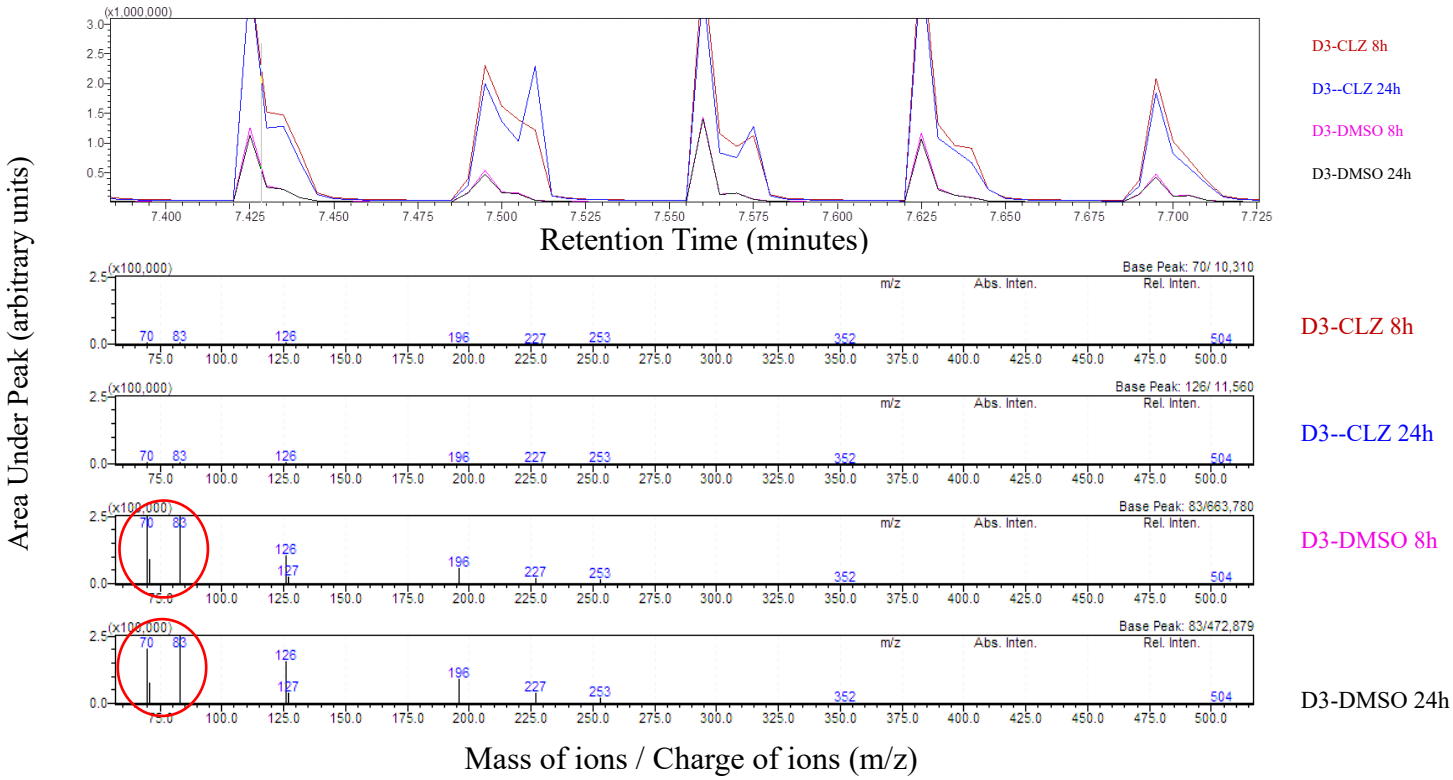


Figure 41. Chromatogram and mass spectra of human samples including donor 3 faeces incubated with clozapine for 8 hours (D3-CLZ 8h), and for 24 hours (D3-CLZ 24h), donor 3 faeces incubated with DMSO for 8 hours (D3-DMSO 8h) and for 24 hours (D3-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.51 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for clozapine; 70, 71 and 83 m/z.

It is clear from the chromatogram in Figure 41 that there is minimal clozapine in the Donor3-DMSO control condition (pink and black lines) whereas in the Donor 3 samples incubated with clozapine, the drug can be detected (red and blue). This is also evident in the mass spectra, where red circles denote the presence of qualifying ions for clozapine, 70, 71 and 83 m/z, in spectra 3 and 4, for the samples incubated with clozapine, but not in 1 and 2, for the samples incubated with DMSO.

Quantitative

The clozapine levels in the donor 3 samples incubated with clozapine for 8 hours were found to have an unequal variance to the clozapine levels in the samples with media and clozapine ($p = 0.05$), with samples containing faeces showing a lot less variation than the samples containing media. This is clear from the error bars in the bar graph in Figure 42. The two-sample t test of unequal variance did not meet significance ($p = 0.46$).

The clozapine levels in the donor 3 samples incubated with clozapine for 8 hours were found to have an equal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours

(p = 0.75). Both conditions have low variance, indicated by the small error bars in Figure 42. The two-sample t test of equal variance was close to meeting significance (p = 0.07).

Table 11. Analysis of Equal Variance F tests and T tests of Clozapine Levels in Donor 3 Samples and Controls. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor3-Clozapine 8h	**Donor3-Clozapine 24h**	**Donor3-DMSO 8h**	**Donor3-DMSO 24h**
Media-Clozapine	0.05, 0.46	0.37, 0.03	x	x
Autoclaved-Clozapine 8h	0.75, 0.07	x	x	x
Autoclave-Clozapine 24h	x	0.02, 0.05	x	x
Donor3-Clozapine 8h	x	x	0.01	x
Donor3-Clozapine 24h	0.23	x	x	0.03
Donor3-DMSO 8h	x	x	x	0.16

A paired t test was conducted on the clozapine levels in the donor 3 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 3 samples incubated with DMSO for 8 hours and this met significance (p = 0.01).

A paired t test was conducted on the clozapine levels in the donor 3 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 3 samples incubated with clozapine for 24 hours and this was not significant (p = 0.23).

The clozapine levels in the donor 3 samples incubated with clozapine for 24 hours were found to have an equal variance to the clozapine levels in the samples with media and clozapine (p = 0.37) and the two-sample t test of equal variance was not significant (p = 0.42).

The clozapine levels in the donor 3 samples incubated with clozapine for 24 hours were found to have an unequal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 24h (p = 0.02). This is evident from the large variation in the non-autoclaved samples, compared to the low variation in the autoclaved samples – from the error bars in the bar graph in Figure X. The two-sample t test of unequal variance was significant (p = 0.05).

A paired t test was conducted on the clozapine levels in the donor 3 samples incubated with clozapine for 24 hours and the clozapine levels in the donor 3 samples incubated with DMSO for 24 hours and this did not meet significance (p = 0.03).

A paired t test was conducted on the clozapine levels in the donor 3 samples incubated with DMSO for 8 hours and the clozapine levels in the donor 3 samples incubated with DMSO for 24 hours was not significant ($p = 0.16$).

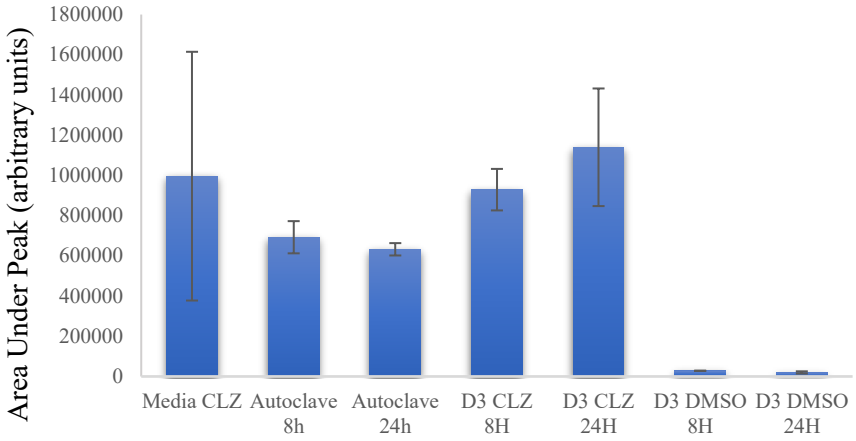


Figure 41. Bar graph displaying the clozapine levels in samples using human donor 3 (D3) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 3 faeces incubated with clozapine for 8 hours (D3-CLZ 8h), and for 24 hours (D3-CLZ 24h), donor 3 faeces incubated with DMSO for 8 hours (D3-DMSO 8h) and for 24 hours (D3-DMSO 24h) and media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

Norclozapine Levels

Qualitative

Figure 42 illustrates the GCMS output of Donor 3 samples and controls.

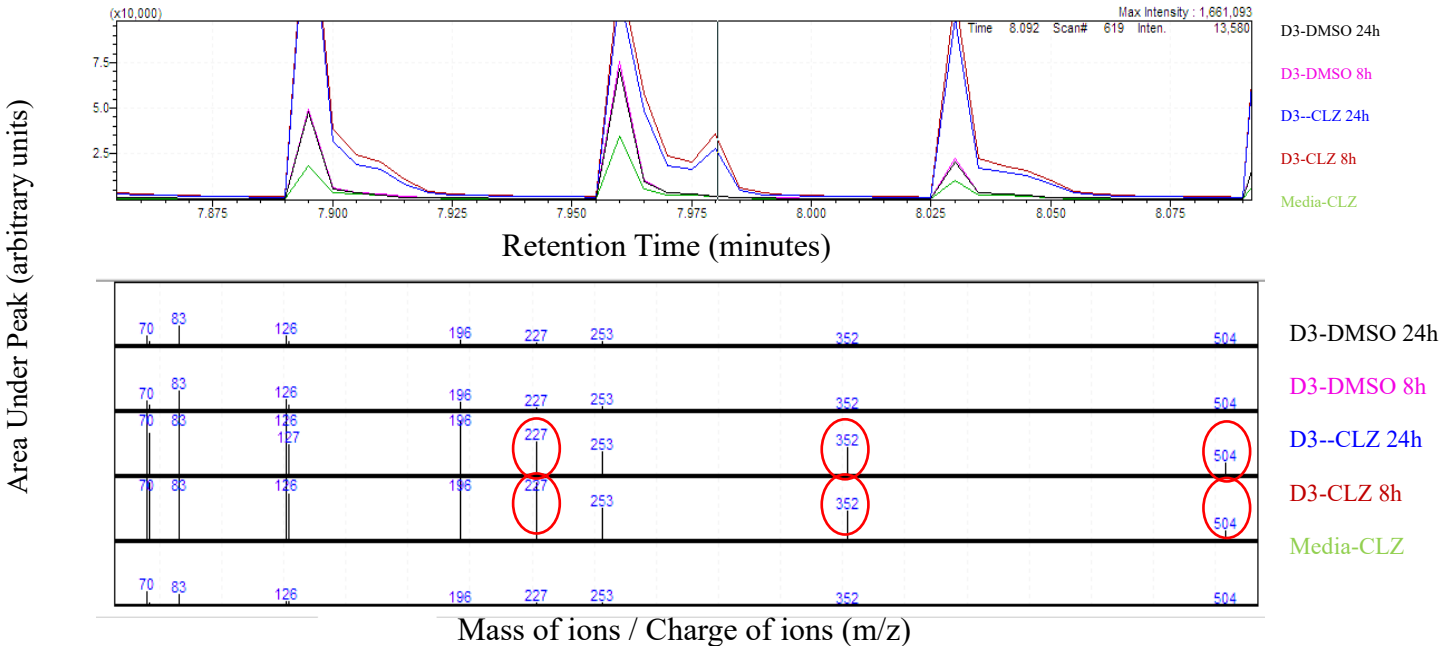


Figure 42. Chromatogram and mass spectra of human samples including donor 3 faeces incubated with clozapine for 8 hours (D3-CLZ 8h), and for 24 hours (D3-CLZ 24h), donor 2 faeces incubated with DMSO for 8 hours (D3-DMSO 8h) and for 24 hours (D3-DMSO 24h) and media incubated with clozapine (media-

CLZ), at 7.98 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for norclozapine; 227, 352 and 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in large quantities in the samples with faeces incubated with clozapine – the blue and red lines on the chromatogram and the presence of ions 227, 352, and 504 m/z on spectra 3 and 4.

All the other lines of the chromatogram show minimal/no levels of norclazpine - the pink, black and green lines signifying the donor 3 faeces incubated with DMSO and sample with media and clozapine. Their spectra (1, 2 and 5, respectively) also do not contain the qualifying ions for norclozapine

Quantitative

The norclozapine levels in the donor 3 samples incubated with clozapine for 8 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.005$) and the two-sample t test of unequal variance met significance ($p = 0.001$).

The norclozapine levels in the donor 3 samples incubated with clozapine for 8 hours were found to have an equal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.08$) and the two-sample t test of equal variance did not meet significance ($p = 0.48$)

Table 12. Analysis of Equal Variance F tests and T tests of Norclozapine levels in Donor 3 Samples and controls. Variance not conducted on paired samples. results were reported using the format of (f test p value, t test p value).

	Donor3-Clozapine 8h	**Donor3-Clozapine 24h**	**Donor3-DMSO 8h**	**Donor3-DMSO 24h**
Media-Clozapine	0.005, 0.001	<0.0001 , 0.1	x	x
Autoclaved-Clozapine 8h	0.08, 0.48	x	x	x
Autoclave-Clozapine 24h	x	0.03, 0.35	x	x
Donor3-Clozapine 8h	x	x	0.001	x
Donor3-Clozapine 24h	0.21	x	x	0.10
Donor3-DMSO 8h	x	x	x	0.16

A paired t test was conducted on the norclozapine levels in the donor 3 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 3 samples incubated with DMSO for 8 hours and this met significance ($p = 0.001$).

A paired t test was conducted on the norclozapine levels in the donor 3 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 3 samples incubated with clozapine for 24 hours and this was not significant ($p = 0.21$).

The norclozapine levels in the donor 3 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p < 0.0001$) and the two-sample t test of unequal variance was not significant ($p = 0.10$).

The norclozapine levels in the donor 3 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 24h ($p = 0.03$). This can be seen by the large variance in the non-autoclaved group, by the error bar in Figure 43. The two-sample t test of unequal variance was not significant ($p = 0.35$).

A paired t test was conducted on the norclozapine levels in the donor 3 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 3 samples incubated with DMSO for 24 hours, which was not significant ($p = 0.001$).

A paired t test was conducted on the norclozapine levels in the donor 3 samples incubated with DMSO for 8 hours and the norclozapine levels in the donor 3 samples incubated with DMSO for 24 hours and this was not significant ($p = 0.16$).

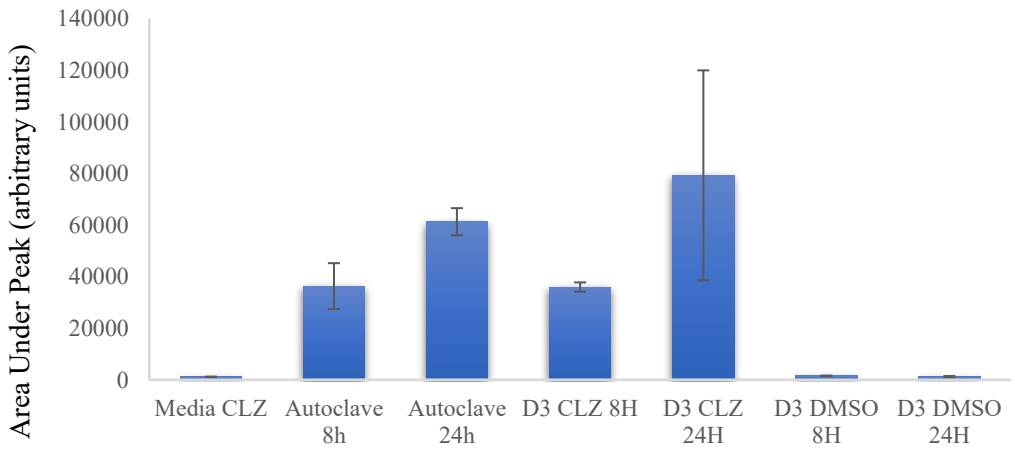


Figure 43. Bar graph displaying the norclozapine levels in samples using human donor 3 (D3) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 3 faeces incubated with clozapine for 8 hours (D3-CLZ 8h), and for 24 hours (D3-CLZ 24h), donor 3 faeces incubated with DMSO for 8 hours (D3-DMSO 8h) and for 24 hours (D3-DMSO 24h) and media incubated with clozapine (media-CLZ). DMSO = dimethyl sulfoxide. CLZ = clozapine.

Norclozapine:clozapine Ratio

Figure 44 displays the ratio of norclozapine:clozapine in the human donor 3 samples.

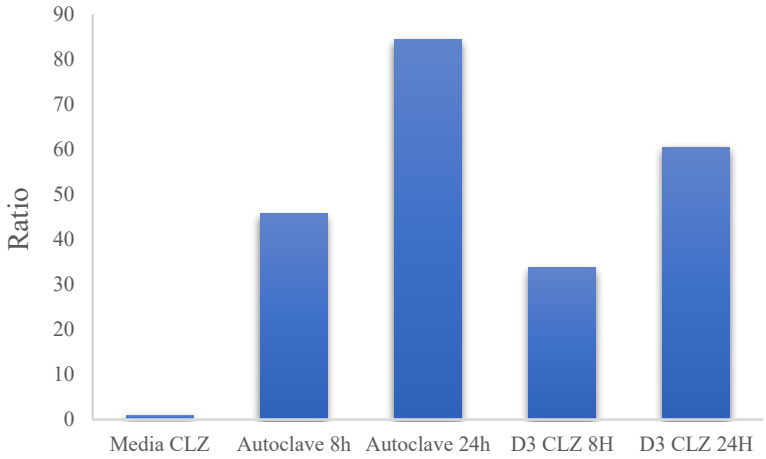


Figure 44. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 3, including autoclaved faeces incubated with clozapine, human donor 3 faeces incubated with clozapine samples for 8 hours (D3-CLZ 8h), and for 24 hours (D3-CLZ 24h), donor 3 faeces incubated with DMSO for 8 hours (D3-DMSO 8h) and for 24 hours (D3-DMSO 24h), as well as media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

For donor 3, the faeces incubated with clozapine showed similar clozapine-metabolism to donor 2, as the norclozapine:clozapine ratios are also lower than the ratios produced by the autoclaved samples, with 8 hours of incubation demonstrating a ratio of around 30, and 24 hours of incubation displaying a ratio of around 65.

Donor 4

Clozapine levels

Figure 45 illustrates the GCMS output of Donor 4 samples and controls.

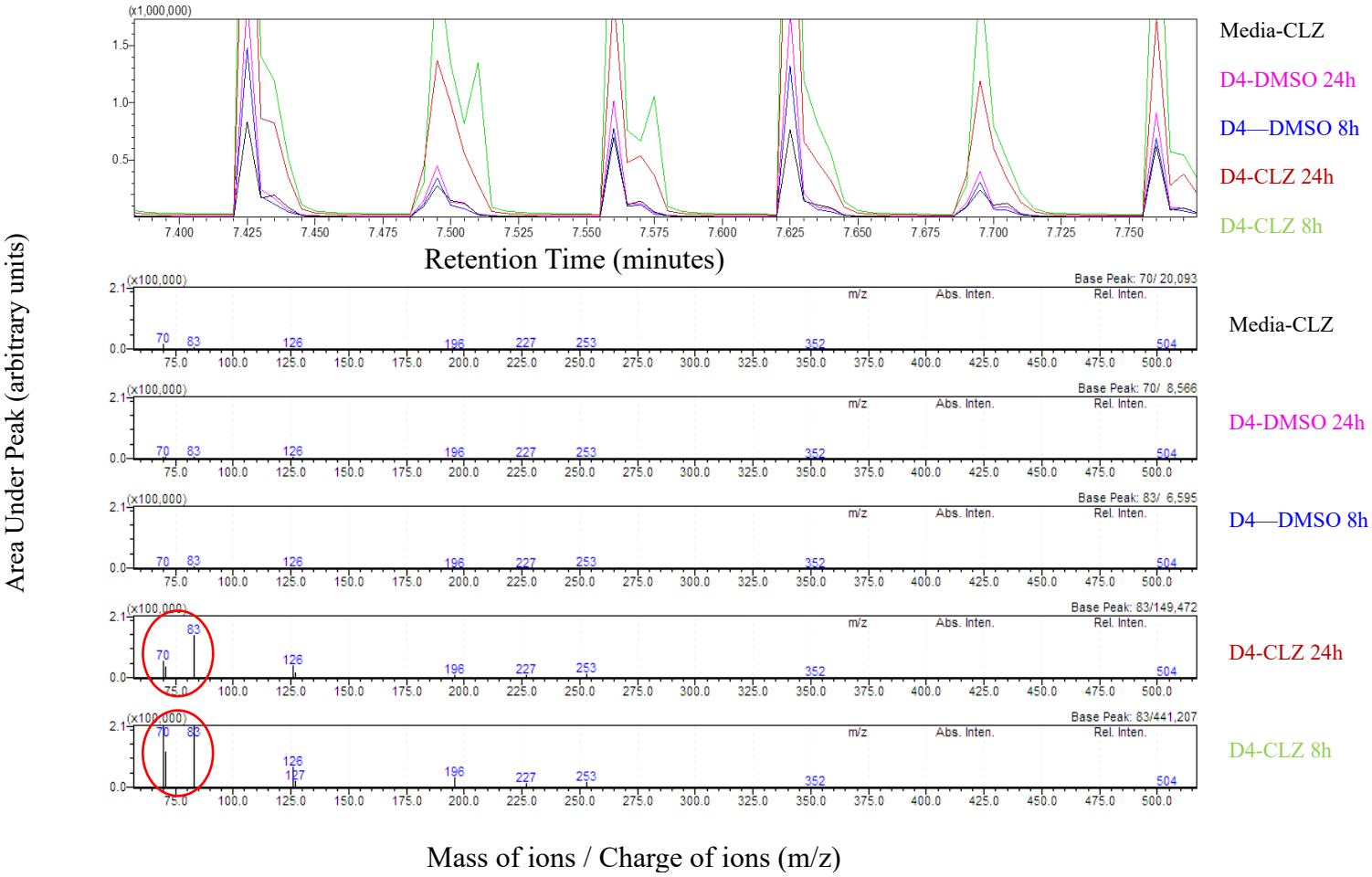


Figure 45. Chromatogram and mass spectra of human samples including donor 4 faeces incubated with clozapine for 8 hours (D4-CLZ 8h), and for 24 hours (D4-CLZ 24h), donor 4 faeces incubated with DMSO for 8 hours (D4-DMSO 8h) and for 24 hours (D4-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.575 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for clozapine; 70, 71 and 83 m/z.

It is clear from the chromatogram in Figure 45 that there is minimal clozapine in the Donor4-DMSO samples (pink and blue lines), and media-clozapine sample (black line). However, the Donor 3 samples incubated with clozapine, the drug can be detected (red and green). This is also evident in the mass spectra, where red circles denote the presence of qualifying ions for clozapine, 70, 71 and 83 m/z, in spectra 4 and 5, for the samples incubated with clozapine, but not in 1, 2 and 3.

Quantitative

The clozapine levels in the donor 4 samples incubated with clozapine for 8 hours were found to have an unequal variance to the clozapine levels in the samples that with media and clozapine ($p = 0.001$). This is evident from the error bars on the bar graph in Figure 46, which vary in size. The two-sample t test of unequal variance did not meet significance ($p = 0.17$).

The clozapine levels in the donor 4 samples incubated with clozapine for 8 hours were found to have an equal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.08$) and the two-sample t test of equal variance did meet significance ($p = 0.003$).

A paired t test was conducted on the clozapine levels in the donor 4 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 4 samples incubated with clozapine for 24 hours was not significant ($p = 0.09$).

Table 13. Analysis of Equal Variance F tests and T tests of Clozapine Levels in the Donor 4 Samples and controls. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor4-Clozapine 8h	**Donor4-Clozapine 24h**	**Donor4-DMSO 8h**	**Donor4-DMSO 24h**
Media-Clozapine	0.001, 0.17	0.004,0.18	x	x
Autoclaved-Clozapine 8h	0.08, 0.003	x	x	x
Autoclave-Clozapine 24h	x	0.83, <0.0001	x	x
Donor4-Clozapine 8h	x	x	x, 0.003	x
Donor4-Clozapine 24h		x	x	x, 0.006
Donor4-DMSO 8h	x	x	x	x, 0.33

The clozapine levels in the donor 4 samples incubated with clozapine for 24 hours were found to have an unequal variance to the clozapine levels in the samples with media and clozapine ($p = 0.004$), which can be seen from the error bars. The two-sample t test of unequal variance did not meet significance ($p = 0.18$).

