ELUCIDATION OF THE IN-VITRO PRODUCTS OF 25H-NBOMe USING S9 LIVER PREPARATION: A PRELIMINARY INVESTIGATION

Honours Thesis

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Table of Contents

Declaration vi
Acknowledgements vii
List of Figures and Tables viii
List of Abbreviations xi
Abstract xiii

Chapter 1: Introduction

1.1 Introduction to NBOMe and rationale for the study 1
1.2 Legislation surrounding Novel Psychoactive Substances 5
1.3 Novel Psychoactive Substances: A problem faced by forensic laboratories 9
1.4 Drug metabolism in the human body: an overview 11
1.5 Phase I metabolism 12
1.6 Phase II metabolism 14
1.7 NBOMe-type drugs as hallucinogens 17
1.8 The analysis of NPS: Evolution and evaluation of methods over time 19
1.9 Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry 24
1.10 Aims 27
1.11 Hypothesis 27

Chapter 2: Materials

2.1 Incubation assays 30
2.2 Sample extraction and reconstitution 30
Chapter 3: Methods

3.1 Preparation of parent xenobiotic
3.2 Preparation of incubation mixtures
3.3 Phase I metabolism: Incubation of 25H-NBOMe with S9
3.4 Phase II metabolism: Incubation of 25H-NBOMe with S9 and phase II conjugating compounds
3.5 Sample extraction and reconstitution
3.6 Incubation Mixture Protocol
3.7 Variable 1: Incubation time
3.8 Variable 2: Effect of enzyme concentration
3.9 Variable 3: Extraction efficiency
3.10 Phase I 25H-NBOMe metabolism
3.11 Phase II 25H-NBOMe metabolism

Chapter 4: Results and Discussion
Method development

4.1 Tramadol: Identification of the parent compound
4.2 Demethylated tramadol
4.3 JWH18: Identification of parent compound
4.4 Monohydroxylated JWH18
4.5 Incubation time
4.6 Effect of enzyme concentration
4.7 Extraction efficiency

25H-NBOMe Phase I metabolism
4.8 25H-NBOMe: Identification of parent compound 51
4.9 Demethylated 25H-NBOMe 53
4.10 Hydroxylated 25H-NBOMe 56

25H-NBOMe Phase II metabolism

4.11 25H-NBOMe Acetylation 58
4.12 25H-NBOMe Glucuronidation 59
4.13 25H-NBOMe Sulphation 59
4.14 Search for Phase II conjugating enzymes 60
4.15 Failure to produce or detect Phase II metabolites of 25H-NBOMe 61
4.16 Absence of soluble enzymes required to catalyse the conjugation of the activated compound with the xenobiotic 61

Chapter 5: Recommendations and Conclusions

5.1 Enzyme profiling studies 67
5.2 Improving the detection of Phase II conjugates 68
5.3 Comparison of this investigation to other studies 69
5.4 Significance of this study 71

Chapter 6: References 74
Appendices

A  PCDL spectra of Tramadol  78
B  PCDL spectra of JWH18   78
C  PCDL spectra of 25H-NBOMe  78
Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

CLeTessier
(Christine Le Tessier)
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List of Figures and Tables

Figure 1.1  The structures of 25B, 25H, 25C and 25I-NBOMe  4
Figure 1.3  Chromatographic separation of 25B and 25I-NBOMe-d3 in the patient’s serum sample  11
Figure 1.5.1  Example of the Phase I process of demethylation for 25B-NBOMe  14
Figure 1.5.2  Example of the Phase I process of hydroxylation for 25B-NBOMe  14
Figure 1.6.1  Activation of acetate to Acetyl CoA preceding the process of acetylation  15
Figure 1.6.2  Formation of active UDP glucuronic acid prior to Phase II glucuronidation  16
Figure 1.6.3  Activation of PAPS required before conjugation of the xenobiotic with sulphate  17
Figure 1.8  How S9 and HLMs are derived from hepatocytes  23
Figure 1.9.1  The process of LC-QTOF-MS  25
Table 3.6  Concentration of reagents in preliminary incubation mixtures  35
Table 3.8  Incubation assays carried out to investigate the effect of enzyme concentration  35
Table 3.10  Contents of the incubation mixtures to assess Phase I 25H-NBOMe metabolism  36
Table 3.11  Incubations carried out for Phase II metabolism  37
Figure 4.1.1  Chromatogram derived from incubations containing tramadol  39
Figure 4.1.2  Mass spectrum obtained from the MSMS of tramadol  40
Figure 4.2.1  Chromatogram of demethylated tramadol obtained from incubation mixtures  41
Figure 4.2.2  Mass spectra of demethylated tramadol indicating the loss of a methyl group  41
Figure 4.2.3  Mass spectrum of demethylated tramadol obtained after 4.9 minutes  42

Figure 4.2.4  Fragmentation of tramadol and its demethylation sites as deduced from the mass spectra  42

Figure 4.3.1  Chromatogram obtained of incubation mixtures containing parent JWH18  43

Figure 4.3.2  Mass spectra of parent JHW18 from incubation mixtures  44

Figure 4.4.1  Chromatogram of monohydroxylated JWH18  45

Figure 4.4.2  Mass spectra after MSMS of monohydroxylated JWH18 obtained after 8.9 minutes  45

Figure 4.4.3  Proposed fragmentation and monohydroxylation of JWH18 at three suggested sites  46