The clozapine levels in the donor 4 samples incubated with clozapine for 24 hours were found to have an equal variance to the clozapine levels in the samples that were autoclaved with clozapine for 24 hours ($p = 0.83$) and the two-sample t test of unequal variance did meet significance ($p < 0.0001$).

A paired t test was conducted on the clozapine levels in the donor 4 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 4 samples incubated with DMSO for 8 hours was significant ($p = 0.003$).

A paired t test was conducted on the clozapine levels in the donor 4 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 4 samples incubated with DMSO for 8 hours was significant ($p = 0.006$). A paired t test was conducted on the clozapine levels in the donor 4 samples incubated with DMSO for 8 hours and the clozapine levels in the donor 4 samples incubated with DMSO for 24 hours was not significant ($p = 0.33$).

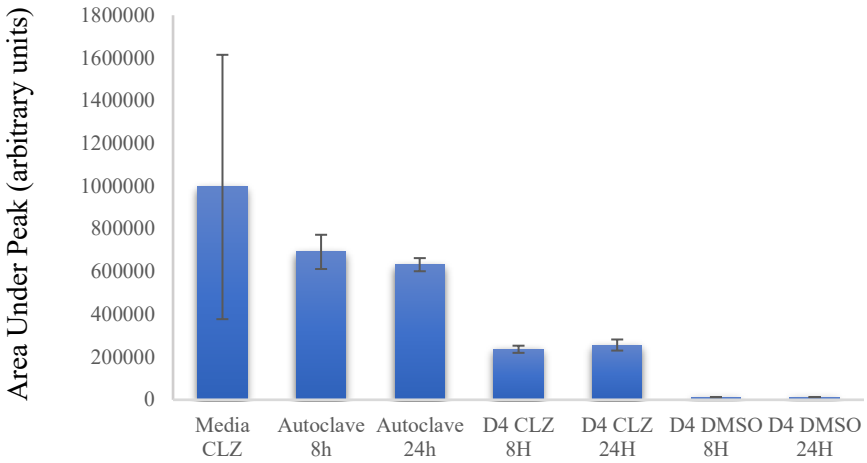


Figure 46. Chromatogram and mass spectra of human samples including donor 4 faeces incubated with clozapine for 8 hours (D4-CLZ 8h), and for 24 hours (D4-CLZ 24h), donor 4 faeces incubated with DMSO for 8 hours (D4-DMSO 8h) and for 24 hours (D4-DMSO 24h) and media incubated with clozapine (media-CLZ). DMSO = dimethyl sulfoxide, CLZ = clozapine.

Norclozapine Levels

Figure 47 illustrates the GCMS output of Donor 4 samples and controls.

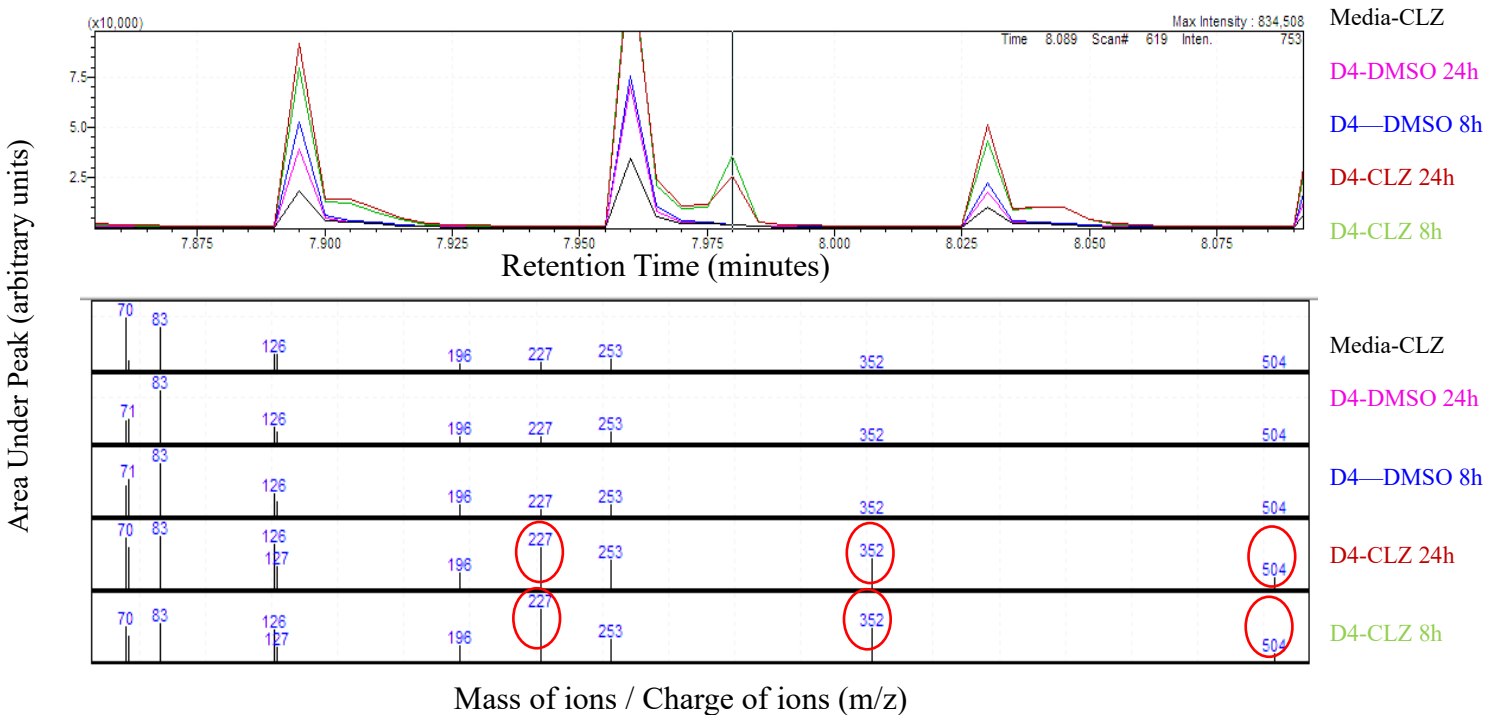


Figure 47. Chromatogram and mass spectra of human samples including donor 4 faeces incubated with clozapine for 8 hours (D4-CLZ 8h), and for 24 hours (D4-CLZ 24h), donor 4 faeces incubated with DMSO for 8 hours (D4-DMSO 8h) and for 24 hours (D4-DMSO 24h) and media incubated with clozapine (media-

CLZ), at 7.98 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for norclozapine; 227, 352 and 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in large quantities in the samples with faeces incubated with clozapine – the red and green lines on the chromatogram and the presence of ions 227, 352, and 504 m/z on spectra 4 and 5.

All the other lines of the chromatogram show minimal/no levels of norclazpine – the black, pink, and blue lines signifying the donor 4 faeces incubated with DMSO and sample with media and clozapine. Their spectra (1, 2 and 3, respectively) also do not contain the qualifying ions for norclozapine

Quantitative

The norclozapine levels in the donor 4 samples incubated with clozapine for 8 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.0004$). This evident from the error bars in the bar graph in Figure 48, showing that the media condition shows minimal variance. The two-sample t test of unequal variance met significance ($p = 0.02$).

The norclozapine levels in the donor 4 samples incubated with clozapine for 8 hours were found to have an equal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.69$). The error bars of these two conditions are similar sizes (Figure 48). The two-sample t test of equal variance did not meet significance ($p = 0.34$).

A paired t test was conducted on the norclozapine levels in the donor 4 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 4 samples incubated with DMSO for 8 hours and this met significance ($p = 0.02$).

Table 14. Analysis of Equal Variance F tests and T tests of Norclozapine Levels in Donor 4 Samples and Controls. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor4-Clozapine 8h	**Donor4-Clozapine 24h**	**Donor4-DMSO 8h**	**Donor4-DMSO 24h**
Media-Clozapine	0.0004, 0.02	0.004, 0.02	x	x
Autoclaved-Clozapine 8h	0.69, 0.34	x	x	x
Autoclave-Clozapine 24h	x	0.83, <0.0001	x	x
Donor4-Clozapine 8h	x	x	x, 0.02	x
Donor4-Clozapine 24h	0.2	x	x	x, 0.001
Donor4-DMSO 8h	x	x	x	x, 0.45

A paired t test was conducted on the norclozapine levels in the donor 4 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 4 samples incubated with clozapine for 24 hours and this was not significant ($p = 0.20$).

The norclozapine levels in the donor 4 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.004$) and the two-sample t test of unequal variance was significant ($p = 0.02$).

The norclozapine levels in the donor 4 samples incubated with clozapine for 24 hours were found to have an equal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 24h ($p = 0.83$) and the two-sample t test of equal variance was significant ($p < 0.001$).

A paired t test was conducted on the norclozapine levels in the donor 4 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 4 samples incubated with DMSO for 24 hours, which was significant ($p = 0.001$).

A paired t test was conducted on the norclozapine levels in the donor 4 samples incubated with DMSO for 8 hours and the norclozapine levels in the donor 4 samples incubated with DMSO for 24 hours and this was not significant ($p = 0.45$).

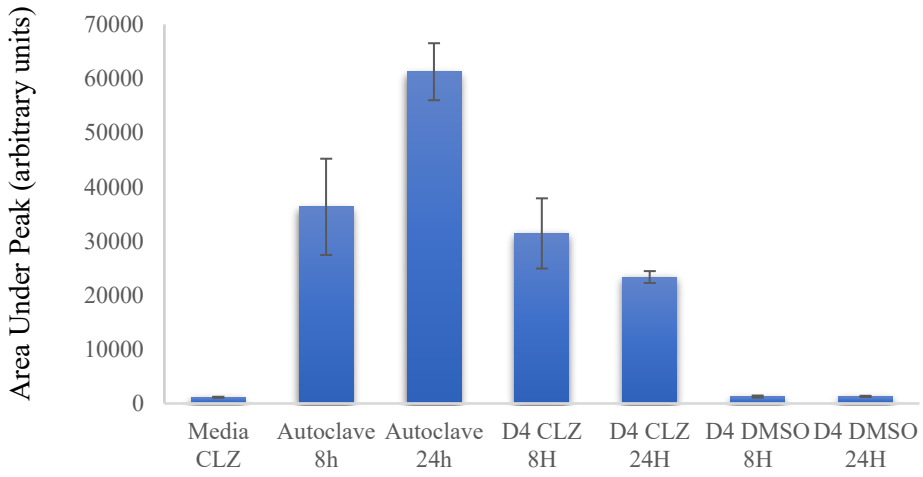


Figure 48. Bar graph displaying the norclozapine levels in samples using human donor 4 (D4) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 4 faeces incubated with clozapine for 8 hours (D4-CLZ 8h), and for 24 hours (D4-CLZ 24h), donor 4 faeces incubated with DMSO for 8 hours (D4-DMSO 8h) and for 24 hours (D4-DMSO 24h) and media incubated with clozapine (media-CLZ). DMSO = dimethyl sulfoxide. CLZ = clozapine.

Norclozapine:clozapine Ratio

Figure 49 displays the ratio of norclozapine:clozapine in the human donor 4 samples.

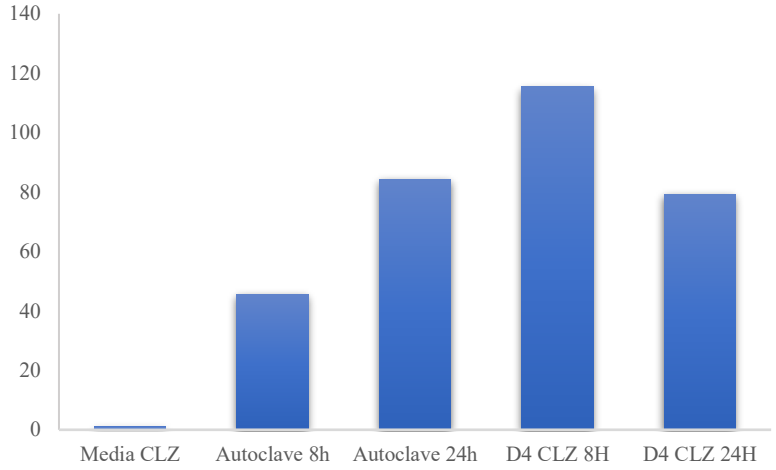


Figure 49. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 4, including autoclaved faeces incubated with clozapine, human donor 4 faeces incubated with clozapine samples for 8 hours (D4-CLZ 8h), and for 24 hours (D4-CLZ 24h), donor 4 faeces incubated with DMSO for 8 hours (D4-DMSO 8h) and for 24 hours (D4-DMSO 24h), as well as media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

For donor 4, the faeces incubated with clozapine showed slightly higher clozapine-metabolism than donors 2 and 3, with the norclozapine:clozapine ratios also higher those produced by the autoclaved samples. D4 CLZ 8h demonstrated a ratio of around 110, and 24 hours around 80.

Donor 5

Clozapine levels

Figure 50 illustrates the GCMS output of Donor 1 samples and controls.

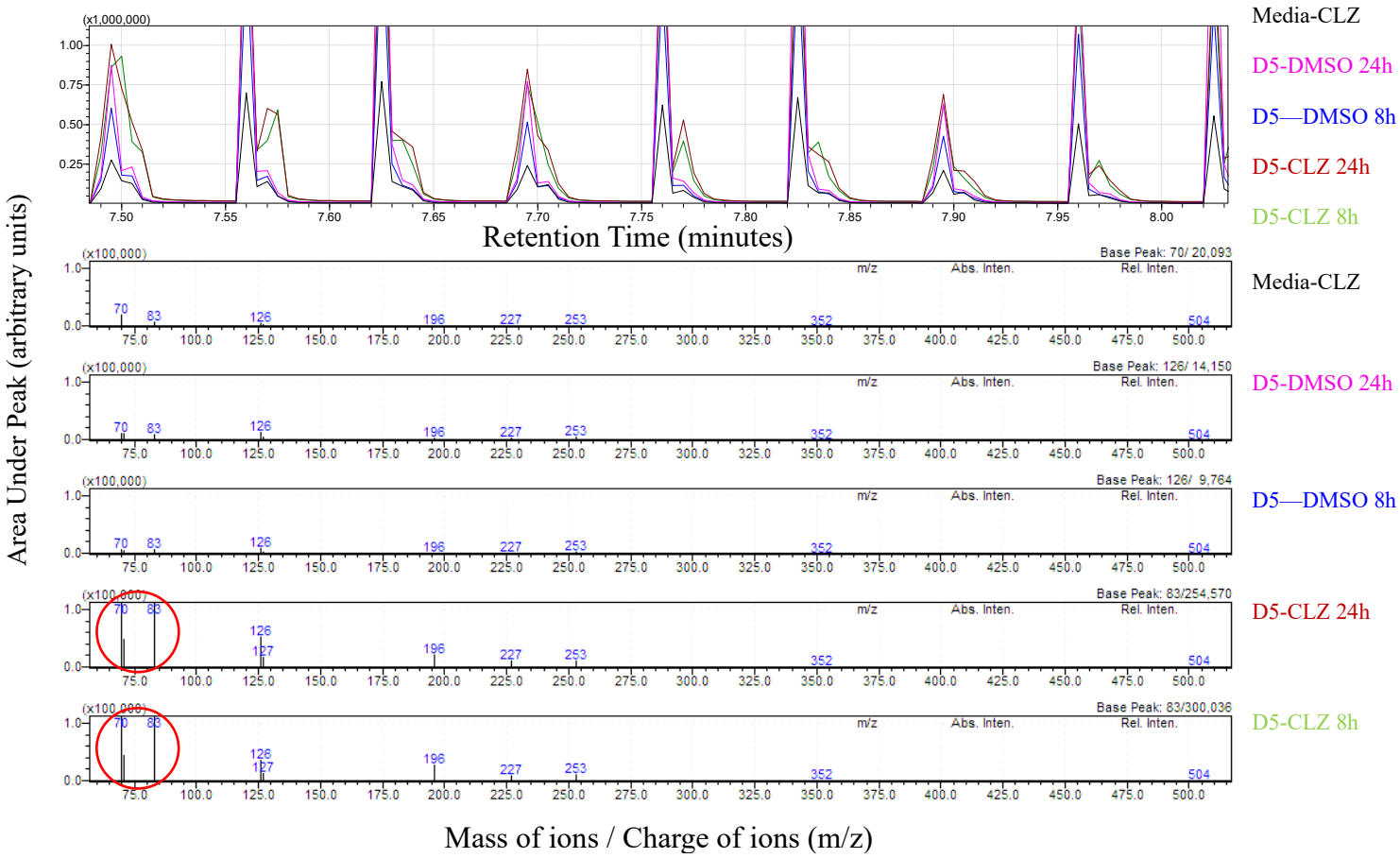


Figure 50. Chromatogram and mass spectra of human samples including donor 4 faeces incubated with clozapine for 8 hours (D4-CLZ 8h), and for 24 hours (D4-CLZ 24h), donor 4 faeces incubated with DMSO for 8 hours (D4-DMSO 8h) and for 24 hours (D4-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.775 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for clozapine; 70, 71, and 83 m/z.

It is clear from the chromatogram in Figure X that there is minimal clozapine in the Donor5-DMSO samples (pink and blue lines), and media-clozapine sample (black line). However, the Donor 5 samples incubated with clozapine, the drug can be detected (red and green). This is also evident in the mass spectra, where red circles denote the presence of qualifying ions for clozapine, 70, 71 and 83 m/z, in spectra 4 and 5, for the samples incubated with clozapine, but not in 1, 2 and 3.

Quantitative

The clozapine levels in the donor 5 samples incubated with clozapine for 8 hours were found to have an unequal variance to the clozapine levels in the samples with media and clozapine ($p = 0.03$). This can be seen in the error bars in the bar graph (Figure 51) – the donor condition has vary small variation, whereas the media condition has large variation. The two-sample t test of unequal variance did not meet significance ($p = 0.16$).

The clozapine levels in the donor 5 samples incubated with clozapine for 8 hours were found to have an equal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.93$). Both have similar sized error bars (see Figure 51). The two-sample t test of equal variance did not meet significance ($p = 0.01$)

A paired t test was conducted on the clozapine levels in the donor 5 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 5 samples incubated with DMSO for 8 hours did not meet significance ($p = 0.06$).

A paired t test was conducted on the clozapine levels in the donor 5 samples incubated with clozapine for 8 hours and the clozapine levels in the donor 5 samples incubated with clozapine for 24 hours was not significant ($p = 0.11$).

Table 15. Analysis of Equal Variance F tests and T tests of Clozapine Levels in Donor 5 Samples and controls. Variance not conducted on paired samples.

	Donor5-Clozapine 8h	**Dono5-Clozapine 24h**	**Donor5-DMSO 8h**	**Donor5-DMSO 24h**
Media-Clozapine	0.03, 0.16	0.004,0.20	x	x
Autoclaved-Clozapine 8h	0.93, 0.01	x	x	x
Autoclave-Clozapine 24h	x	0.05, 0.002	x	x
Donor5-Clozapine 8h	x	x	0.06	x
Donor5-Clozapine 24h	0.11	x	x	< 0.001
Donor5-DMSO 8h	x	x	x	0.16

The clozapine levels in the donor 5 samples incubated with clozapine for 24 hours were found to have an unequal variance to the clozapine levels in the samples with media and clozapine ($p = 0.004$). There was extremely low variation in the donor samples (error bars, Figure 51). The two-sample t test of unequal variance did not meet significance ($p = 0.20$).

The clozapine levels in the donor 5 samples incubated with clozapine for 24 hours were found to have an unequal variance to the clozapine levels in the samples that were autoclaved and incubated with clozapine for 24h ($p = 0.05$) and the two-sample t test of unequal variance did not meet significance ($p = 0.002$).

A paired t test was conducted on the clozapine levels in the donor 5 samples incubated with clozapine for 24 hours and the clozapine levels in the donor 5 samples incubated with DMSO for 24 hours did not meet significance ($p < 0.001$).

A paired t test was conducted on the clozapine levels in the donor 5 samples incubated with DMSO for 8 hours and the clozapine levels in the donor 5 samples incubated with DMSO for 24 hours was not significant ($p = 0.16$).

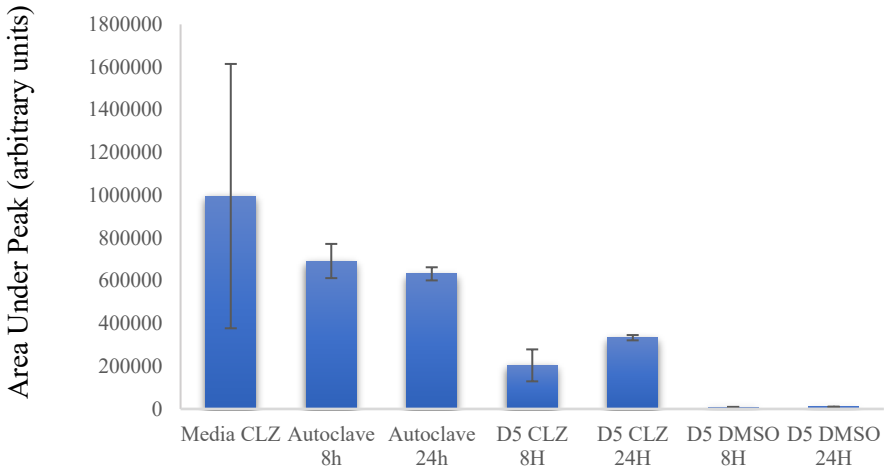


Figure 51. Bar graph displaying the clozapine levels in samples using human donor 5 (D5) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 5 faeces incubated with clozapine for 8 hours (D5-CLZ 8h), and for 24 hours (D5-CLZ 24h), donor 4 faeces incubated with DMSO for 8 hours (D5-DMSO 8h) and for 24 hours (D5-DMSO 24h) and media incubated with clozapine (media-CLZ). DMSO = dimethyl sulfoxide. CLZ = clozapine.

Norclozapine Levels

Figure 52 illustrates the GCMS output of Donor 5 samples and controls.

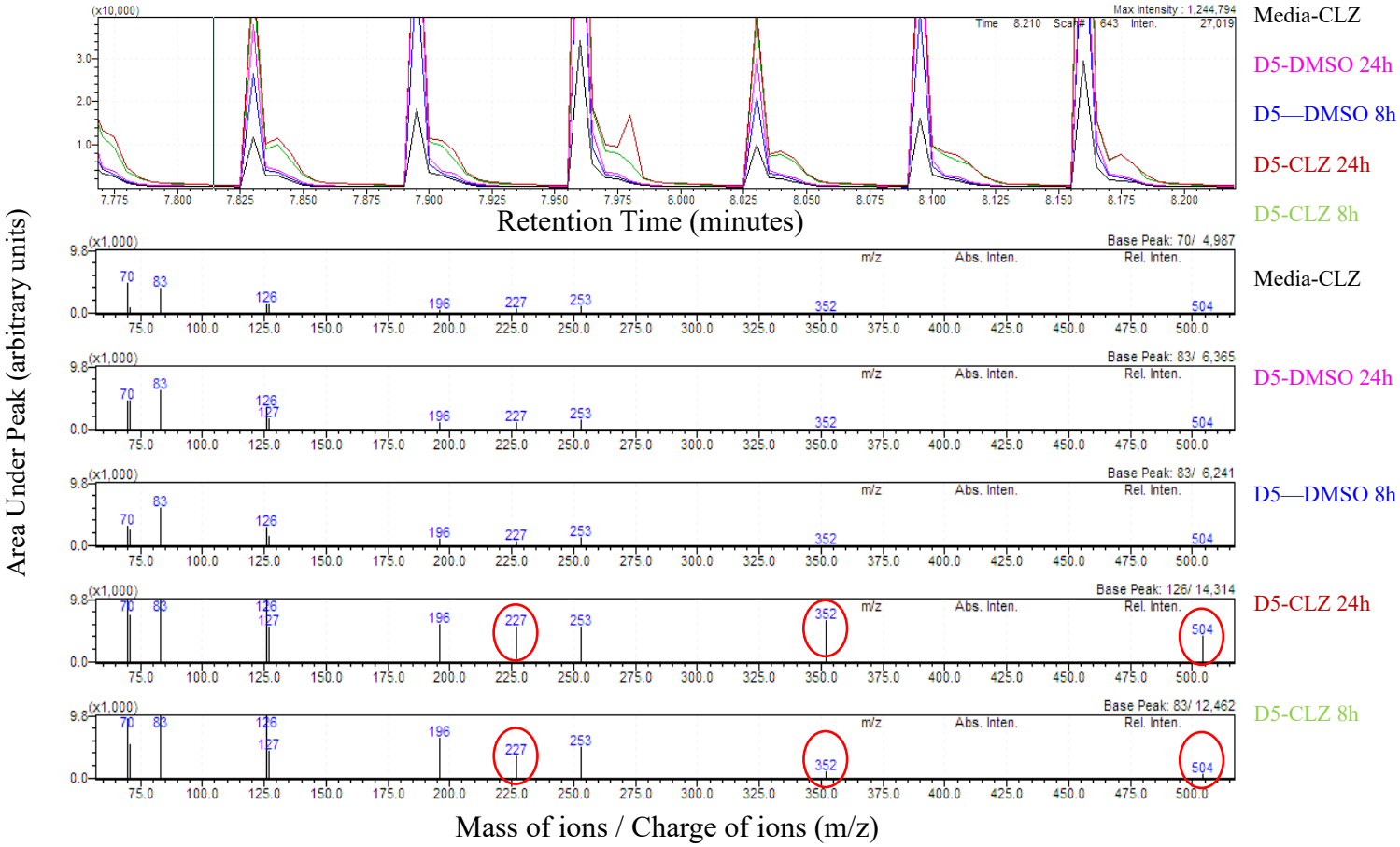


Figure 52. Chromatogram and mass spectra of human samples including donor 5 faeces incubated with clozapine for 8 hours (D5-CLZ 8h), and for 24 hours (D5-CLZ 24h), donor 5 faeces incubated with DMSO for 8 hours (D5-DMSO 8h) and for 24 hours (D5-DMSO 24h) and media incubated with clozapine (media-CLZ), at 7.98 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for norclozapine; 227, 352 and 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in large quantities in the samples with faeces incubated with clozapine – the red and green lines on the chromatogram and the presence of ions 227, 352, and 504 m/z on spectra 4 and 5. The quantities of norclozapine in the D5-CLZ 8 hour condition appear smaller than in the 8 hour condition.

All the other lines of the chromatogram show minimal/no levels of norclozapine – the black, pink, and blue lines signifying the donor 4 faeces incubated with DMSO and sample with media and clozapine. Their spectra (1, 2 and 3, respectively) also do not contain the qualifying ions for norclozapine

Quantitative

The norclozapine levels in the donor 4 samples incubated with clozapine for 8 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.0002$). This evident

from the error bars in the bar graph in Figure 53, showing that the media condition shows minimal variance. The two-sample t test of unequal variance met significance ($p = 0.01$).

The norclozapine levels in the donor 5 samples incubated with clozapine for 8 hours were found to have an equal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 8 hours ($p = 0.97$), evident by the equally sized error bars in Figure 53. The two-sample t test of equal variance did not meet significance ($p = 0.10$)

A paired t test was conducted on the norclozapine levels in the donor 5 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 5 samples incubated with DMSO for 8 hours and this did not met significance ($p = 0.06$).

A paired t test was conducted on the norclozapine levels in the donor 5 samples incubated with clozapine for 8 hours and the norclozapine levels in the donor 5 samples incubated with clozapine for 24 hours and this was significant ($p = 0.01$).

Table 16. Analysis of Equal Variance F tests and T tests Norclozapine Levels in Donor 5 Samples and controls. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor5-Clozapine 8h	**Donor5-Clozapine 24h**	**Donor5-DMSO 8h**	**Donor5-DMSO 24h**
Media-Clozapine	0.0002, 0.01	0.01, 0.0001	x	x
Autoclaved-Clozapine 8h	0.97, 0.10	x	x	x
Autoclave-Clozapine 24h	x	0.03, 0.18	x	x
Donor5-Clozapine 8h	x	x	x, 0.06	x
Donor5-Clozapine 24h	x, 0.01	x	x	x, 0.0002
Donor5-DMSO 8h	x	x	x	0.33

The norclozapine levels in the donor 5 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.01$). The two-sample t test of unequal variance was highly significant ($p = 0.0001$).

The norclozapine levels in the donor 5 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples that were autoclaved and incubated with clozapine for 24h ($p = 0.03$). The donor 5 condition had very low variance (see error bars Figure 53). The two-sample t test of unequal variance was not significant ($p = 0.18$).

A paired t test was conducted on the norclozapine levels in the donor 5 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 5 samples incubated with DMSO for 24 hours, which was not significant ($p = 0.0002$).

A paired t test was conducted on the norclozapine levels in the donor 5 samples incubated with DMSO for 8 hours and the norclozapine levels in the donor 5 samples incubated with DMSO for 24 hours and this was not significant ($p = 0.33$).

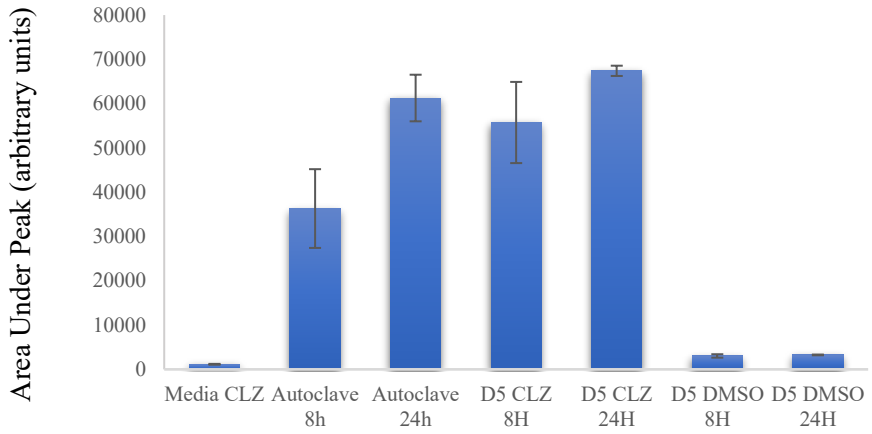


Figure 53. Bar graph displaying the norclozapine levels in samples using human donor 5 (D5) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 5 faeces incubated with clozapine for 8 hours (D5-CLZ 8h), and for 24 hours (D5-CLZ 24h), donor 5 faeces incubated with DMSO for 8 hours (D5-DMSO 8h) and for 24 hours (D5-DMSO 24h) and media incubated with clozapine (media-CLZ). DMSO = dimethyl sulfoxide. CLZ = clozapine.

Norclozapine:Clozapine Ratio

Figure 54 displays the ratio of norclozapine:clozapine in the human donor 5 samples.

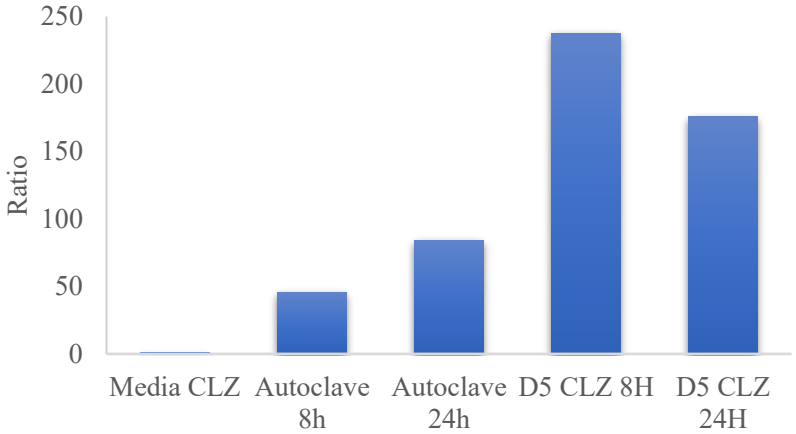


Figure X. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 5, including autoclaved faeces incubated with clozapine, human donor 5 faeces incubated with clozapine samples for 8 hours (D5-CLZ 8h), and for 24 hours (D5-CLZ 24h), donor 5 faeces incubated with DMSO for 8 hours (D5-DMSO 8h) and for 24 hours (D5-DMSO 24h), as well as media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

For donor 5, the faeces incubated with clozapine shows very high clozapine-metabolism, with the norclozapine:clozapine ratios far higher those produced by the autoclaved samples. D5 CLZ 8h demonstrated a ratio of around 230, and 24 hours around 170.

All Donors

Norclozapine:clozapine Ratio

Figure 55 shows bar graphs containing the ratios of all the experimental donor samples, compared to the media and autoclaved controls, at 8 hours incubation time (Fig. 55A) and 24 hours incubation time (Fig. 55B).

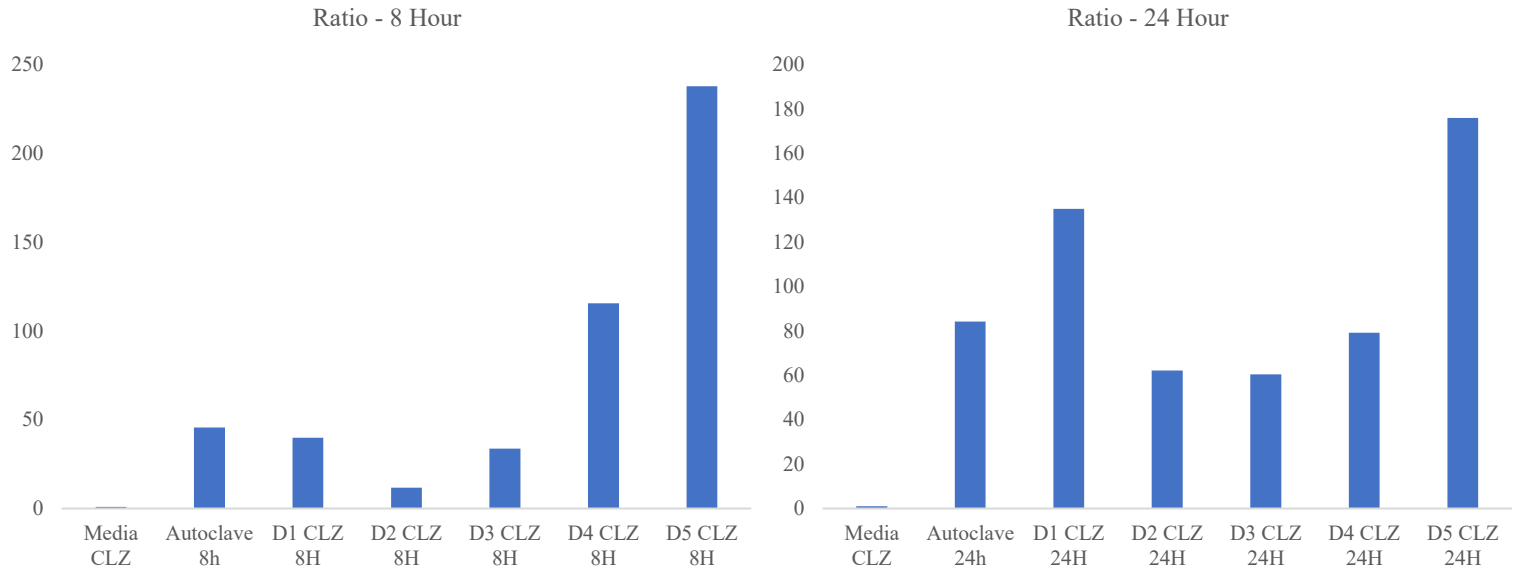


Figure 55: Comparative Analysis of Norclozapine:Clozapine Ratios in Donor Samples and Controls. Graph A shows samples incubated for 8 hours and Graph B shows samples incubated for 24 hours. CLZ = clozapine.

These bar graphs facilitate a clearer comparison of the variations in the ability of faeces from each donor to metabolise clozapine into norclozapine. At 8 hours, the ratios for samples from donors 1, 2 and 3 are all low, and lower than the autoclaved control. Samples from donors 4 and 5 however display significantly higher ratios of approximately 110 and 230, respectively.

At 24 hours, again samples from donors 2 and 3 exhibit a low level of conversion – lower than the autoclaved control. However, at this incubation point donor 1 displays a much higher ratio than at the 8 hour incubation time point, with a ratio of around 130. Interestingly, samples from donor 4 appear to lose ability to metabolise clozapine, displaying a lower ratio than the autoclaved samples. Donor 5, again, has a high ratios, albeit lower than its ratio at the 8 hour time point.

Microbial Derived Metabolism Analysis

The figure below shows a visual representation of the pearson correlation test between levels of clozapine and norclozapine across all donors.

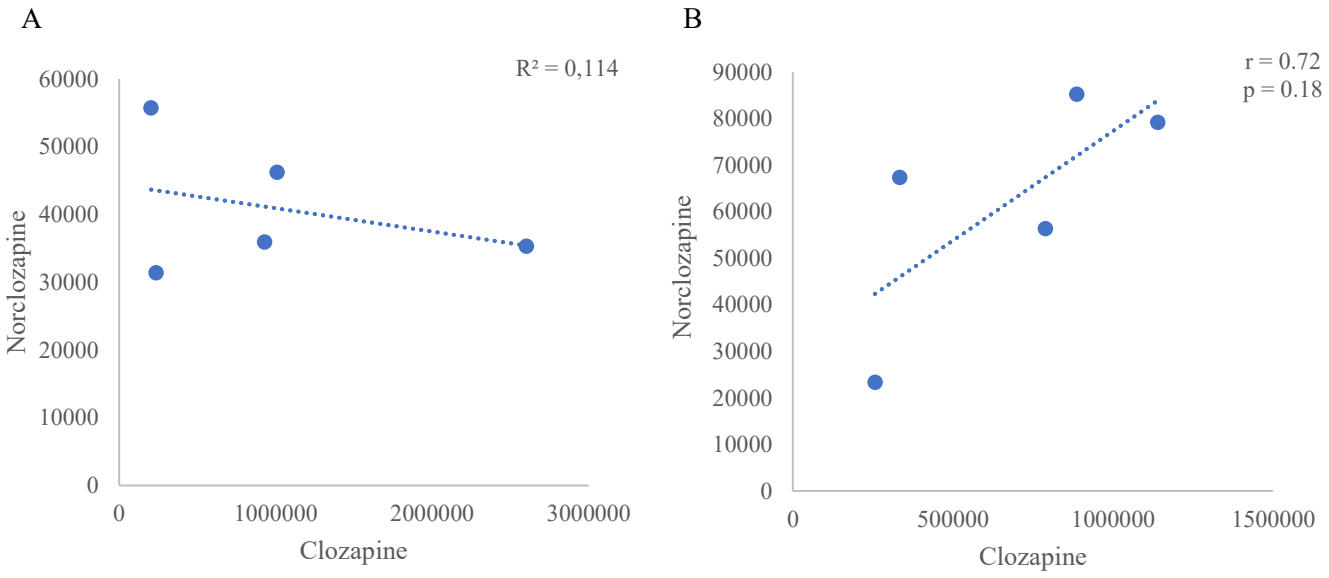


Figure 56. Correlational analysis between clozapine and norclozapine levels in all donors at A = 8 hours and B = 24 hours. Pearson r and corresponding p value are shown in right hand corner. Dotted line indicates linear trend.

The provided data indicate significant differences in norclozapine to clozapine ratios in faeces at different time points. At the 8-hour mark, there is a negative Pearson correlation between clozapine and norclozapine ($r = -0.338$). A t test showed that this was not statistically significant ($p = 0.536$). By 24 hours, there is a positive Pearson correlation ($r = 0.72$). This was also not statistically significant ($p = 0.18$).

DISCUSSION

Initial Validation of GCMS Methodology

The initial analysis, demonstrated that the optimised GC-MS methodology was capable of detecting both clozapine and its primary metabolite, norclozapine, in human faeces. The chromatograms and mass spectra obtained from human donor samples exhibited characteristic peaks corresponding to clozapine and norclozapine, which were consistent with their respective standards. These findings confirmed the method's suitability for quantifying clozapine and norclozapine levels in complex faeces matrices, and therefore justified its selection for further analyses.

FDA hydrolysis test

The negative controls were valid in this study, exhibiting statistically significantly low metabolic activity at both time points. This aligns with the expectation that bacterial metabolism is responsible for much of the hydrolytic activity and demonstrates a reasonable baseline of activity in our samples. The donor samples displayed a range of absorbance values at 8 hours, demonstrating individual variability in hydrolytic activity. Across the donor samples, light absorbance decreased from 8 hours to 24 hours, suggesting a time-dependent decline in metabolic activity. The lack of significant effect of time may be due to the low power of the test. However, the variation between donors emphasises that individual metabolic profiles vary and that the results are specific to hydrolytic enzyme activity.

These human faeces demonstrated a greater magnitude of hydrolytic activity, as determined by the FDA assay, in comparison to mouse faeces in the previous experiment. This observation is congruent with the extant literature, which documents a higher degree of complexity and diversity within the human gut microbiome relative to those in mice. The enhanced metabolic repertoire of the human gut, which has evolved to accommodate the digestion of a more varied and intricate range of dietary substrates, is likely a contributing factor to this disparity. Furthermore, the comparatively larger intestinal volume and prolonged transit time observed in humans may facilitate a more extensive and sustained period of metabolic processing.

Optimised In-Vitro Samples

Controls

There were no significant differences in clozapine levels across the control samples (the media-clozapine compared to the autoclaved-clozapine). This is to be expected as both have no live bacteria that can digest clozapine. There was, however, a high variance of clozapine levels within the media-clozapine samples, which

could be attributed to a possible pipetting error. Minimal levels of norclozapine were observed also observed in the media samples, which is again expected. Ratios were also minimal in samples with media only (as expected). Additionally, there were no significant differences in clozapine levels across the DMSO samples over time (the donor-DMSO samples incubated for 8 hours and those incubated for 24 hours). There were also no significant differences in norclozapine levels over time (the donor-DMSO samples incubated for 8 hours and those incubated for 24 hours). Again, this is to be expected as both should have no clozapine in them. However, significantly higher levels of norclozapine were observed in the autoclaved controls, which signifies either contamintation of these samples with live bacteria, an alternative factor within the killed-faeces sample that can break down clozapine, thermostable enzymes or chemical reactions induced by autoclaving. This also led to the presence of elevated ratios in autoclaved samples. This activity was interestingly undetected by the FDA test, which failed to detect enzymatic activity in these samples. This prompted the further investigation of the origin of this norclozapine-production with the proteinase K test and the dilutions, which will be reported in the next section.

Donors

The observed data highlight significant variability in the norclozapine to clozapine ratios across different donor samples and incubation periods, with ratios ranging from nearly negligible to over 200. Donors 1, 2 and 3 produced low ratios at the 8 hour incubation point, whereas donors 4 and 5 produced high ratios. A negative Pearson correlation between clozapine and norclozapine was demonstrated at this time point, which would his suggests that a substantial amount of clozapine is being converted to norclozapine, indicating active bacterial metabolism. However, this correlation did not meet significance, and therefore is likely due to random chance rather than a true relationship. Therefore, these correlation results *cannot be directly used* to inform clinical procedures at this time.

At the 24 hour incubation point, Donor 1 also produced high ratios, along with Donors 4 and 5. High ratios indicate presence of a mechanism in their faeces capable of metabolising clozapine. The Pearson correlation also suggests a different dynamic. A positive correlation means that as the concentration of clozapine increases, the concentration of norclozapine also increases, and vice versa. This shift from a negative to a positive correlation implies that the relationship between clozapine and norclozapine has changed over time. However this relationship also failed to meet significance.

Several factors could contribute to these changes in patterns over time. Initially, bacterial enzymes responsible for converting clozapine are highly active, resulting in high ratios and a negative correlation at the 8-hour mark. However, as time progresses, the availability of clozapine may decrease, reducing the substrate for the bacteria to act upon. Additionally, bacterial growth dynamics may shift, with bacteria entering a stationary phase where metabolic activity slows down due to nutrient limitations or the accumulation of metabolic by-products. Furthermore, the enzymes responsible for the conversion might become less active or degrade over time, contributing to the reduced conversion rates. Changes in the sample environment, such as pH shifts or the presence of inhibitory compounds, might also negatively impact bacterial activity and enzyme function.

Although the Pearson correlations did not reach statistical significance, the change from a negative to a positive correlation between clozapine and norclozapine from 8 to 24 hours *implies* that there may be a change in the process that facilitates the clozapine:norclozapine ratio. Specifically, the negative correlation at 8 hours may indicate ongoing conversion, while the positive correlation at 24 hours *suggests* a decrease in active conversion. This demonstrates that clozapine metabolism is not a static process and can vary across different time points.

To gain a more comprehensive understanding of these findings, several steps should be undertaken. Firstly, further investigation is required to confirm the presence of thermostable enzymes that might remain active post-autoclaving. Specific biochemical assays targeting various enzymatic activities should be employed to identify any such enzymes. It would also be beneficial to conduct further time-point analyses, investigate the impact of varying clozapine concentrations, and perform enzyme activity assays at different time points.

Finally, validating these findings through additional metabolic activity tests using different substrates and indicators is essential. This will help confirm the absence of metabolic activity and ensure the observed conversions are not influenced by biological factors.

Through these comprehensive investigations, we can elucidate the mechanisms driving the unexpected high ratios in the autoclaved samples and ensure accurate interpretations of the metabolic conversion process in the experimental donor samples.

Comparison of FDA Results and Metabolomic Results

Dsiciprenancies are observed between the results of the FDA test of metabolic activity with the observed norclozapine:clozapine ratios. Despite donors 1 and 2 showing the highest metabolic activity at 8 hours, these samples exhibited the lowest ratios of norclozapine:clozapine at the 8 hour time point. Conversely, the samples from donors 4 and 5, which exhibited comparitevly lower metabolic activity in the FDA test, demonstrated the highest norclozapine:clozapine ratios at the 8 hour time point. Additionally, the autoclaved controls exhibited higher metabolic activity in the FDA test at 8 hours, than 24 hours, but the ratios reflect the opposite – with a higher norclozapine:clozapine ratio demonstrated at 24 hours. This inconsistency in metabolic results is also demonstrated in 24 hours time point for the donor samples. All the donor samples demonstrated lower metabolic activity in the FDA test at 24 hours, yet donors 1 and 2 exhibited a 6 fold higher norclozapine:clozapine ratio, and donor 3 a 2-fold increase, at this time point compared to 8 hours. Donors 4 and 5 however exhibited lower norclozapine:clozapine ratios at the 24 hour time point compared to the 8 hour time point, consistent with their FDA tests. This generally inverse relationship of FDA output and norclozapine:clozapine ratios is a strong indication that the FDA assay is not measuring the specific enzymes or pathways responsible for clozapine metabolism. The fact that only donors 4 and 5 exhibit consistent drops in both measures of activity may indicate that these donors metabolise clozapine differently than the others. The fact that the discrepancies vary across donors suggests that there are individual differences in how clozapine is metabolised, highlighting the complexity of individual variation in drug metabolism.

Limitations

The small sample size and limited number of time points reduce the power of the statistical analysis. A larger donor cohort and more frequent timepoints to enhance statistical power and resolution of metabolic processes over time. Variation observed in media samples should also be addressed by repeating this method.

The discrepenacies between the FDA test and the metabolomic output indicate that it is not a direct measure of clozapine metabolism. This encourages the implementation of a more optimised approach to measuring metabolic activity. This could involve attempting to normalise the samples by FDA result, in order to achieve a similar metabolic capacity so that differences in clozapine metabolism will not be confounded by differences in general metabolic vigour.

By pursuing these lines of inquiry, we can elucidate the mechanisms driving the observed variations in norclozapine to clozapine ratios and gain insights into the bacterial metabolism of clozapine over time.

Conclusion

This experiment successfully validated a GC-MS method capable of detecting and quantifying clozapine and norclozapine in human faeces, thus confirming its suitability for further in-vitro analysis. Several key findings were elucidated.

Significant variability in clozapine metabolism was observed between human donors and across different incubation periods, demonstrating that clozapine metabolism is a dynamic process that varies considerably based on individual gut microbiome composition and environmental factors within the sample. Additionally, the autoclaved controls unexpectedly showed the production of norclozapine, indicating the presence of a yet-unidentified mechanism independent of live bacteria, which could have implications for future experimental design. Most importantly, discrepancies were observed between the FDA assay, a measure of overall hydrolytic activity, and clozapine-specific metabolism. These findings strongly suggest that the FDA assay needs further optimisation, and investigation needs to be conducted into the origin of the norclozapine production observed in the autoclaved samples.

Chapter 4:
Human Sample Analysis with Further Investigations

Introduction

Building on the previous experiments, the third experiment was conducted using samples from the same human donors, with additional optimisation steps to enhance the accuracy and reliability of the findings. This experiment aimed to replicate the methodology applied to mouse samples while incorporating further refinements to address specific challenges presented by human samples.

Based on the discrepancy between FDA and metabolomic output, it was decided a more optimised approach was needed. Normalising the samples by FDA result could achieve a similar metabolic capacity across the samples, which would help to control for variations in activity affecting variations in clozapine metabolism.

Furthermore, additional steps were implemented to determine the metabolic origin of the observed activity in the control samples. This included creating dilutions and assessing their metabolomic output, as well as employing proteinase K treatment to identify proteinaceous contributions to the observed metabolic activity. These steps were essential for isolating the specific factors contributing to clozapine metabolism and distinguishing between microbial and non-microbial influences.

The same rigorous statistical analysis used in prior experiments, including Pearson correlation and p-value significance testing, was applied to determine the involvement of the microbiome in clozapine metabolism. By replicating the experimental design with human samples and incorporating these additional optimisation steps, this study aims to validate and extend the findings from mouse models, ultimately contributing to a more comprehensive understanding of clozapine metabolism in human physiology.

Methodology

Normalising by Metabolic Activity

Analysis of human faeces was optimised by adding additional protocol. This included weighing samples prior to culturing. Additionally, after 20 hours of culturing, an FDA test identified the sample with the lowest metabolic activity (lowest absorbance), which served as a benchmark. This benchmark was used to calculate the volumes of culture and B3HI medium required to dilute the other samples to achieve equal concentration across cultures.

Determining Origin of Enzymes Responsible for Clozapine Metabolism

It could be possible that the enzymes resulting in the conversion of clozapine to norclozapine originate from human enzymes in the faeces, instead of any bacteria. To assess this, the donor faeces with the highest norclozapine production must first be determined via GCMS. Then, this sample will be diluted to produce a

1:10 dilution and 1:100 dilution. If norclozapine is produced via human enzymes, the 10- and 100-fold dilutions will show decreasing norclozapine, by a factor of 10 and 100 respectively, when analysed by GCMS. However, if norclozapine is produced via bacterial enzymes, the 10- and 100- fold dilutions will not impact the levels of norclozapine.

Proteinase K

To investigate the source of the enzymes that may contribute to production of norclozapine, proteinase K will be spiked into some of the samples with killed bacteria. Proteinase K can be used to digest proteins, which can serve many purpose, including the removal of proteinaceous templates from the binding sites of polymers (Erdőssy et al., 2017). In the case of this experiment, proteinase K will digest any proteases in the samples with killed bacteria, that could be responsible for norclozapine production, and these samples should not yield any norclozapine. If the samples that have not received the proteinase K treatment do yield norclozapine, then this will suggest that there are protease enzymes in these bacteria-killed samples that are responsible for this, which will imply that these enzymes originate from a source other than the bacteria.

All conditions used triplicate repeats. An extra autoclave condition was introduced with proteinase K treatment, which involved the addition of 10 uL of 0.05 mg/mL proteinase K per sample, followed by incubation for an hour at 55 °C, under aerobic conditions. Once complete, all autoclaved samples received clozapine as per the previous protocol. All samples were subsequently incubated at 37 °C for 24 hours and spun down.

Results

Metabolic Activity Results

This figure shows the results of the FDA hydrolysis test.

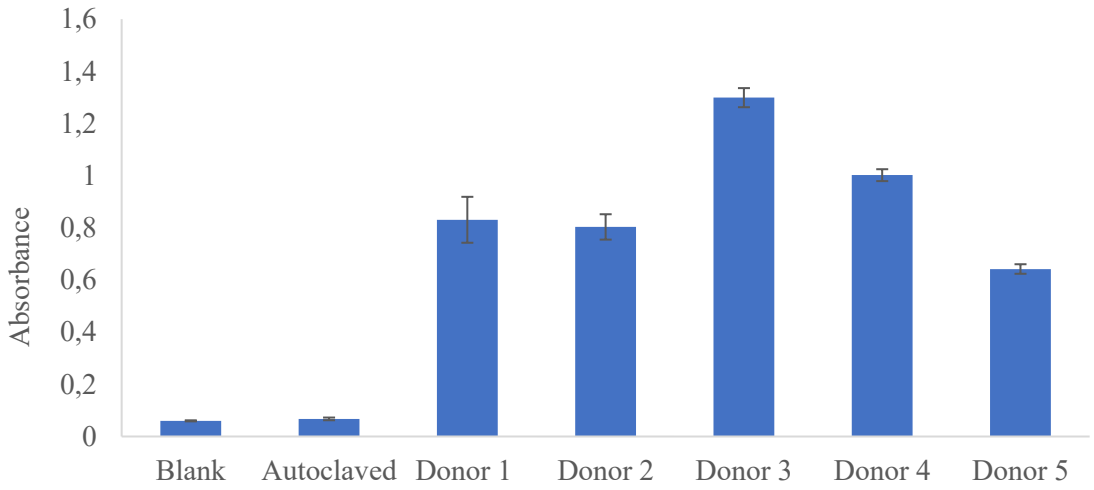


Figure 57. Results of FDA test of metabolic activity, with values of samples given in Absorbance. Samples include controls – a blank (containing media and FDA reagents in equal volume) and an autoclaved pooled faeces condition. Samples from donors 1 – 5.

It can be seen that Donor 5 is exhibits the lowest metabolic activity compared to the other 4 donors.

Weights of Samples

This figure is the weight calculated from the difference between the weight of the tube with sample and the weight of the tube once sample had been removed.

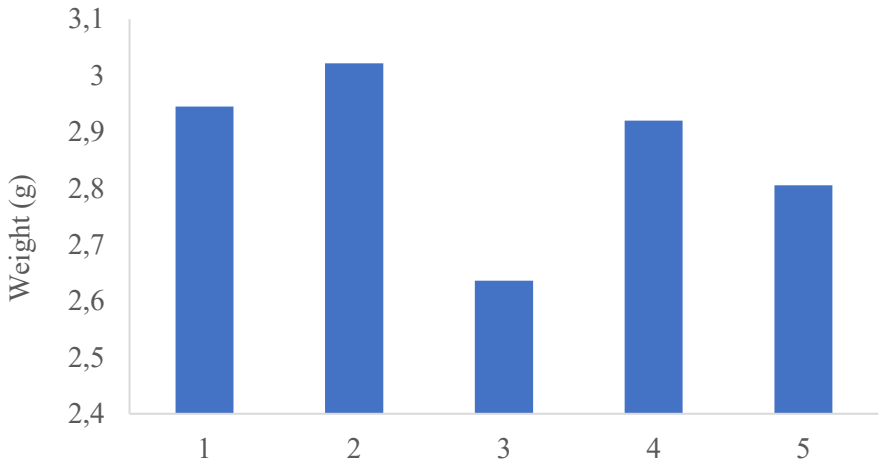


Figure 58: Initial fecal sample weights (grams), calculated as the difference between the weight of the sample tube with sample and the weight of the same tube after sample removal, for each of the five human donors.

Donor 1

Clozapine

Qualitative

Figure 59 illustrates the GCMS output of Donor 1 samples and controls.

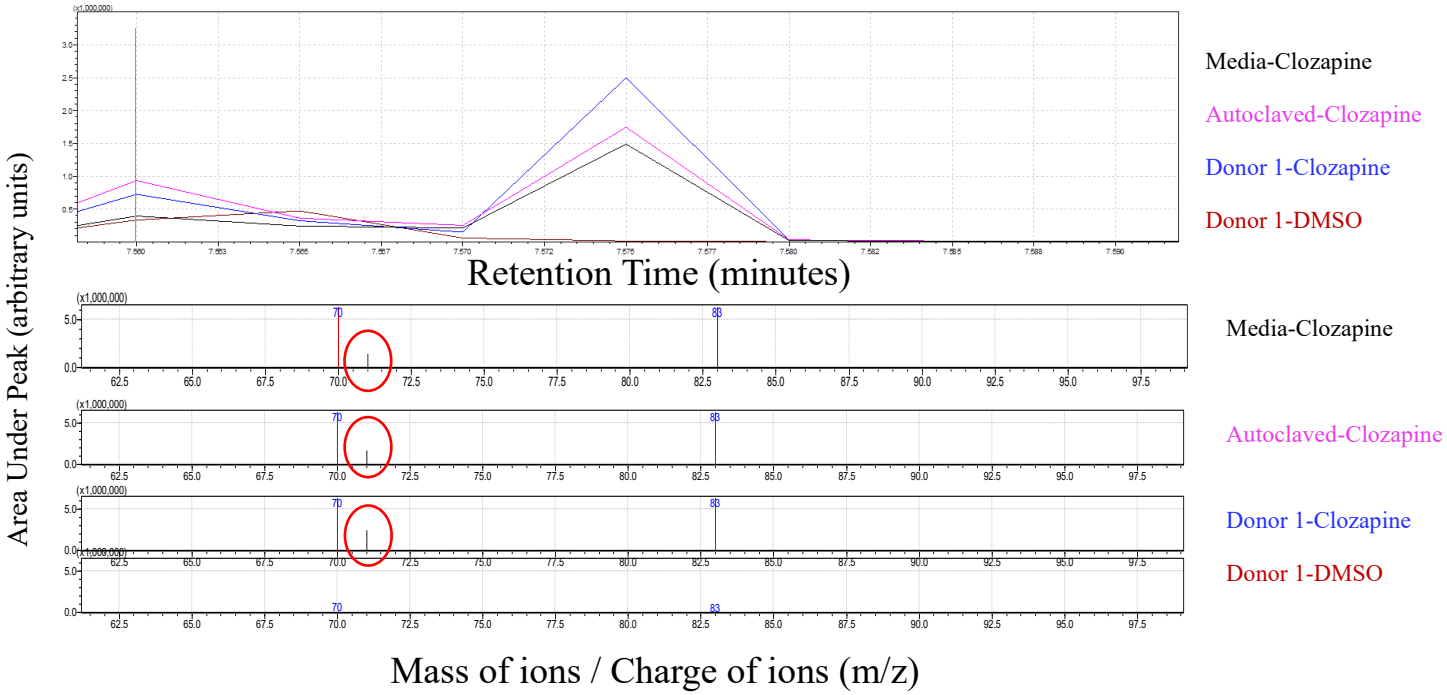


Figure 59. Chromatogram and mass spectra of media, autoclave, Donor 1-clozapine and Donor 1-DMSO at 7.575 minutes. Red circles indicate presence of qualifying ions. Red peaks indicate saturation.

There is minimal clozapine in the Donor1-DMSO control condition, whereas in the other samples incubated with clozapine, the drug can be detected. This is clear from the chromatogram in Figure 59 at 7.575 minutes where the samples are differentiated by colour, with the red line indicating the DMSO sample is close to zero. There is varying clozapine levels in the other samples, with clozapine levels highest in the Donor1-Clozapine condition (blue line chromatogram), and similarly lower levels in the control-clozapine conditions, media and autoclaved (black line chromatogram and pink line chromatogram, respectively). For the samples containing clozapine (spectra 1-3), the spectra contain red peaks at ions 70 and 83, which were previously used as qualifying ions. This red line indicates that the machine has been saturated for this ion and so cannot be used for qualification. The ion 71 m/z will be used, and is present in the samples containing clozapine, but not the sample containing DMSO (spectra 4), as indicated with red circles.

Quantitative

The repeated analysis are shown in Table 17. The clozapine levels in the donor 1 samples incubated with clozapine for 24 hours were found to have an equal variance to the clozapine levels in the samples with media and clozapine ($p = 0.724$). This variance can be identified by the similar sizes of the error bars in Figure 60. The two-sample t-test of equal variance was significantly higher in the donor samples ($p = 0.0037$).

A paired t-test was conducted on the clozapine levels in the donor 1 samples incubated with clozapine for 24 hours and the clozapine levels in the donor 1 samples incubated with DMSO for 24 hours. Clozapine levels were significantly higher in the donor1-clozapine samples (p = 0.00257).

Table17. Analysis of Equal Variance F tests and T tests of Clozapine Levels in Repeated Donor 1 Samples and controls. Asterisks indicate the sample in which levels are significantly higher. Results reported using the format of (f test p value, t test p value). Variance not conducted on paired samples.

	Donor1-Clozapine 24h	**Donor1-DMSO 24h**
Media-Clozapine	0.724, 0.0037 (**D)	x
Donor1-Clozapine 24h	x	0.00257 (**C)

Figure 60 displays the levels of clozapine in the donor 1 samples, compared to media and DMSO controls.

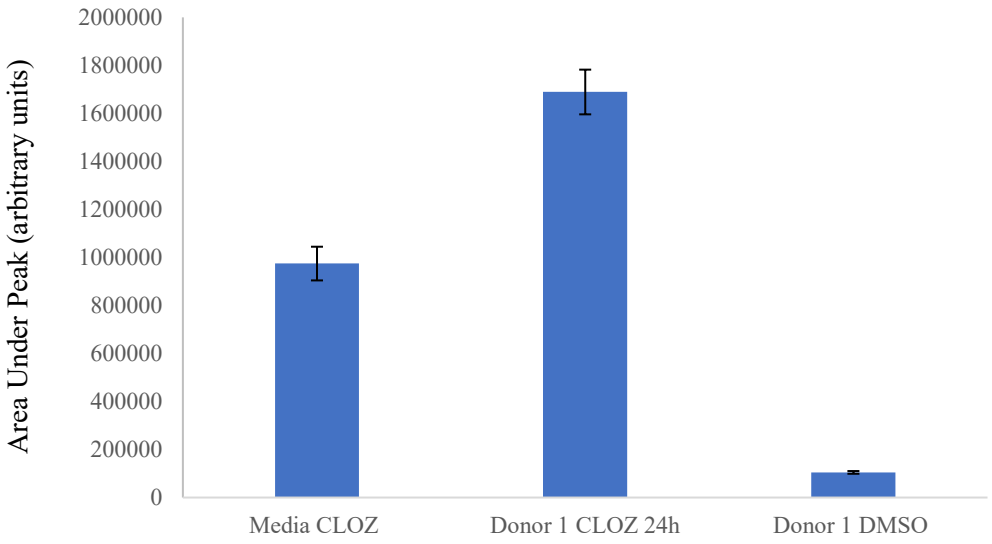


Figure 60. Bar graph displaying the clozapine levels in samples using human donor 1 (D1) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 1 faeces incubated with clozapine for 24 hours (Donor CLOZ 24h) and donor 1 faeces incubated with DMSO 24 hours (Donor 1 DMSO) and media incubated with clozapine (media-CLOZ). DMSO = dimethyl sulfoxide. CLOZ = clozapine. Error bars show standard error of the mean.

Comparison to Batch 1

The previous batch had much lower levels of clozapine in donor 1 CLOZ 24h samples. The levels in the Donor 1 DMSO samples were similar.

Norclozapine

Figure 61 illustrates the GCMS output of Donor 1 samples and controls.

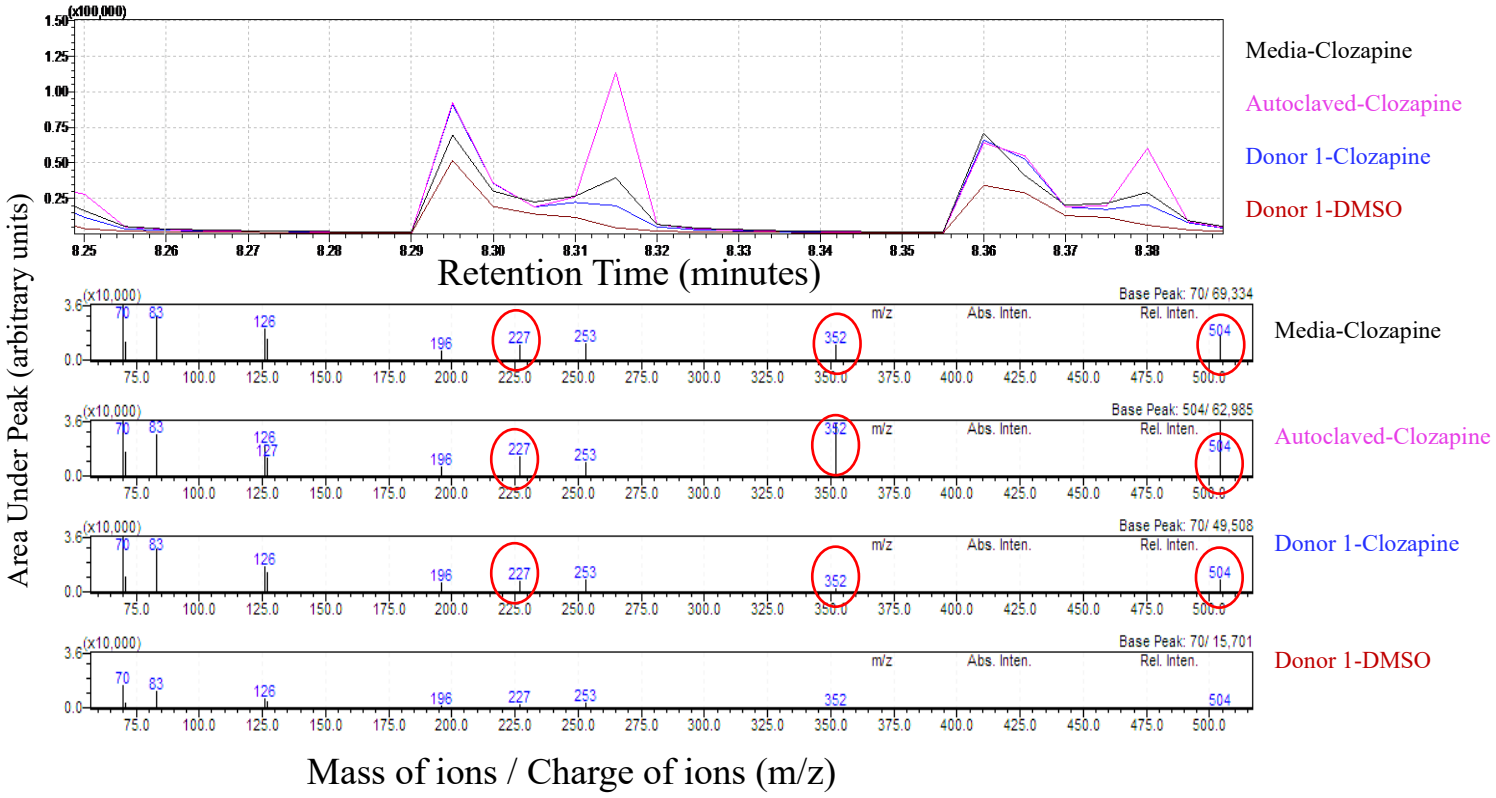


Figure 61. Chromatogram and mass spectra of human samples including donor 1 faeces incubated with clozapine and incubated with DMSO, and media and autoclaved faeces incubated with clozapine at 8.315 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for norclozapine; 227, 352 and 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in large quantities in the autoclaved sample with clozapine (pink line, spectra 2), using the ions 227, 352 and 504 m/z to qualify. Norclozapine is also present in the media-clozapine sample (black line, spectra 1) and the donor1-clozapine sample (blue line, spectra 3), in smaller quantities. The donor1-DMSO sample appears to contain no norclozapine (red line, spectra 4).

Quantitative

The repeated analysis of donor 1 samples can be seen in Table 18. The norclozapine levels in the donor 1 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.0377$). This evident from the large variance in the donor 1 samples, as indicated by the tall error bar in Figure 62. The media samples appear to have very small variance. The two-sample t-test of unequal variance was not significant ($p = 0.483$).

A paired t-test was conducted on the norclozapine levels in the donor 1 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 1 samples incubated with DMSO for 24 hours, which was not significant (p = 0.0666).

Table 18. Analysis of Equal Variance F tests and T tests of Norclozapine Levels in Repeated Donor 1 Samples and controls. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor1-Clozapine 24h	**Donor1-DMSO 24h**
Media-Clozapine	0.0377, 0.483	x
Donor1-Clozapine 24h	x	0.0666

Figure 62 shows the bar chart representing the norclozapine levels in the media and DMSO controls, compared to the Donor sample incubated with clozapine.

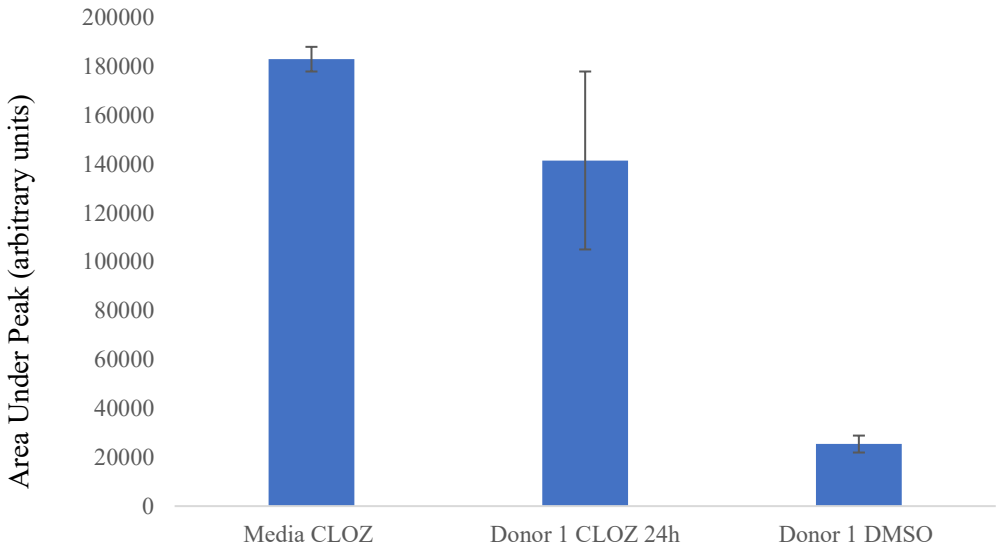


Figure 62. Bar graph displaying the norclozapine levels in samples using human donor 1 (D1) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 1 faeces incubated with clozapine for 24 hours (Donor 1 CLOZ 24h) and donor 1 faeces incubated with DMSO 24 hours (Donor 1 DMSO) and media incubated with clozapine (media-CLOZ). DMSO = dimethyl sulfoxide. CLOZ = clozapine. Error bars show standard error of the mean.

Comparison to Batch 1

The norclozapine levels in these donor 1 samples were similar to the previous batch of donor 1 samples.

Norclozapine:Clozapine Ratio

Figure 63 displays the ratio of norclozapine:clozapine in the human donor 1 samples.

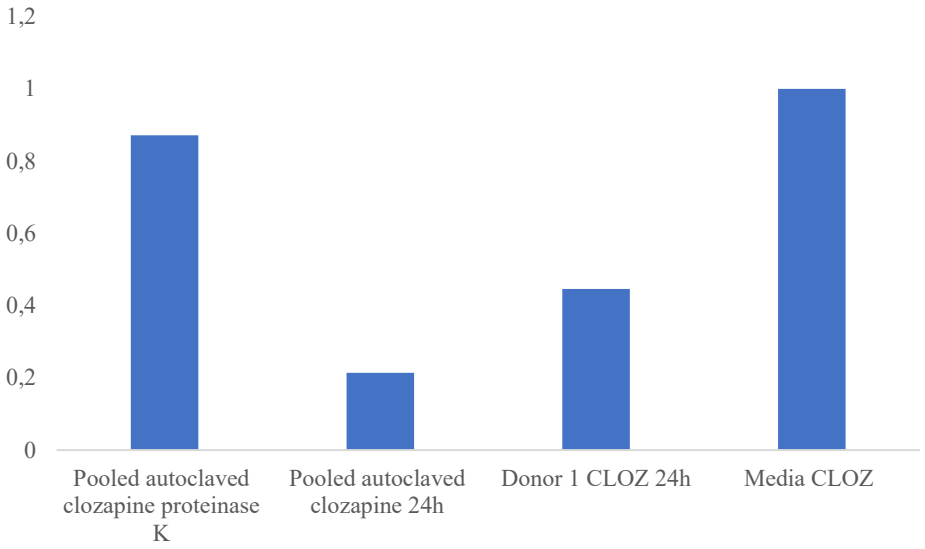


Figure 63. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 1, including autoclaved faeces incubated with clozapine, autoclaved faeces incubated with clozapine and proteinase K, human donor 1 faeces incubated with clozapine for 24 hours, as well as media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

The media-clozapine samples and the samples with pooled autoclaved faeces, incubated with clozapine and proteinase K have the highest norclozapine:clozapine ratio. Donor 1 has a moderate ratio, and the standard autoclaved samples exhibits a ratio which is approximately ½ of the donor.

Comparison to Batch 1

Proportionally, the pooled autoclaved and donor 1 clozapine samples appear to have a similar relationship, with a relatively similar increase in ratio observed in the donor 1 samples. However, these are well below the ratio in the media sample, which was not observed in the previous batch.

Donor 2

Clozapine

*F*igure 64 illustrates the GCMS output of Donor 2 samples and controls.

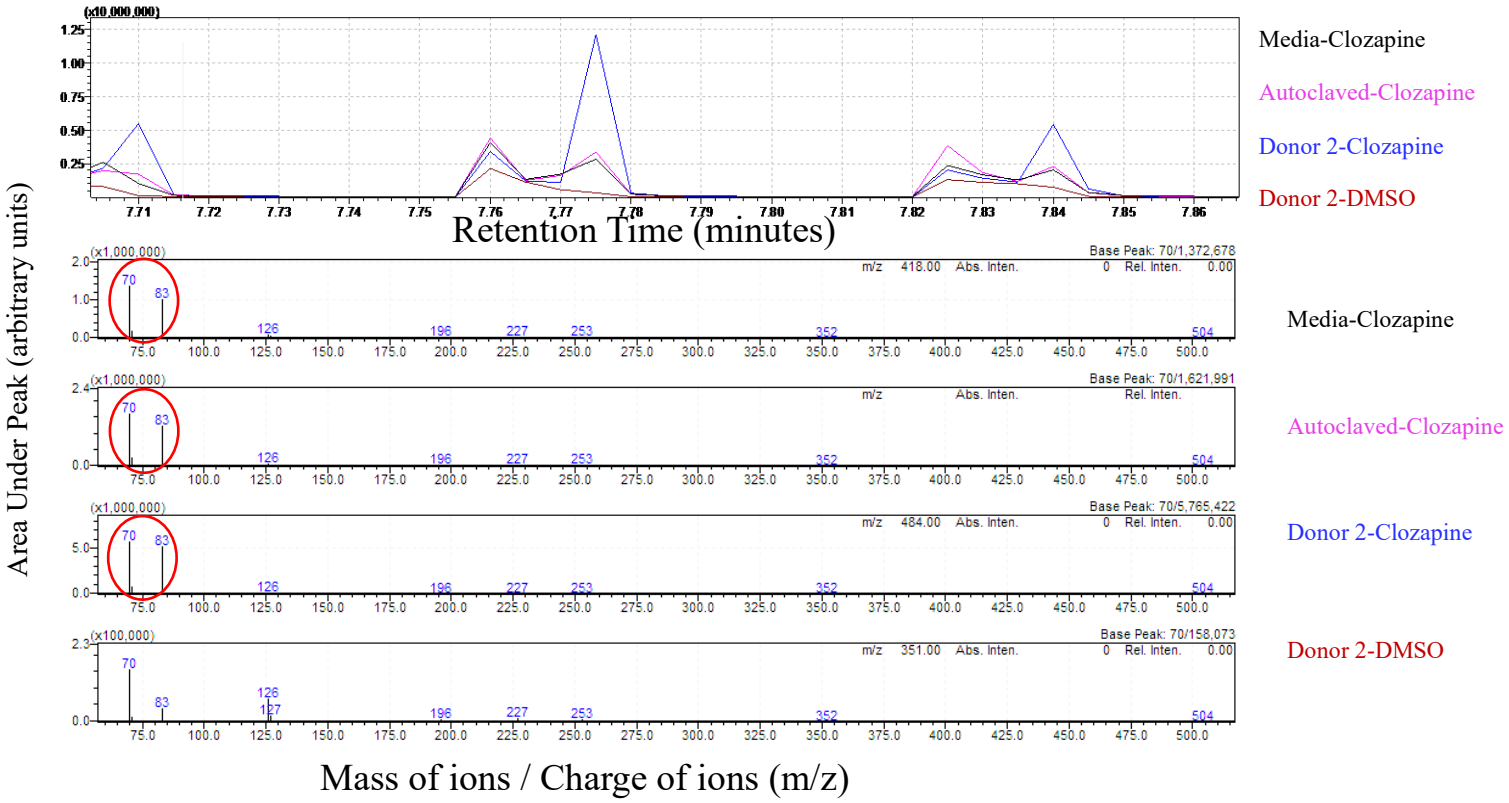


Figure 64. Chromatogram and mass spectra of human samples including donor 2 faeces incubated with clozapine and incubated with DMSO, and media and autoclaved faeces incubated with clozapine at 7.775 minutes. DMSO = dimethyl sulfoxide. Red circles denote presence of qualifying ions for clozapine; 71 m/z.

Both the chromatogram and the spectra reveal that clozapine is present in large quantities in the donor 1 sample with clozapine (blue line, spectra 3), using the ion 71 m/z to qualify. Clozapine is also present in the media-clozapine sample (black line, spectra 1) and the autoclaved-clozapine sample (pink line, spectra 2), in smaller quantities. The donor1-DMSO sample appears to contain no norclozapine (red line, spectra 4).

Quantitative

The repeat analyses are shown in Table 19. The clozapine levels in the donor 2 samples incubated with clozapine for 24 hours were found to have an equal variance to the clozapine levels in the samples with media and clozapine ($p = 0.345$) and the two-sample t-test of equal variance showed that these levels were significantly higher in the donor-clozapine sample ($p < 0.001$).

A paired t-test was conducted on the clozapine levels in the donor 2 samples incubated with clozapine for 24 hours and the clozapine levels in the donor 2 samples incubated with DMSO for 24 hours, and these levels were significantly higher in the donor 2 clozapine 24h samples (p = 0.00404).

Table 19. Analysis of Equal Variance F tests and T tests of Clozapine Levels in Repeated Donor 2 Samples and controls. Asterisks indicate the sample in which levels are significantly higher. Results reported using the format of (f test p value, t test p value).Variance not conducted on paired samples.

	Donor2-Clozapine 24h	**Donor2-DMSO 24h**
Media-Clozapine	0.345, <0.001 (**M)	x
Donor2-Clozapine 24h	X	0.00404 (**C)

Figure 65 shows the levels of clozapine in the donor 2 samples and controls.

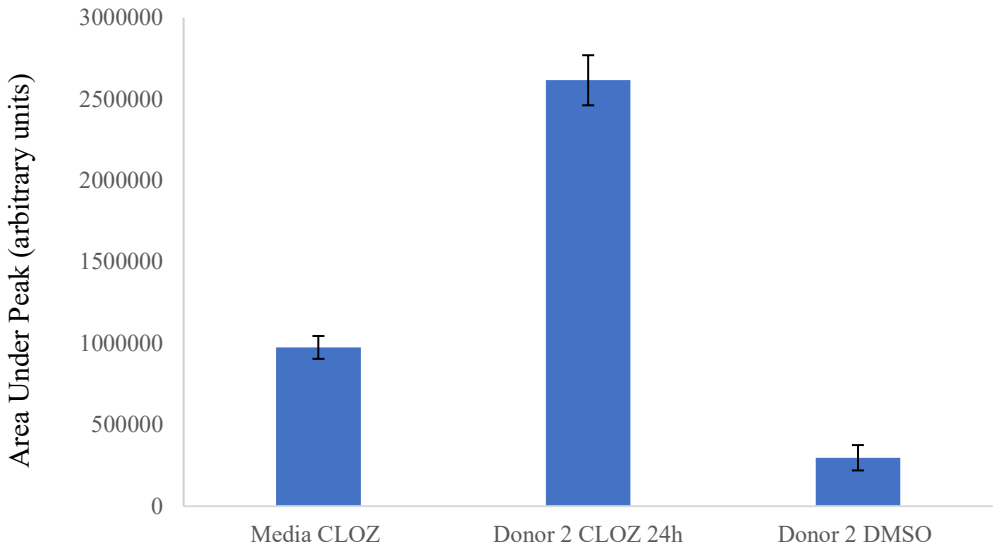


Figure 65. Bar graph displaying the clozapine levels in samples using human donor 2 (D2) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 2 faeces incubated with clozapine for 24 hours (Donor CLOZ 24h) and donor 2 faeces incubated with DMSO 24 hours (Donor 2 DMSO) and media incubated with clozapine (media-CLOZ). DMSO = dimethyl sulfoxide. CLOZ = clozapine. Error bars show standard error of the mean.

Comparison to Batch 1

The donor 2 clozapine samples appear to contain three times as much clozapine as the previous donor 2 samples, and the donor 2 DMSO samples almost double.

Norclozapine Levels

Figure 66 illustrates the GCMS output of Donor 2 samples and controls.

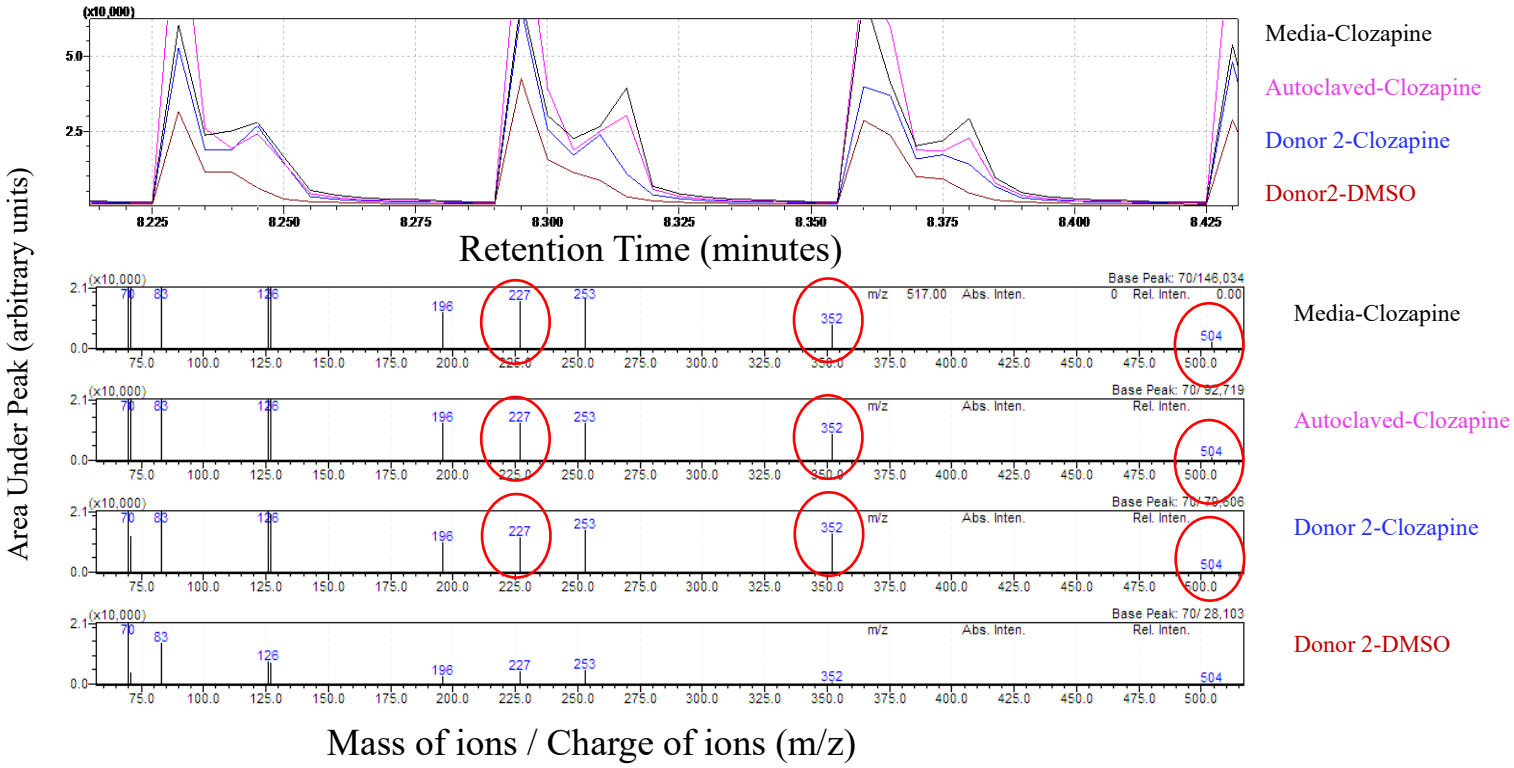


Figure 66. Chromatogram and mass spectra of human samples including donor 2 faeces incubated with clozapine and incubated with DMSO, and media and autoclaved faeces incubated with clozapine at 8.315 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for norclozapine; 227, 352, 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in the media-clozapine sample (black line, spectra 1) the autoclaved sample with clozapine (pink line, spectra 2), and the donor1-clozapine sample (blue line, spectra 3), using the ions 227, 352 and 504 m/z to qualify. The donor1-DMSO sample appears to contain minimal norclozapine (red line, spectra 4).

Quantitative

The repeated analysis are shown in Table 20. The norclozapine levels in the donor 2 samples incubated with clozapine for 24 hours were found to have an equal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.964$), as can be seen by the similar sizes of error bars in Figure 67. The two-sample t-test of equal variance showed that these levels were significantly higher in the media-clozapine sample ($p < 0.001$).

A paired t-test was conducted on the norclozapine levels in the donor 2 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 2 samples incubated with DMSO for 24 hours, which was not significant ($p = 0.0564$).

Table 20. Analysis of Equal Variance F tests and T tests of Norclozapine Levels in Repeated Donor 2 Samples and controls. Asterisks indicate the sample in which levels are significantly higher. Results reported using the format of (f test p value, t test p value).Variance not conducted on paired samples.

	Donor2-Clozapine 24h	**Donor2-DMSO 24h**
Media-Clozapine	0.964, <0.001 (**M)	x
Donor2-Clozapine 24h	x	0.0564

Figure 67 displays the norclozapine levels in the donor 2 samples.

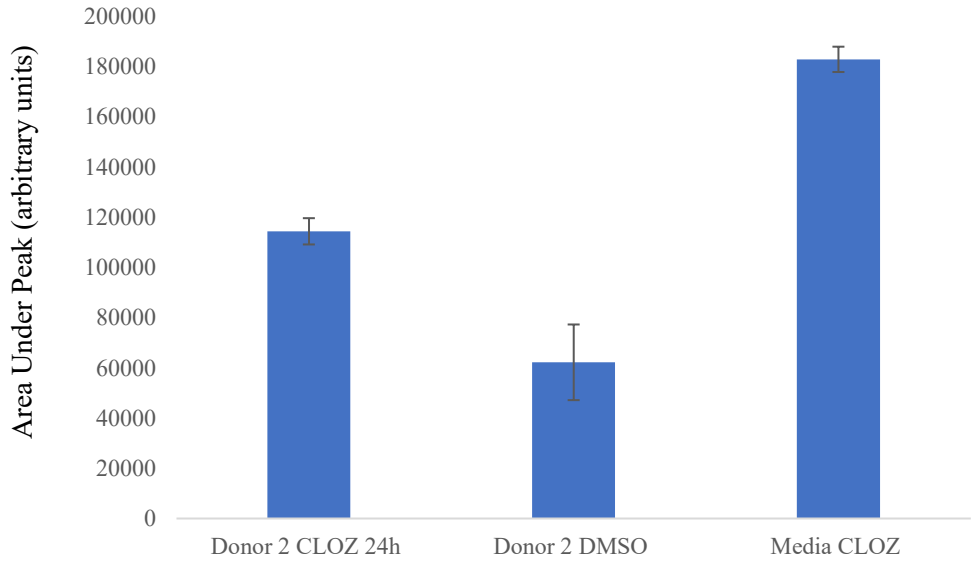


Figure 67. Bar graph displaying the norclozapine levels in samples using human donor 2 (D2) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 2 faeces incubated with clozapine for 24 hours (Donor 2 CLOZ 24h) and donor 2 faeces incubated with DMSO 24 hours (Donor 2 DMSO) and media incubated with clozapine (media-CLOZ). DMSO = dimethyl sulfoxide. CLOZ = clozapine. Error bars show standard error of the mean.

Comparison to Batch 1

The donor 2 clozapine samples exhibit almost double the amount of norclozapine than the previous batch, the donor 2 DMSO exhibit approximately 30 fold amounts, and the media exhibit exponentially higher.

Norclozapine:Clozapine Ratio

Figure 68 displays the ratio of norclozapine:clozapine in the human donor 2 samples.

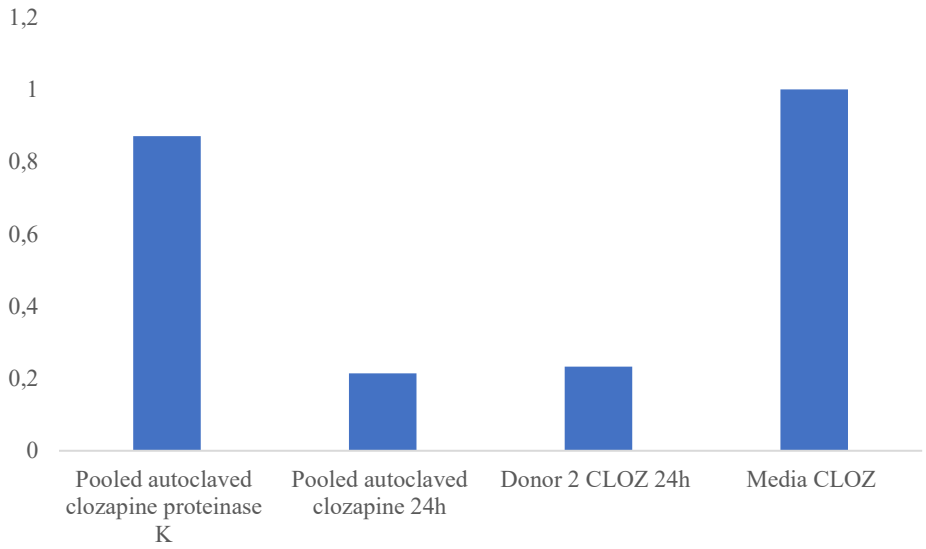


Figure 68. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 2, including autoclaved faeces incubated with clozapine, autoclaved faeces incubated with clozapine and proteinase K, human donor 2 faeces incubated with clozapine for 24 hours, as well as media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

As previously identified, the media-clozapine samples and the proteinase K autoclaved samples have the highest norclozapine:clozapine ratio. Donor 2 has a lower ratio than donor 1, with similar a level to the standard autoclaved samples.

Comparison to Batch 1

This relationship between donor 2 and donor 1 is consistent with previous samples.

Donor 3

Clozapine Levels

Figure 69 illustrates the GCMS output of Donor 1 samples and controls .

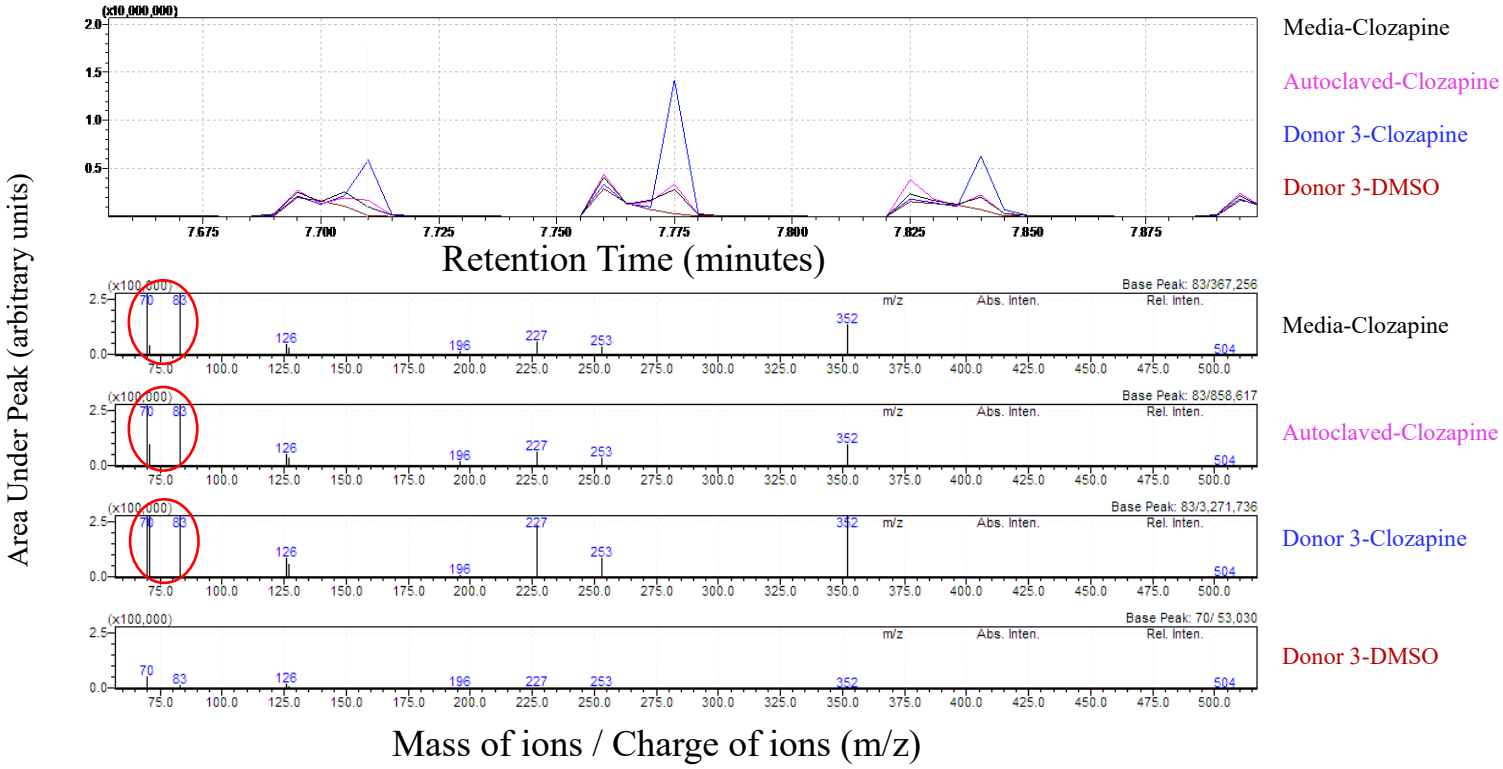


Figure 69. Chromatogram and mass spectra of human samples including donor 3 faeces incubated with clozapine and incubated with DMSO, and media and autoclaved faeces incubated with clozapine at 7.775 minutes. DMSO = dimethyl sulfoxide. Red circles denote presence of qualifying ions for clozapine; 71 m/z.

There is minimal clozapine in the Donor3-DMSO control condition, whereas in the other samples incubated with clozapine, the drug can be detected. This is clear from the chromatogram in Figure 69 at 7.775 minutes, where the samples are differentiated by colour, with the red line indicating the levels of clozapine in the DMSO sample, which is close to zero. There is varying clozapine levels in the other samples, with clozapine levels similarly high in the Media-Clozapine (black line chromatogram) and Autoclaved-Clozapine (pink line chromatogram) conditions, and lower levels in the Donor 3-Clozapine (blue line chromatogram). These is also evident in the mass-spectra of the four samples, in which the samples incubated with clozapine, shown on mass spectra 1-3, contain peaks at 71 m/z, denoted by red circles. The fourth spectrum, indicative of the Donor 3-DMSO sample, however, shows no peak at this value.

Quantitative

The repeated analysis are shown in Table 21. The clozapine levels in the donor 3 samples incubated with clozapine for 24 hours were found to have an unequal variance to the clozapine levels in the samples with media and clozapine (p = 0.015). This is evident from the error bars in the bar graph (Figure 70), where the donor 3 clozapine sample has a tall error bar, indicating high variation. The two-sample t-test of unequal variance showed that these clozapine levels were not significantly different (p = 0.0812).

A paired t-test was conducted on the clozapine levels in the donor 3 samples incubated with clozapine for 24 hours and the clozapine levels in the donor 3 samples incubated with DMSO for 24 hours, which was not significantly different (p = 0.0671).

Table 21. Analysis of Equal Variance F tests and T tests of Clozapine Levels in Donor 3 Samples and Controls. Results reported using the format of (f test p value, t test p value). Variance not conducted on paired samples.

	Donor3-Clozapine 24h	**Donor3-DMSO 24h**
Media-Clozapine	0.015, 0.0812	x
Donor3-Clozapine 24h	X	0.0671

Figure 70 displays the levels of clozapine in the donor 3 samples, compared to media and DMSO controls.

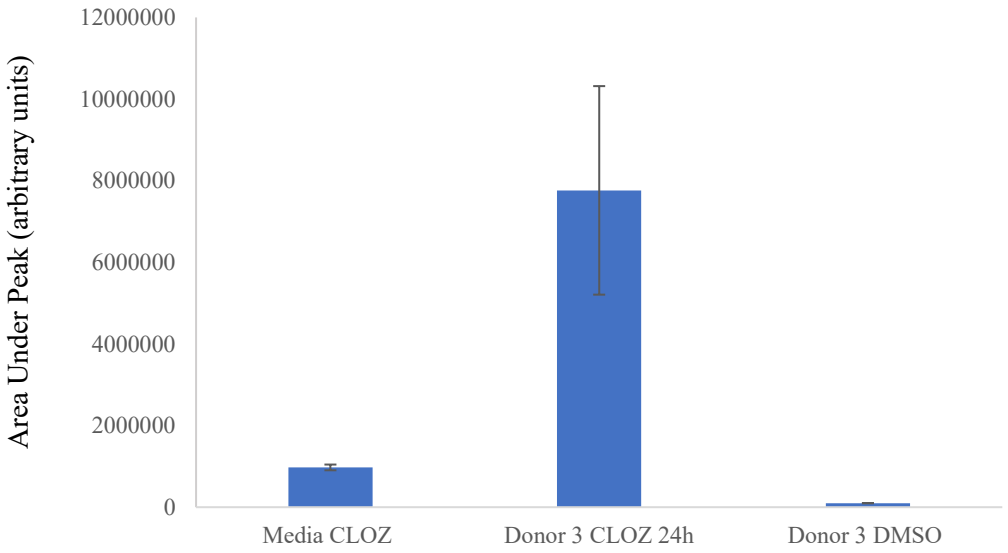


Figure 70. Bar graph displaying the clozapine levels in samples using human donor 3 (D3) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 3 faeces incubated with clozapine for 24 hours (Donor 3 CLOZ 24h) and donor 3 faeces incubated with DMSO 24 hours (Donor 3 DMSO) and media incubated with clozapine (media-CLOZ). DMSO = dimethyl sulfoxide. CLOZ = clozapine. Error bars show standard error of the mean.

Comparison to Batch 1

The donor 3 clozapine samples exhibit over 6 fold the levels of clozapine as the previous batch. The donor 3 DMSO exhibit similar amounts.

Norclozapine Levels

*F*igure 71 illustrates the GCMS output of Donor 1 samples and controls

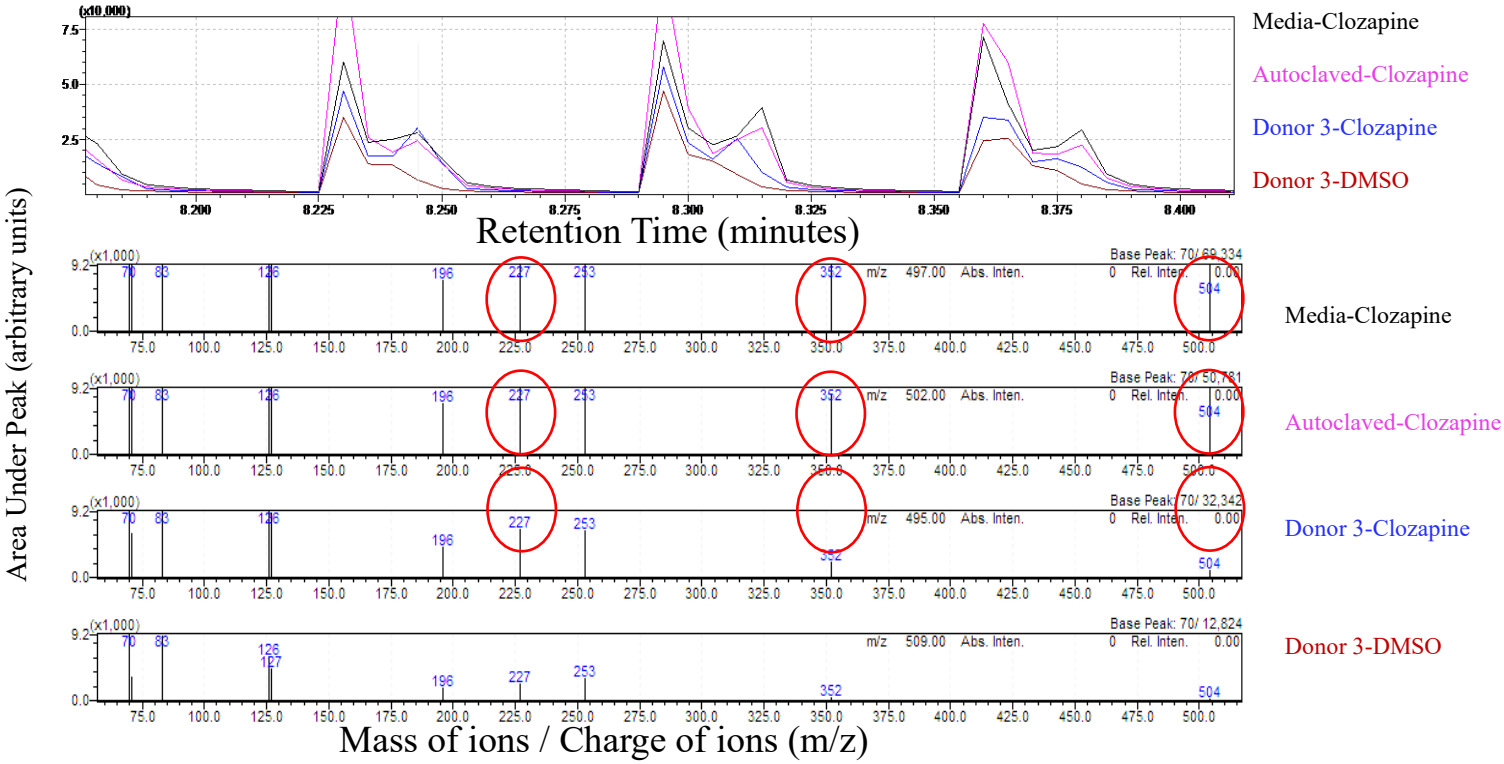


Figure 71. Chromatogram and mass spectra of human samples including donor 3 faeces incubated with clozapine and incubated with DMSO, and media and autoclaved faeces incubated with clozapine at 8.315 minutes. DMSO = dimethyl sulfoxide. Red circles denote presence of qualifying ions for norclozapine; 227, 352, 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in the media-clozapine sample (black line, spectra 1) the autoclaved sample with clozapine (pink line, spectra 2), and the donor3-clozapine sample (blue line, spectra 3), using the ions 227, 352 and 504 m/z to qualify. The donor3-DMSO sample appears to contain no norclozapine (red line, spectra 4).

Quantitative

The repeated analysis are shown in Table 22. The norclozapine levels in the donor 3 samples incubated with clozapine for 24 hours were found to have an unequal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.005$). This is evident from the large variation in the error bars in Figure 72. The

two-sample t-test of unequal variance showed that these levels were significantly higher in the donor 3-clozapine sample (p = 0.00736).

A paired t-test was conducted on the norclozapine levels in the donor 3 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 3 samples incubated with DMSO for 24 hours, which was not significantly different (p = 0.0718).

Table 22. Analysis of Equal Variance F tests and T tests of Norclozapine Levels in Repeated Donor 3 Samples and controls. Asterisks indicate the sample in which levels are significantly higher. Results reported using the format of (f test p value, t test p value). Variance not conducted on paired samples.

	Donor3-Clozapine 24h	**Donor3-DMSO 24h**
Media-Clozapine	0.005, 0.00736 (**D)	x
Donor3-Clozapine 24h	x	0.0718

Figure 72 displays the levels of clozapine in the donor 3 samples, compared to media and DMSO controls.

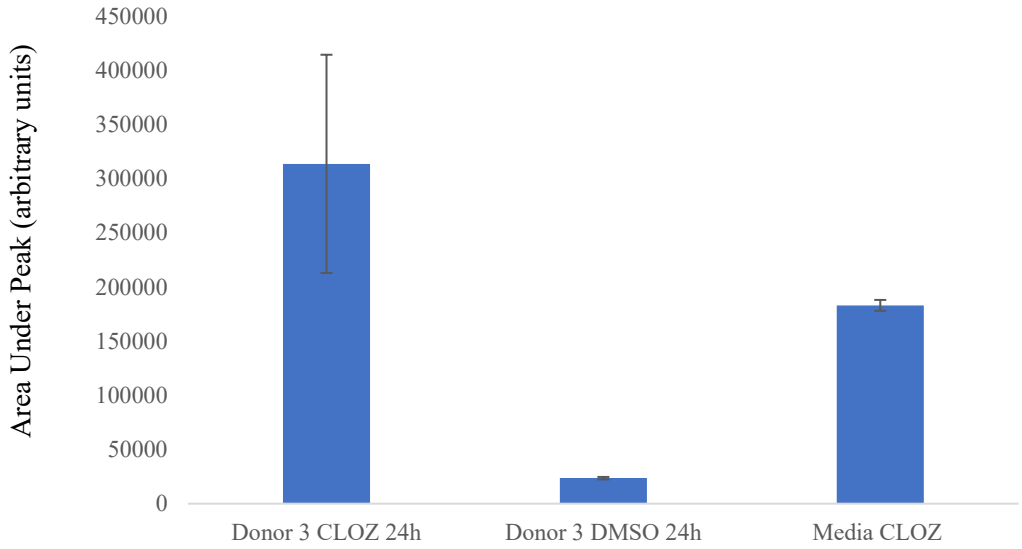


Figure 72. Bar graph displaying the norclozapine levels in samples using human donor 3 (D3) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 3 faeces incubated with clozapine for 24 hours (Donor 3 CLOZ 24h) and donor 3 faeces incubated with DMSO 24 hours (Donor 3 DMSO) and media incubated with clozapine (media-CLOZ). DMSO = dimethyl sulfoxide. CLOZ = clozapine. Error bars show standard error of the mean.

Comparison to Batch 1

The donor 3 clozapine samples exhibit almost 4 fold the levels of norclozapine as the previous batch. The donor 3 DMSO exhibit almost 10 times the amount of norclozapine.

Norclozapine:Clozapine Ratio

Figure 73 displays the ratio of norclozapine:clozapine in the human donor 3 samples.

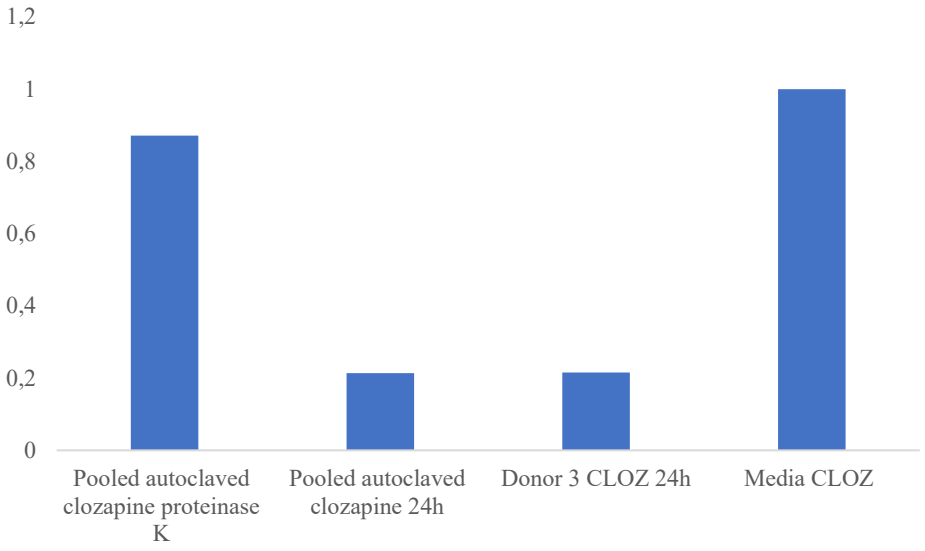


Figure 73. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 3, including autoclaved faeces incubated with clozapine, autoclaved faeces incubated with clozapine and proteinase K, human donor 3 faeces incubated with clozapine for 24 hours, as well as media incubated with clozapine (media-CLZ). CLZ = clozapine, DMSO = dimethyl sulfoxide.

As previously identified, the media-clozapine samples and the proteinase K autoclaved samples have the highest norclozapine:clozapine ratio. Donor 3 has a similar ratio to donor 2 and the standard autoclaved samples.

Comparison to Batch 1

This relationship between the donors and autoclaved samples is consistent with the samples from the previous batch.

Donor 4

Clozapine Levels

Figure 74 illustrates the GCMS output of Donor 4 samples and controls

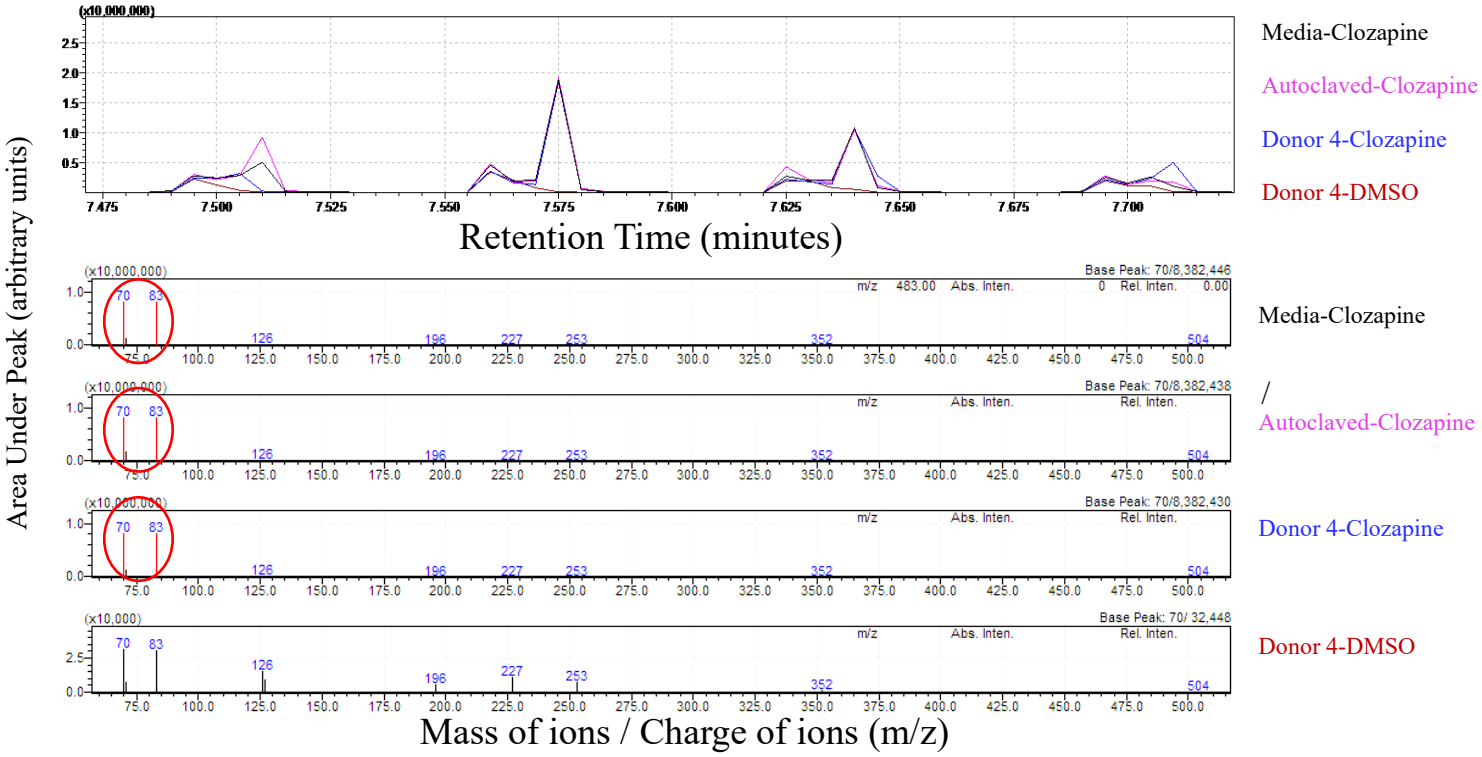


Figure 74. Chromatogram and mass spectra of human samples including donor 4 faeces incubated with clozapine and incubated with DMSO, and media and autoclaved faeces incubated with clozapine at 7.575 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ion for clozapine; 71 m/z.

There is minimal clozapine in the Donor4-DMSO control condition, whereas in the other samples incubated with clozapine, the drug can be detected. This is clear from the chromatogram in Figure X at 7.775 minutes, where the samples are differentiated by colour, with the red line indicating the levels of clozapine in the DMSO sample, which is close to zero. There are similar clozapine levels in the other samples, which are all high in the Media-Clozapine (black line chromatogram) and Autoclaved-Clozapine (pink line chromatogram) conditions, and the Donor 4-Clozapine (blue line chromatogram). These is also evident in the mass-spectra of the four samples, in which the samples incubated with clozapine, shown on mass spectra 1-3, contain peaks at 71 m/z, denoted by red circles. The fourth spectrum, indicative of the Donor 4-DMSO sample, however, shows no peak at this value.

Quantitative

The repeated analysis are shown in Table 23. The clozapine levels in the donor 4 samples incubated with clozapine for 24 hours were found to have an equal variance to the clozapine levels in the samples with media and clozapine ($p = 0.874$). This is evident from the similar size of the error bars in the bar graph in Figure75. The two-sample t-test of equal variance showed that these levels were significantly higher in the donor-clozapine sample ($p < 0.001$).

A paired t-test was conducted on the clozapine levels in the donor 4 samples incubated with clozapine for 24 hours and the clozapine levels in the donor 4 samples incubated with DMSO for 24 hours. This showed that the clozapine levels were significantly higher in the donor 4-clozapine sample ($p = 0.00653$).

Table 23. Analysis of Equal Variance F tests and T tests of Clozapine Levels in Repeated Donor 4 Samples and controls. Asterisks indicate the sample in which levels are significantly higher. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor4-Clozapine 24h	**Donor4-DMSO 24h**
Media-Clozapine	0.874, <0.001 (**D)	x
Donor4-Clozapine 24h	x	0.00653 (**C)

Figure 75 displays the levels of clozapine in the donor 4 samples, compared to media and DMSO controls.

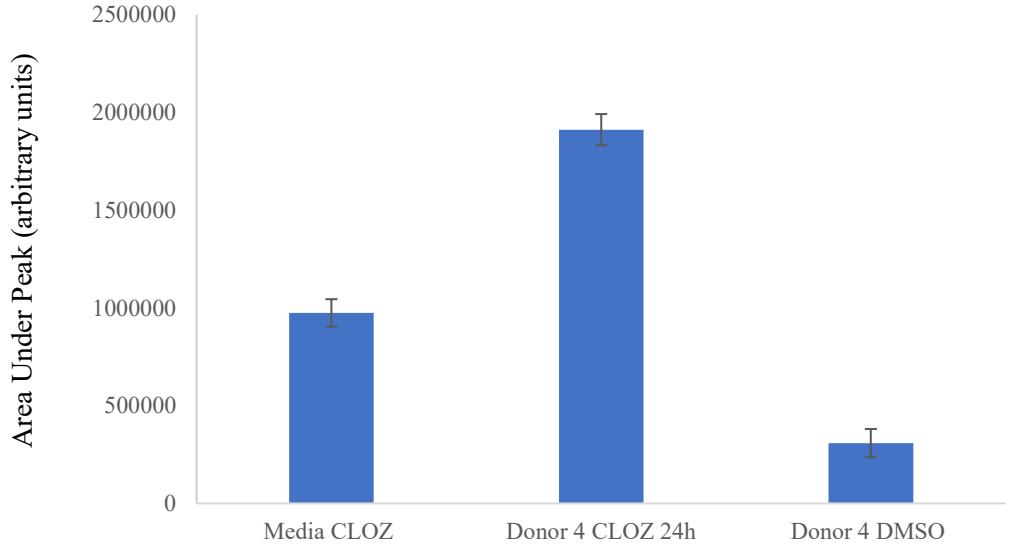


Figure 75. Bar graph displaying the clozapine levels in samples using human donor 4 (D4) faeces, including samples with autoclaved human faeces incubated with clozapine, donor 4 faeces incubated with clozapine for 24 hours (Donor 4 CLOZ 24h) and donor 4 faeces incubated with DMSO 24 hours (Donor 4 DMSO) and media incubated with clozapine (media-CLOZ). DMSO = dimethyl sulfoxide. CLOZ = clozapine. Error bars show standard error of the mean.

Comparison to Batch 1

The donor 4 clozapine samples exhibit over 7 fold the levels of clozapine as the previous batch. The donor 4 DMSO exhibit exponentially larger amounts.

Norclozapine Levels

Figure 76 illustrates the GCMS output of Donor 4 samples and controls

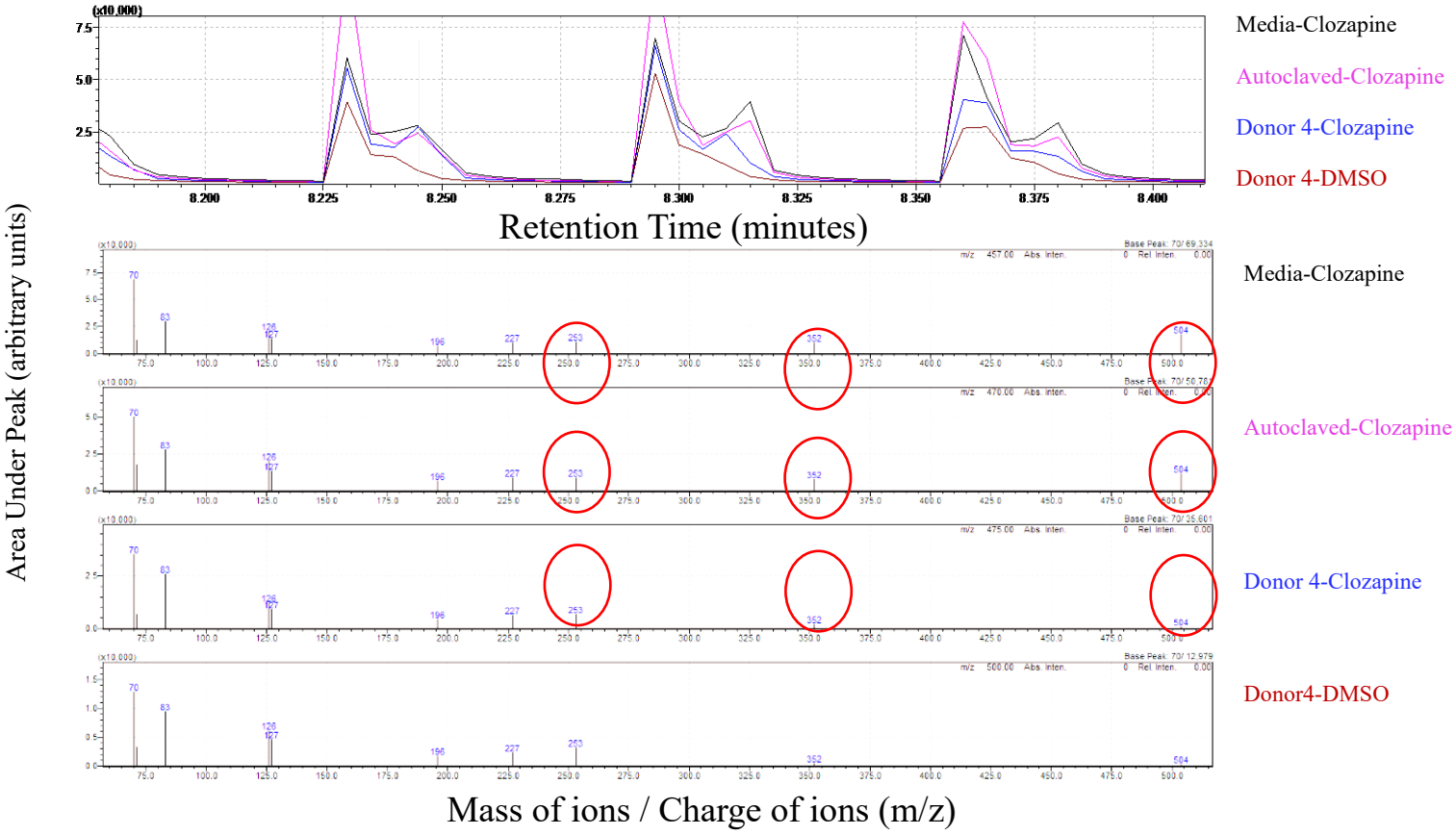


Figure 76. Chromatogram and mass spectra of human samples including donor 4 faeces incubated with clozapine and incubated with DMSO, and media and autoclaved faeces incubated with clozapine at 8.315 minutes. DMSO = dimethyl sulfoxide, CLZ = clozapine. Red circles denote presence of qualifying ions for norclozapine; 227, 352, 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in the media-clozapine sample (black line, spectra 1) the autoclaved sample with clozapine (pink line, spectra 2), and the donor4-clozapine sample (blue line, spectra 3), using the ions 227, 352 and 504 m/z to qualify. The donor4-DMSO sample appears to contain no norclozapine (red line, spectra 4).

Quantitative

The repeated analysis are shown in Table 24. The norclozapine levels in the donor 4 samples incubated with clozapine for 24 hours were found to have an equal variance to the norclozapine levels in the samples with

media and clozapine ($p = 0.976$) and the two-sample t-test of equal variance showed that these levels were significantly higher in the media-clozapine sample ($p < 0.001$).

A paired t-test was conducted on the norclozapine levels in the donor 4 samples incubated with clozapine for 24 hours and the norclozapine levels in the donor 4 samples incubated with DMSO for 24 hours, which was not significantly different ($p = 0.380$).

Table 24. Analysis of Equal Variance F tests and T tests of Norclozapine Levels in Repeated Donor 4 Samples and controls. Asterisks indicate the sample in which levels are significantly higher. Results reported using the format of (f test p value, t test p value). Variance not conducted on paired samples.

	Donor4-Clozapine 24h	**Donor4-DMSO 24h**
Media-Clozapine	0.975, <0.001 (**M)	x
Donor4-Clozapine 24h	x	0.380

Figure 77 displays the levels of norclozapine in the donor 4 samples, compared to media and DMSO controls.

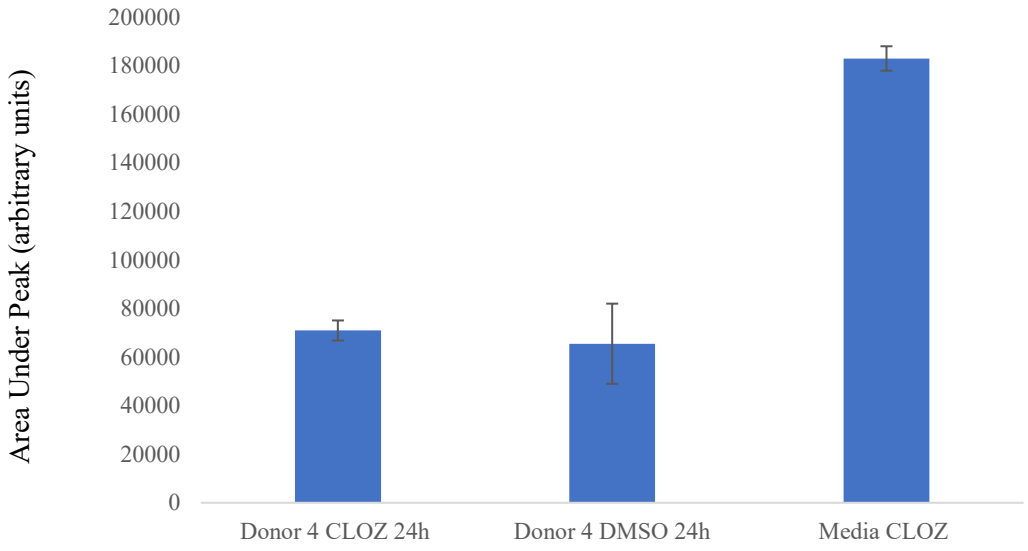


Figure 77. Bar graph displaying the norclozapine levels in samples using human donor 4 faeces, including samples with autoclaved human faeces incubated with clozapine, donor 4 faeces incubated with clozapine for 24 hours (Donor 4 CLOZ 24h) and donor 4 faeces incubated with DMSO 24 hours (Donor 4 DMSO) and media incubated with clozapine (media-CLOZ). DMSO = dimethyl sulfoxide. CLOZ = clozapine. Error bars show standard error of the mean.

The donor 4 clozapine samples have 3 times the amount of clozapine compared to the donor 4 samples from the previous batch. The DMSO samples have an exponentially higher amount.

Norclozapine:Clozapine Ratio

Figure 78 depicts the norclozapine:clozapine ratios of the donor 4 samples compared to controls.

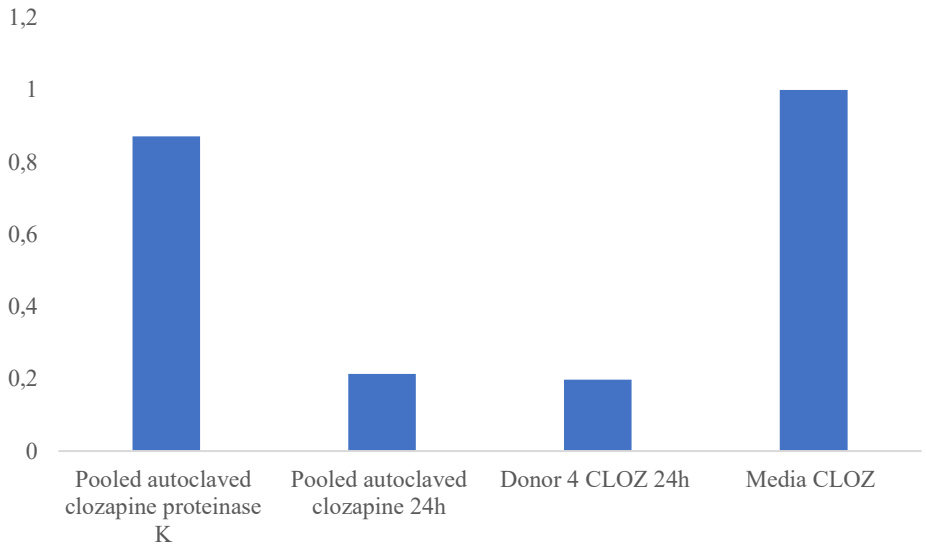


Figure 78. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 4, including autoclaved faeces incubated with clozapine, autoclaved faeces incubated with clozapine and proteinase K, human donor 4 faeces incubated with clozapine for 24 hours, as well as media incubated with clozapine (media-CLOZ). CLOZ = clozapine, DMSO = dimethyl sulfoxide.

As previously identified, the media-clozapine samples and the proteinase K autoclaved samples have the highest norclozapine:clozapine ratio. Donor 4 has a low ratio, similar to donors 3 and 2, as well as to the standard autoclaved samples. This relationship between the donors is consistent with previous samples.

Donor 5

Clozapine Levels

Figure 79 shows the GCMS output for donor 5 samples and controls.

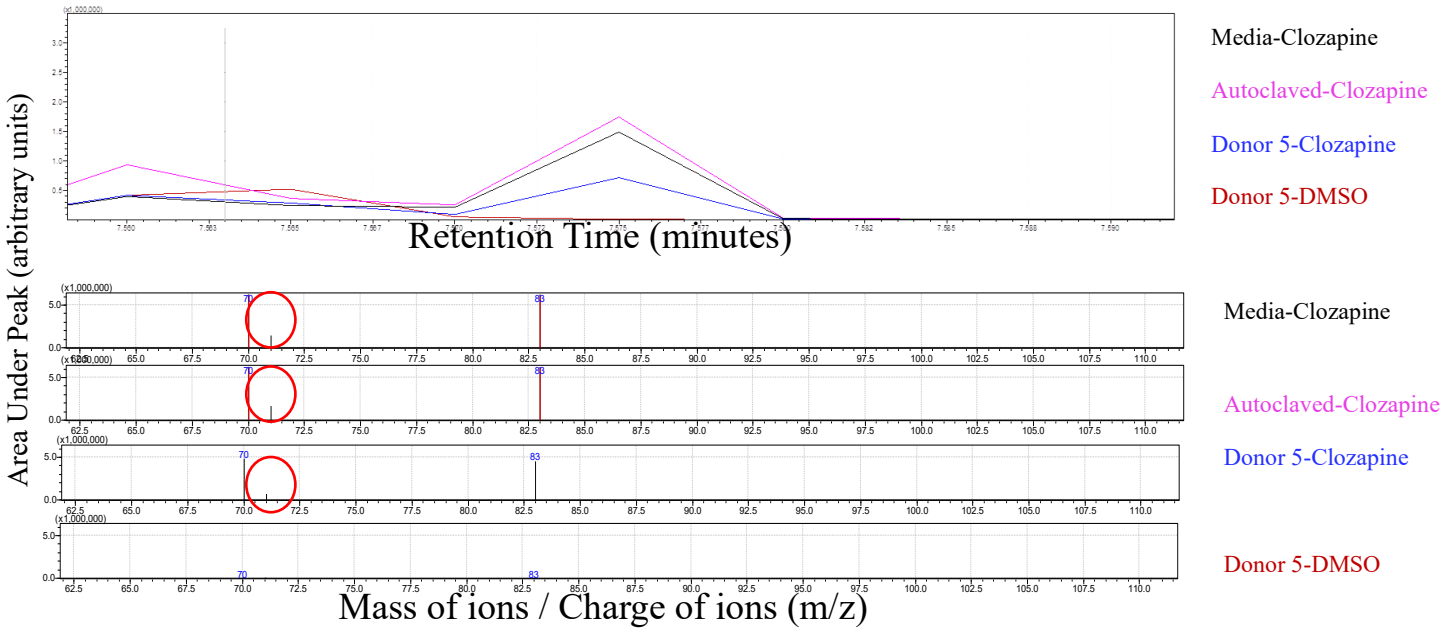


Figure 79. Chromatogram and mass spectra of media, autoclave, Donor 5-clozapine and Donor 5-DMSO at 7.575 minutes.

There is minimal clozapine in the Donor5-DMSO control condition, whereas in the other samples incubated with clozapine, the drug can be detected. This is clear from the chromatogram in Figure 79 at 7.575 minutes, where the samples are differentiated by colour, with the red line indicating the levels of clozapine in the DMSO sample, which is close to zero. There is varying clozapine levels in the other samples, with clozapine levels similarly high in the Media-Clozapine (black line chromatogram) and Autoclaved-Clozapine (pink line chromatogram) conditions, and lower levels in the Donor 5-Clozapine (blue line chromatogram). These differences are also arbitrarily quantified in the mass-spectra of the four samples, in which the samples incubated with clozapine, shown on mass spectra 1-3, contain peaks at 71 m/z. It can also be noted that peak is almost half as high in the third mass spectrum, the Donor 5-Clozapine sample. The fourth spectrum, indicative of the Donor 5-DMSO sample, however, shows no peak at this value.

Quantitative

The repeated analysis are shown in Table 25. The clozapine levels in the donor 5 samples incubated with clozapine for 24 hours were found to have an equal variance to the clozapine levels in the samples with media and clozapine ($p = 0.836$), and the two-sample t-test of equal variance showed that these levels were significantly higher in the media-clozapine sample ($p < 0.001$).

The same analyses was conducted on the diluted samples. With the 1:10 dilution, similar results were observed – an equal variance with the media samples ($p = 0.429$) and significantly higher levels in the donor samples (p

< 0.001). Similarly, there was an equal variance with the 1:100 dilution and the media samples (p = 0.140) and the donor sample had significantly higher levels of clozapine (p < 0.001).

A comparison was conducted between the donor sample and the dilutions. The clozapine levels were significantly higher in both dilution samples than the original sample, with the 1:10 dilution (p = 0.00270) and the 1:100 dilution (p = 0.00627).

Table 25. Analysis of Equal Variance F tests and T tests of Clozapine Levels in Repeated Donor 5 Samples and controls. Asterisks indicate the sample in which levels are significantly higher. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor5-Clozapine 24h	**Donor5-DMSO 24h**	**Donor5-Clozapine 24h 1:10**	**Donor5-Clozapine 24h 1:100**
Media-Clozapine	0.836, <0.001 (**D)	x	0.429, <0.001 (**D)	0.140, <0.001 (**D)
Donor5-Clozapine 24h	x	<0.001 (**C)	x	x
Donor5-Clozapine 24h 1:10	0.00270 (**10)		x	
Donor5-Clozapine 24h 1:100	0.00627 (**100)			

Figure 80 depicts the clozapine levels observed in the donor 5 samples and controls.

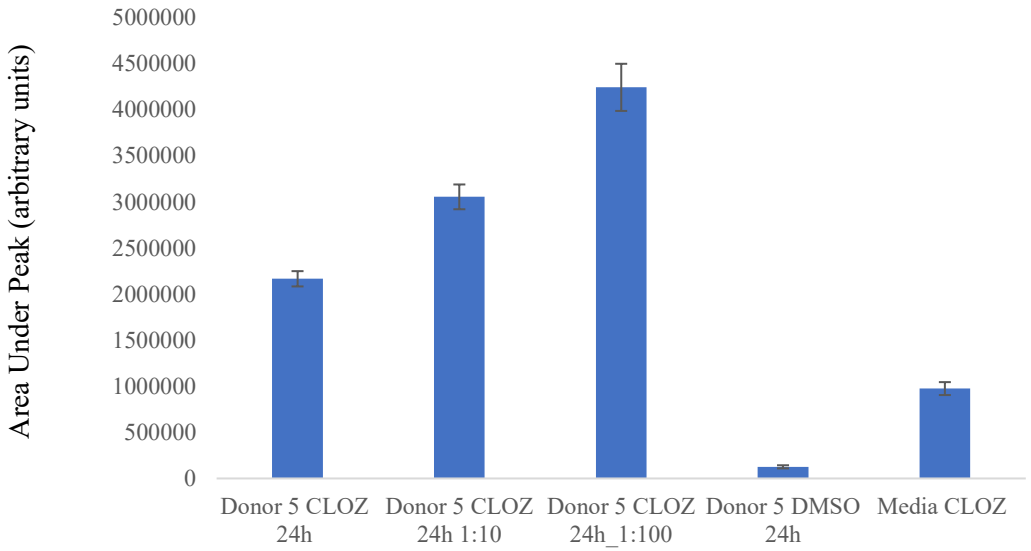


Figure 80. Clozapine levels observed in the donor 5 samples and controls, with donor 5 CLOZ 24h representing donor 5 faeces incubated with clozapine for 24 hours, donor 5 CLOZ 24h 1:10 representing the same sample diluted using a 1:10 ratio, and donor 5 CLOZ 24h 1:100 diluted using a 1:100 ratio. The controls

are donor 4 DMSO 24h and media incubated with clozapine (CLOZ). Error bars show standard error of the mean.

Norclozapine Levels

Qualitative

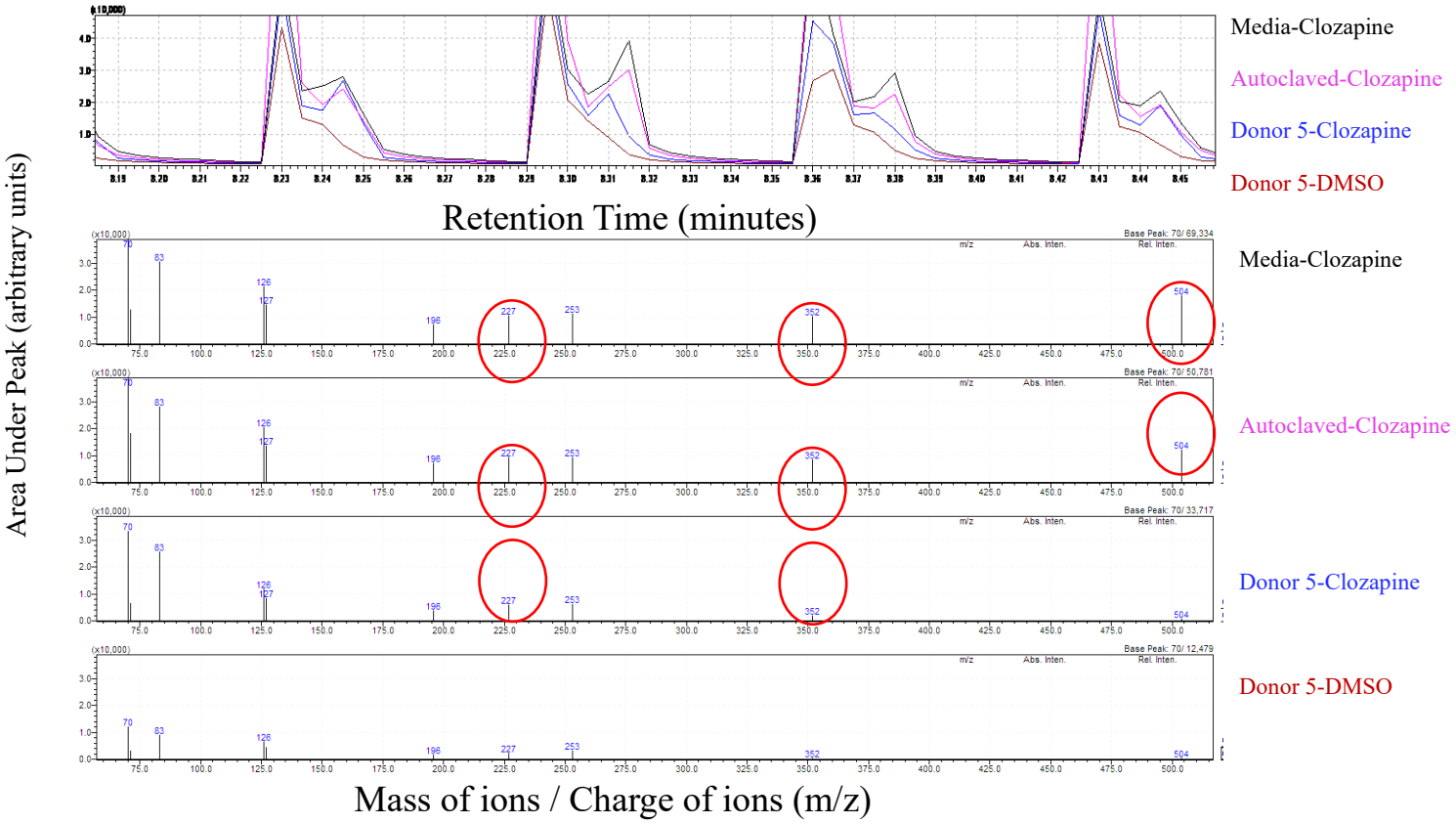


Figure 81. Chromatogram and mass spectra of media, autoclave, Donor 5-clozapine and Donor 5-DMSO at 8.315 minutes. Red circles denote presence of qualifying ions 227, 352 and 504 m/z.

Both the chromatogram and the spectra reveal that norclozapine is present in the media-clozapine sample (black line, spectra 1) the autoclaved sample with clozapine (pink line, spectra 2), and the donor3-clozapine sample (blue line, spectra 3), using the ions 227, 352 and 504 m/z to qualify. The donor3-DMSO sample appears to contain no norclozapine (red line, spectra 4).

Quantitative

The repeated analysis are shown in Table 26. The norclozapine levels in the donor 5 samples incubated with clozapine for 24 hours were found to have an equal variance to the norclozapine levels in the samples with media and clozapine ($p = 0.745$) and the two-sample t-test of equal variance showed that these levels were significantly higher in the media-clozapine sample ($p < 0.001$).

The same analyses was conducted on the diluted samples. With the 1:10 dilution, similar results were observed – an equal variance with the media samples ($p = 0.557$) and significantly higher levels in the donor samples (p

< 0.001). Similarly, there was an equal variance with the 1:100 dilution and the media samples (p = 0.491) and the donor sample had significantly higher levels of clozapine (p < 0.001).

A comparison was conducted between the donor sample and the dilutions. The t test did not reach significance with either dilution, with the 1:10 dilution (p = 0.0785) or the 1:100 dilution (p = 0.382).

Table 26. Analysis of Equal Variance F tests and T tests of Clozapine Levels in Repeated Donor 5 Samples and controls. Asterisks indicate the sample in which levels are significantly higher. Variance not conducted on paired samples. Results reported using the format of (f test p value, t test p value).

	Donor5-Clozapine 24h	**Donor5-DMSO 24h**	**Donor5-Clozapine 24h 1:10**	**Donor5-Clozapine 24h 1:100**
Media-Clozapine	0.745, <0.001 (***M)	x	0.557, <0.001 (**M)	0.491, <0.001 (**M)
Donor5-Clozapine 24h	x	0.0123 (**C)	x	x
Donor5-Clozapine 24h 1:10	0.0785		x	
Donor5-Clozapine 24h 1:100	0.382			

Figure 82 depicts the norclozapine levels observed in the donor 5 samples and controls.

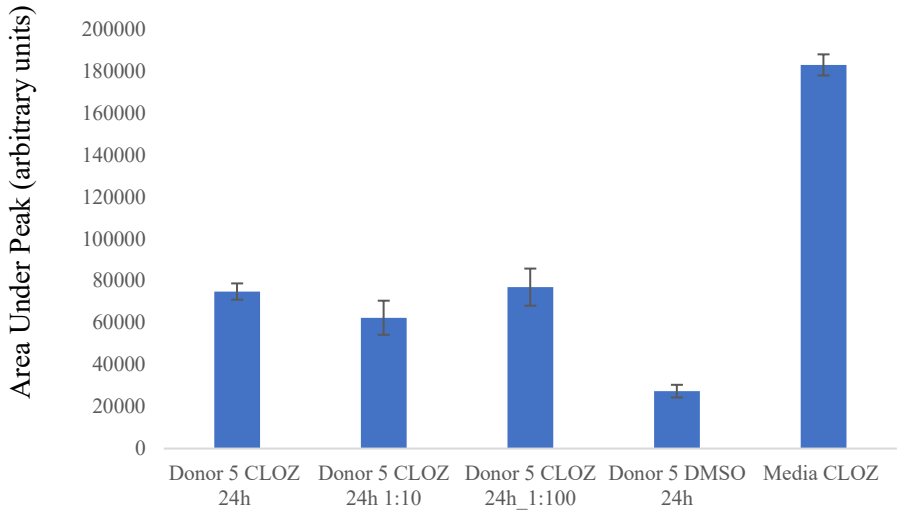


Figure 82. Clozapine levels observed in the donor 5 samples and controls, with donor 5 CLOZ 24h representing donor 5 faeces incubated with clozapine for 24 hours, donor 5 CLOZ 24h 1:10 representing the same sample diluted using a 1:10 ratio, and donor 5 CLOZ 24h 1:100 diluted using a 1:100 ratio. The controls are donor 5 DMSO 24h and media incubated with clozapine (CLOZ). Error bars show standrd error of the mean.

Norclozapine:Clozapine Ratio

Figure 83 depicts the norclozapine:clozapine levels observed in the donor 5 samples and controls.

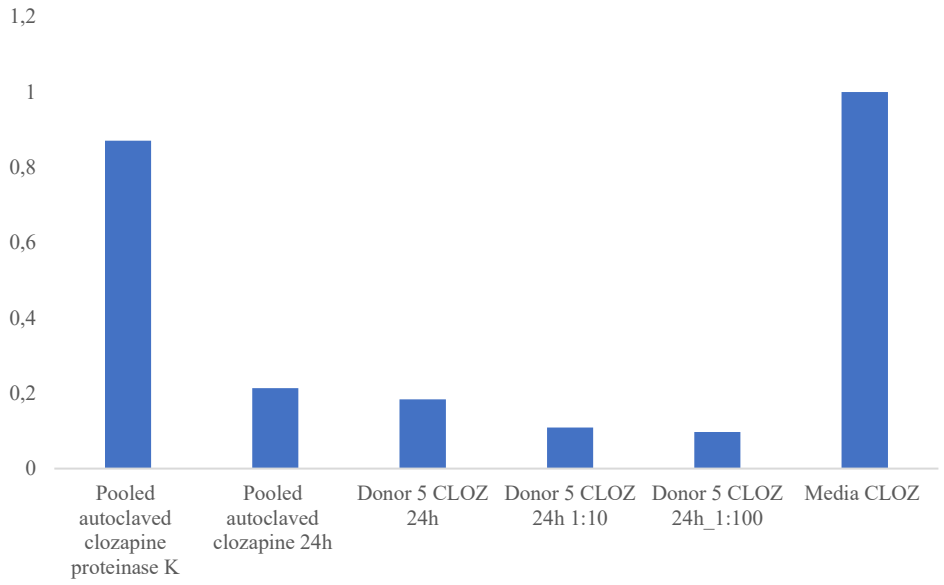


Figure 83. Bar graph displaying the norclozapine:clozapine ratios in samples using faeces from human donor 5 including autoclaved faeces incubated with clozapine, autoclaved faeces incubated with clozapine and proteinase K, human donor 5 faeces incubated with clozapine for 24 hours, donor 5 CLOZ 24h 1:10 representing the same sample diluted using a 1:10 ratio, and donor 5 CLOZ 24h 1:100 diluted using a 1:100 ratio, as well as media incubated with clozapine (media-CLOZ). CLOZ = clozapine, DMSO = dimethyl sulfoxide.

Donor 5 has a similar ratio to the other donors and similar level to the standard autoclaved samples. The ratio of the diluted donor 5 samples shows a slight decrease. This similarity in donors is not consistent with the samples from the previous batch. The previous analysis of donor 5 showed the highest ratio of norclozapine:clozapine, higher than donor 1.

All Donors Ratio

Figure 84 shows a bar graph depicting the ratio of norclozapine:clozapine across all donors and relevant controls.

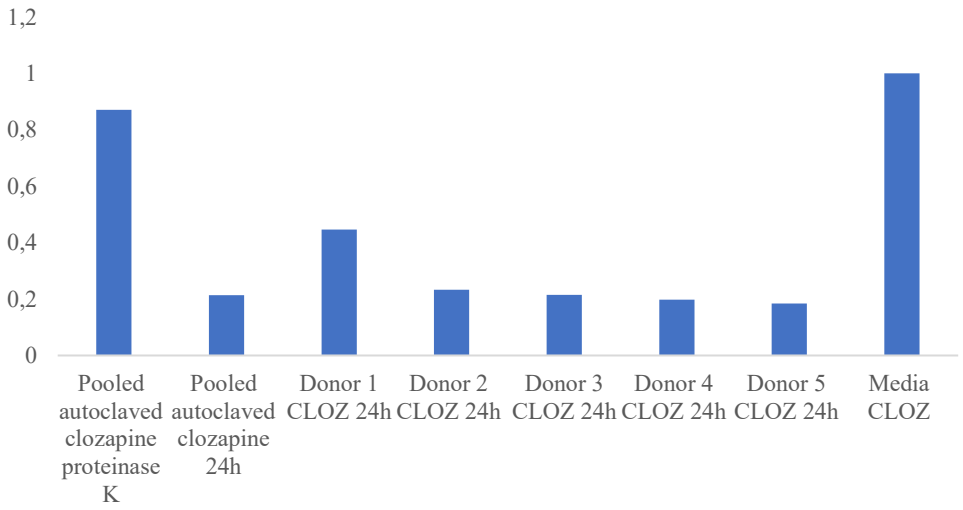


Figure 84. Relative norclozapine:clozapine ratios for various sample types after 24-hour incubation. Includes pooled autoclaved samples treated with and without proteinase K, samples from five individual donors, and a media control sample. CLOZ = clozapine.

As previously described, the donors exhibit similar ratios, with donor 1 displaying the ighest. Most of these are higher than the autoclaved control, bur lower than the proteinase K treated autoclaved control and media control.

Microbial Derived Metabolism Analysis

The figure below shows a visual representation of the pearson correlation test between levels of clozapine and norclozapine across all donors.

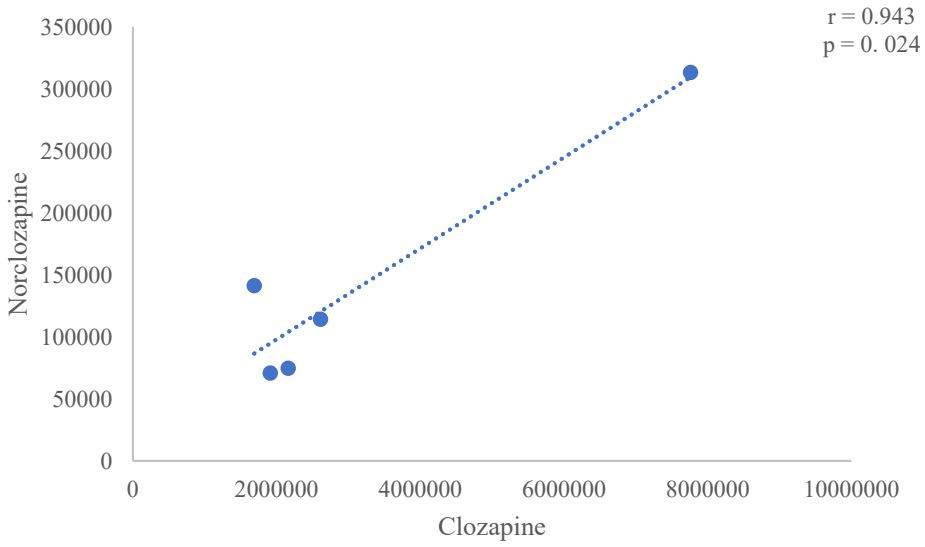


Figure 85. Correlational analysis between clozapine and norclozapine levels in all donors at 24 hours. Pearson r and corresponding p value are shown in right hand corner. Dotted line indicates linear trend.

The provided data indicate that there is a strong positive relationship between clozapine and norclozapine ($r = 0.943$). A t test showed that this was statistically significant ($p = 0.024$).

Discussion

Test of Metabolic Activity

The variability in the consistency in the repeat FDA test is an interesting result. The differences were only present in two of the donors – only donors 3 and 4 showed higher metabolic activity on the repeat, whilst the remaining three donors exhibited a similar absorbance output. The results on the first test are likely to be the inaccurate results, potentially due to a human error in pipetting. However, it would be expected that an error of this kind would be systematic, with inconsistency observed across all samples, which did not occur. This variation could be due to other methodological inconsistencies, including slight differences in the timing of specific steps for individual or micro-environmental factors, including plate edge effects and measurement noise.

Proteinase K

The introduction of an additional autoclave condition with proteinase K treatment was a novel aspect of this study. It was hypothesised that the addition of proteinase K would break down enzymes contributing to norclozapine production in the autoclaved samples, and thus these samples would exhibit low ratios. However, the opposite was observed in these samples, where a higher ratio was exhibited by these samples compared to the standard autoclaved samples, and all the other donors. This suggests that the addition of this treatment, as well as the incubation of 55 degrees contributed to norclozapine production.

1:10 and 1:100 dilutions comment

There is no statistical difference in the norclozapine levels or the ratios between the dilutions and the original donor 5 samples. This implies that norclozapine could be produced via bacterial enzymes, which is a novel finding.

Full Experiment

Controls

It is evident that the controls in this analysis produced highly unexpected results, strongly suggesting that this protocol needs review. The autoclaved and media samples are intended to act as negative controls due to their lack of bacteria. Therefore, the positive results raise serious concern about the

validity of this protocol. With the autoclaved samples, there are many factors that could be producing this positive result (as previously explained in the last discussion section), but the results from the media samples are harder to explain. The most probable cause for this is a potential change in the media environment in this repeat analysis that resulted in an increased susceptibility for to be converted to norclozapine during the timeframe of the experiment or storage. Factors like pH, temperature, or dissolved oxygen levels might play a role, especially if there was a subtle change in the media composition or preparation method. To explore this, a series of media-clozapine samples should be incubated and analysed at various time points to assess the rate of norclozapine formation over time. The media should also be constantly plated on agar and assessed for growth. A test of metabolic activity should also be assessed. Of concern also, is the unexpectedly high levels of clozapine and norclozapine observed in the DMSO controls. Whilst these were not statistically significant, detection of them at all indicates a systematic issue and contamination.

Comparison to Previous Batch

Controls

The consistency of the levels of clozapine in the media and DMSO controls implies that these are valid. However, the increased production of norclozapine in the media and autoclaved samples is concerning, as it implies an alternative factor is producing norclozapine, other than live bacteria. This increased norclozapine production has skewed the ratios, making them far higher in the media samples. With the autoclaved samples, this could be associated with the addition of proteinase K, which also involved incubation to 55 degrees.

Donors

The previous batch had much lower levels of clozapine in donor 1 CLOZ 24h samples. However, these samples exhibited similar norclozapine levels to the previous batch. The levels of clozapine in Donor 1 DMSO samples were also similar. The proportional levels of the ratios between these samples and the autoclaved samples, and the previous batch do imply consistency in the repeat. The previous batch also had lower levels of clozapine and norclozapine in the Donor 2 24h clozapine samples. Donor 2 exhibited a lower ratio than donor 1, which was consistent with previous results. Donors 3 and 4 exhibited a similar ratio to donor 2, which was also consistent with previous results. However, in the previous analysis, donor 5 exhibited the highest ratio, but this was not observed in the repeat. Donor 5 exhibited a similar ratio to donors 2, 3 and 4. This could be due to the use of donor 5 as a benchmark metaboliser for the normalisation, thus the other donors are demonstrating convergence. However, the normalisation did not appear to affect samples from donor 1, with ratios

remaining high in both analyses. Whilst it was expected that normalisation would help to control for variation in metabolic activity, it was also previously identified that the FDA test may not be a useful proxy for clozapine metabolism. Therefore, it is unsurprising that dilution based on FDA result had varying effects on the norclozapine:clozapine ratios produced. This data proved further evidence that the FDA assay and the norclozapine:clozapine ratios are measuring different, and at times, opposing aspects of metabolic processes.

Additionally, dilution may not be the most appropriate technique to normalise metabolic activity, especially if the enzymes impacting clozapine metabolism are of bacterial origin. Diluting will likely not affect bacterial concentration, which as previously mentioned is present in billion amounts. It is also not a target or mechanisitic correction, which directly affects enzymes that contribute to clozapine metabolism. As the ability of each individual's gut bacteria to metabolise clozapine differs, dilution will likely not control for varying metabolic activities.

Focus should be shifted towards protocol that are specific to the clozapine metabolic pathway, particularly one that measures CP450 activity.

Both experiments showed that the MDM correlational analysis demonstrated a positive correlation at 24 hours, which fails to meet Javdan's criteria of microbial-derived metabolism. A repeat of the 8 hour incubation analysis should be conducted to ensure if the MDM+ result obtained at that time point was consistent.

Chapter 5: Conclusion

This thesis embarked on an investigation into the intricate relationship between the gut microbiome and the metabolism of clozapine, a antipsychotic medication characterised by its challenging pharmacokinetic profile and variable patient response. Through a series of carefully constructed *in vitro* experiments, the primary aim was to develop a robust model to quantify the impact of microbial activity on clozapine metabolism and to elucidate the role of the microbiome in the clinically significant clozapine to norclozapine (CNR) ratio. While the experimental results were complex and, at times, challenged initial assumptions, they collectively unveil significant insights into the dynamics of drug metabolism and lay the groundwork for future explorations in this field.

The second chapter of this thesis focused on the optimisation of a previously estbalished *in vitro* model using mouse faeces, with a specific emphasis on refining methodologies for the precise detection of clozapine. A critical finding of this chapter was that the heat-killing methods previously used were insufficient, and the employment of autoclaving was necessary for thorough sterilisation of gut microbes. The fluorescein diacetate (FDA) test, proved not to be an accurate measure of the specific metabolic activity under investigation, as FDA results did not correlate with the anticipated changes in clozapine and norclozapine levels. However, gas chromatography-mass spectrometry (GC-MS) was successfully optimised with trifluoroacetic acid (TFAA) and a methanol with methyl tert-butyl ether (MTBE) extraction solvent, yielding a far superior signal detection. The key results from this chapter provided conclusive evidence that the gut microbiome facilitates the conversion of clozapine to its metabolite, norclozapine, as demonstrated by the increased norclozapine to clozapine ratio observed in samples from Specific Pathogen Free (SPF) mice in comparison to autoclaved controls. Critically, this conversion was absent in samples from Germ Free (GF) mice, thereby confirming the essential role of gut microbial activity in this metabolic process.

The third chapter detailed the translation of these optimised methodologies to human samples, where it was found that a method of normalization based on both sample weight and overall metabolic activity (via the FDA assay) was essential. However, as with the mouse samples, the FDA assay failed to correlate with the ratio as expected. This chapter uncovered significant inter-individual variability in clozapine metabolism among the five human donors tested, emphasising the critical influence of individual microbiome variations. Notably, norclozapine was also detected in autoclaved control samples incubated with clozapine, suggesting a non-microbial, heat-stable enzyme source, or a chemical transformation was taking place. These conversions were shown to be independent of overall hydrolytic activity. The complexity of clozapine metabolism was further highlighted by the failure of a Pearson correlation analysis, which could not confirm a statistically significant microbiome-driven metabolism of clozapine. These outcomes underscored the need for a more detailed investigation of experimental conditions and analysis techniques.

The fourth chapter of this thesis focused on refining human sample analysis, concentrating on discrepancies identified in the previous chapter. In order to control for the metabolic variations between samples, a method

was employed to normalise samples based on the output of the FDA assay. Further investigations were conducted to determine the source of the unexpected norclozapine production in control samples by employing additional dilutions and the use of the enzyme inhibitor, proteinase K. Proteinase K was unexpectedly shown to increase, not decrease, the ratio, and dilutions did not affect the ratio, which confirmed that a non-human and non-protease source was responsible. The repeat experiments confirmed a positive correlation between clozapine and norclozapine over time, however this failed to indicate a direct microbial interaction, according to Javdan's classification, and the samples exhibited similar ratios to each other and to controls. This result was due to increased levels of both clozapine and norclozapine that were seen in the controls.

Several key themes arise from these findings. Firstly, the study confirmed that a functional gut microbiome is necessary for the conversion of clozapine to norclozapine, and demonstrated this ability in a complex study of mouse and human faeces, suggesting that the therapeutic outcomes could be influenced by the gut microbiome. Secondly, significant inter-individual variability in clozapine metabolism was uncovered between the human donors, highlighting the personalized nature of drug responses and the need for tailored approaches to clozapine therapy, potentially guided by individual microbiome profiles. The identification of non-microbial contributions, via heat-stable enzymes or alternative chemical pathways, was a completely novel finding, demonstrating that clozapine metabolism is not a function of active microbial activity, and encourages additional exploration into the origin of this conversion. It was also highlighted that limitations exist in current methodology, with discrepancies in results across metabolic assays. The temporal dynamics were highlighted to be complex, with changes in the relationship between clozapine and norclozapine detected across incubation times, demonstrating that additional research is needed to understand the kinetic changes in these samples.

Research Implications

These findings lay the groundwork for future research into the role of the gut microbiome in clozapine metabolism. These results demonstrate that clozapine can be detected following incubation with gut microbiota cultures, and that clozapine metabolism can be quantified. This sets the foundation for subsequent studies to investigate whether specific microbial compositions can predict clozapine metabolism in patients with early-onset schizophrenia.

Furthermore, these findings may inform new psychopharmacological approaches that target the microbiome to enhance clozapine metabolism. Understanding which microbial species are involved in clozapine metabolism could lead to the development of probiotics or prebiotics designed to promote beneficial microbial communities, potentially improving drug efficacy and reducing side effects.

Additionally, incorporating demographic factors such as age, gender, and ethnicity into future studies may help to elucidate how these variables influence the interaction between clozapine and the gut microbiota. Personalized medicine approaches could then be developed to optimise clozapine therapy based on individual microbiome profiles.

Limitations

Discrepancies Between In vivo and In vitro Conditions

There are several limitations of this in vitro model, pertaining to the lack of representation of physiological conditions, which may impact the applicability of these results in a clinical setting. This study is solely focusing on the gut-microbial contributions to clozapine metabolism, without considering the role of the liver and biliary system in producing metabolites. Furthermore, the *in vitro* model does not fully replicate the complex environment of the human gut, including variables like pH, temperature and nutrient gradients, as well as interactions with host tissues, including the immune system (Jones et al., 2015). This discrepancy can lead to variations in growth rates and nutrient utilization, which will in turn affect the proliferation of bacteria (Brown et al., 2012), which limits the relevance of the findings. Studies have evidenced specific discrepancies between *in vitro* model findings and real, *in vivo* conditions, demonstrating that bacterial-derived enzymatic activity is frequent lower *in vitro* (Cole et al., 1985). More novel techniques exist that provide a better representation of *in vivo* microbial-derived metabolism (Tan et al., 2023), including the follow up experiments that Javdan and colleagues (2020) conducted. Future studies should incorporate models that simulate the small intestine better – for example more advanced in vitro models like bioreactors (McDonald et al., 2011), direct sampling from the small intestine (Wang et al., 2024) or liver (Zimmerman et al., 2019) and computational models (Zimmerman et al., 2019).

Variations in Gut Physiology

It is also of note that gut communities, as well metabolism and absorption, vary along the intestine, with the small intestine containing an estimated 10^8 bacterial cells/ml and the large intestine and 10^{11} cells/ml in the large intestine (Sender et al., 2016). Although there a fewer bacterial cells in the small intestine, greater metabolic activity occurs here, including a majority of drug absorption (Zimmerman et al., 2019b), particularly in the duodenum and jejunum. This is due to the larger surface area and presence of transport mechanisms (Hua, 2020) in the small intestine, in contrast to the large intestine. The small intestine also has slightly alkaline pH levels (Hua, 2020) which is more favourable for drug absorption. Therefore, colonic bacteria likely do not encounter much clozapine and therefore are less likely to possess metabolic mechanisms in relation to it. Indeed, it has been evidenced that colonic-transformation of drugs differs from metabolism in other regions of the gut (Iswandana et al., 2018), which is likely due to the spatial heterogeneity of bacteria in the various gut regions (Na Li et al., 2020). For example, Iswandana and colleagues (2018) detailed that the ileum tends to perform phase I metabolic reactions, such as oxidation and reduction reactions which, in the case of testosterone, result in the production of metabolites 6β-hydroxytestosterone and 2β-hydroxytestosterone. This could be linked to higher colonisation of Proteonbacteria, Lactobacillaceae, and Cyanobacteria that tend to be involved in oxygen-dependent reactions (Na Li et al., 2020). Contrastly, phase II reactions tend to occur in the colon (Iswandana et al., 2018), which tend to be performed by saccharolytic anaerobes, which include Bacteroidetes and Ruminococcaceae, that are observed in higher proportions in the colon (Na Li et al., 2020).

In the case of testosterone, this results in the production of metabolites 15α-hydroxytestosterone and 7-hydroxycoumarin glucuronide (Iswandana et al., 2018). As clozapine is metabolised to its metabolite norclozapine via an oxidation reaction, it is unlikely that this can occur via anaerobic colonic bacteria. However, it could be possible that these bacteria could produce reactive oxygen species (ROS) – a mechanism possessed by Bacteroides fragilis and Escherichia coli (Borisov et al., 2021) – which could participate in the oxidation reaction to produce norclozapine in the colon. Further research is warranted to elucidate the specific mechanisms here. Faeces were used in this study, so colonic bacteria will be the primary type that proliferate in vitro. Therefore, these samples will not reflect dynamic changes which occur from diet, medications and overall health (Skonieczena-Zydecka et al., 2019). They also may not reflect the metabolic activity that occurs higher up in the gut. Whilst the samples in this study did show some metabolic activity, this is likely to be an under-representation of the maximum physiological metabolic capability from the bacteria in the rest of the gut.

Analytical Technique

Additionally, an issue encountered by Javdan and colleagues (2020) could be relevant in this study – a large proportion of the drugs were unable to be analysed. Whilst this was mostly due to the reduced sensitivity of LCMS compared to GCMS, another factor for this was the instability of the drugs, particularly after overnight incubation within the no-microbiome control condition, indicating that certain drugs are not fit for this style of investigation. It could be possible that clozapine falls under this category.

Whilst LCMS does have its limitations, there is much more literature, and more recent literature using this technique for the quantification of clozapine and its metabolites. Papers have successfully used LCMS (Rao et al., 2009) and high throughput paper spray mass spectrometry (PS-MS; Saatchi et al., 2024), in conjunction with other techniques, such as in-source collision-induced dissociation (Sato et al., 2021), electrospray ionisation (Aravagiri and Marder, 2001; Rao et al., 2009), paper-spray isotopic internal calibration (Couchman et al., 2013). These techniques may be more successful by expanding the concentration range of the calibration curves, improving linearity and quantitative range (Sato et al., 2021).

It must also be noted that most of these studies used plasma or serum samples. The use of faeces in this study introduces many other confounding components that increase the difficulty of interpreting the metabolomic output. Such confounding components including compounds from the host, from the diet and other microbial products that can be difficult to distinguish from the metabolites. This can lead to misinterpretation of the data. These confounding components also result in higher detection limits for metabolites, making it challenging to detect lower-abundance metabolites (Koek et al., 2011). Plasma and serum samples lack this complexity and are more homogeneous, making it easier to detect and quantify specific metabolites. To remove these confounding components from faeces, unique extraction methods and solvents that have not previously been documented may be required, encouraging experimentation and optimisation prior to conducting the complete experiment, as demonstrated by Erben and colleagues (2021). Whilst this was performed in this study, these techniques are still complicated and this must not be ignored. To overcome this, some studies analysing faeces

have adopted techniques like Rapid Evaporative Ionisation Mass Spectrometry (REIMS) which do not rely on extensive sample preparation (Cameron et al., 2019).

Other Methodological Limitations

Caution is needed in interpreting these findings due to the small sample size, which limits the statistical power and generalisability of the findings (Button et al., 2013). Although triplicate repeats were conducted, there was notable variation within these repeats. A larger sample size would have facilitated more accurate statistical analyses – particularly with the correlational analyses used to identify microbial contributions to metabolism, as described by Javdan and colleagues (2020). A higher number of participants here would have more distinctly highlighted outlined any trends in the clozapine:norclozapine relationship (Gelman and Hill, 2007).

The method of storage and handling of faeces can significantly affect microbial diversity and viability. Bircher and colleagues (2020) evidenced this comparing the differences in bacterial composition of microbiota from plankton and sessile artificial colon, before and after various types of freezing storage. They discovered that there were differences in the bacterial communities before and after freezing, including reduced reestablishment of Bacteroidaceae and reduced propionate formation. Despite all samples in this study receiving the same method of storage (freezing in -70 degrees C), this can still produce variable re-establishment of communities – with Bilinski and colleagues (2022) showing a 55% reduction in alive cell counts between fresh and frozen samples, after culturing. The freeze-thaw cycle of the samples can also affect metabolite detection (Goodman et al., 2021), causing the metabolites to breakdown and produce a weaker signal. However, faeces have been shown to remain stable, even after storage of 11 years at -80 degrees (Gies et al., 2019).

The risk of contamination during the handling and processing of samples is a significant concern in microbiome research, as it can introduce biases and skew the outcomes of the experiments, leading to erroneous conclusions (Eisenhofer et al., 2019). This risk was mitigated by thorough training and the use of proper aseptic techniques, however, human error is inevitable.

A longitudinal study with more frequent analysis intervals may improve insights into temporal changes in microbiome-derived drug metabolism. For example, taking samples from the same donors at different times of day could contribute to generalisability of the results by taking into consideration varying dynamics of the gut microbiome – even in healthy controls (Halfvarson et al., 2017). Additionally, more frequent analysis intervals would provide a more detailed kinetic profile of clozapine metabolism and norclozapine formation (Schadt et al., 2018). A shorter analysis window – such as 8 hours – may have been beneficial to identify the initial interaction of the microbiome and clozapine (Timmerman et al., 2016). These increased data points would further improve the statistical power of the study, allowing for more accurate and reliable findings that better represent in vivo conditions, and can be more confidently applied to clinical settings.

Future Studies

As demonstrated by Javdan and colleagues (2020), additional biological investigations can be conducted that further elucidate the role of the microbiome in clozapine metabolism. Next-generation sequencing can identify specific functional microbial genes, enzymes and specific pathways that influence production of norclozapine.

Javdan and colleagues (2020) also performed transplantation studies with gnotobiotic mice to validate their in vitro findings in vivo. They treated two groups of mice with antibiotics to eliminate their native microbiome, then colonised one group with the microbiome of the pilot donor used in their initial screening. Both groups were administered capecitabine orally and metabolites in their blood and faeces were quantified at various time points via LCMS. They in vivo results confirmed the in vitro findings, showing a direct role of the microbiome in producing a specific capecitabine metabolite. This was evidenced as this metabolite was only detected in the faeces of the group colonised with the pilot donor microbiome. This was not detected in the blood samples of either groups, and there were no significant differences of levels of capecitabine and its other major metabolite between the groups.

Additionally it is important to explore demographic influences on individual bacterial metabolism of clozapine. Yin and colleagues (2023) demonstrated that gender affects the interaction between clozapine and the gut microbiota, particularly in the production of metabolic markers. Other factors including age and ethnic background should also be explored, as their contributions to clozapine metabolism have been elucidated (see section X Introdictoon).

It would also be beneficial to conduct 16S sequencing on the samples before and after incubation with clozapine, particularly as clozapine has been shown to influence the diversity and composition of the gut microbiota (Yin et al., 2023). Analysing the differences in these samples may elucidate which microbial species are involved in clozapine metabolism, which can potentially identify targets for optimising clozapine therapy.

To complete the physiological picture, future studies should include clozapine-naïve patients and follow them across treatment. Their blood plasma and faeces should be taken before, during and after treatment and the clozapine and norclozapine levels assessed via MS. This approach has been done in other studies exploring the interaction between the gut microbiome and other antipsychotics (Cussotto et al., 2019) and would assist with the understanding the pharmacokinetics of clozapine, as well as elucidating the dynamic interaction of the microbiome and clozapine.

Conclusion

This thesis sheds light on the intricate aspects of clozapine metabolism, with a key takeaway being the recognition of the gut microbiome as an essential, but not the sole, contributing factor. Despite unforeseen complexities, particularly in the control conditions, this work has created a strong platform for future studies to more closely examine specific microbial species, metabolic mechanisms, and environmental factors influencing clozapine metabolism. These findings, alongside the future directions and considerations outlined in subsequent sections, will contribute to the progression of precision medicine in psychopharmacology.

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