Table 4.5  Average peak area of tramadol and JHW18 metabolites obtained after 1 and 3 hours  47

Figure 4.6  Effect of enzyme concentration on the rate of metabolite production from 25H-NBOMe over a 1h incubation time  49

Table 4.7  Extraction efficiency: Percentage recovery of parent 25H-NBOMe following one, two, three and four extractions  50

Figure 4.8.1  Chromatogram of parent 25H-NBOMe obtained from incubation mixtures  45

Figure 4.8.2  Mass spectra of parent compound obtained from incubation mixtures containing parent 25H-NBOMe.  52

Figure 4.8.3  Predicted fragmentation of 25H-NBOMe derived from interpretation of its mass spectra  53

Figure 4.9.1  Chromatogram of demethylated 25H-NBOMe obtained from incubation mixtures  53

Figure 4.9.2  Mass spectra of demethylated 25H-NBOMe correlating to the peak obtained at 6.9 minutes  54

Figure 4.9.3  Proposed demethylation sites of 25H-NBOMe  55

Figure 4.10.1  Chromatogram of suspected hydroxylated metabolites of 25H-NBOMe obtained from incubation mixtures  56
| Figure 4.10.2 | Mass spectra of hydroxylated 25H-NBOMe | 57 |
| Figure 4.10.3 | Proposed sites of hydroxylation of 25H-NBOMe | 58 |
| Figure 4.14 | Chromatogram of parent paracetamol | 62 |
| Figure 4.16a | Potential acetylation site of the demethylated metabolite of 25H-NBOMe | 63 |
| Figure 4.16b | Potential glucuronidation site of the demethylated metabolite of 25H-NBOMe | 63 |
| Figure 4.16d | Potential site of sulphation of the demethylated 25H-NBOMe metabolite | 65 |

**Appendix**

| A | PCDL spectra of Tramadol | 78 |
| B | PCDL spectra of JHW18 | 78 |
| C | PCDL spectra of 25H-NBOMe | 78 |
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CYPs</td>
<td>Cytochrome P450 enzymes</td>
</tr>
<tr>
<td>EI</td>
<td>Electron Impact Ionisation</td>
</tr>
<tr>
<td>FMOs</td>
<td>FMOs Flavin-containing monooxygenases</td>
</tr>
<tr>
<td>HLMs</td>
<td>Human Liver Microsomes</td>
</tr>
<tr>
<td>HPLC/MS/MS</td>
<td>High Performance Liquid Chromatography and tandem Mass Spectrometry</td>
</tr>
<tr>
<td>LC-QTOF-MS</td>
<td>Liquid Chromatography-Quadrupole Time of Flight- Mass Spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>NATs</td>
<td>N- acetyltransferases</td>
</tr>
<tr>
<td>NPS</td>
<td>Novel Psychoactive Substances</td>
</tr>
<tr>
<td>PAPS</td>
<td>Phosphoadenosine Phosphosulphate</td>
</tr>
<tr>
<td>PCPI</td>
<td>Positive Ion Chemical Ionisation</td>
</tr>
<tr>
<td>PCDL</td>
<td>Agilent Mass Hunter Personal Compound Database and Library</td>
</tr>
<tr>
<td>SULTs</td>
<td>Sulphotransferases</td>
</tr>
<tr>
<td>UGTs</td>
<td>UDP-glucuronosyltransferases</td>
</tr>
<tr>
<td>UNDOC</td>
<td>United Nations Office on Drugs and Crime</td>
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Abstract
In recent years it has become increasingly important for toxicology laboratories to understand the metabolism of Novel Psychoactive Substances (NPS) to facilitate their identification in forensic and medical contexts. Abuse of the potent hallucinogenic NBOMe-type NPS, colloquially known as “N-bomb” has become prevalent in recent years. There are many different forms of these synthetic phenethylamines including 25I, 25C and 25B-NBOMe. The focus of this research is to establish optimised in vitro methods for the formation of 25H-NBOMe metabolites and liquid chromatography-mass spectrometry techniques to identify them. It was hypothesised that the metabolic transformations undergone by 25H-NBOMe would parallel those previously reported for other NBOMe variants.

25H-NBOMe was incubated with human S9 liver preparation; a liver post-mitochondrial supernatant, thus simulating in vivo xenobiotic metabolism. The metabolic products were extracted, and the chromatographic profile was generated via LC-QTOF-MS. Demethylated and hydroxylated products were identified resulting from Phase I biotransformation. In incubation mixtures containing acetyl CoA, UDP glucuronic acid and phosphoadenosine phosphosulfate, the presence of conjugates generated by the Phase II processes, acetylation, glucuronidation, and sulfation were explored. Neither glucuronides nor sulfate or acetyl conjugates were detected. Potential sites on the NBOMe molecule for demethylation, hydroxylation are discussed.
Chapter 1: Introduction
1.1 Introduction to NBOMe and rationale for the study

Psychoactive substances, like all other drugs and xenobiotics, are prevalent in our society. Xenobiotics are substances foreign to the body, required to be processed and eliminated. As the name suggests, psychoactive substances are compounds which by binding to various receptors in the brain, generate a variety of different mental and physical effects resulting in alterations to brain function\(^1\). Such substances are generally prohibited and used for recreation, including those commonly known as cannabis, LSD, cocaine and ecstasy. These drugs are taken recreationally to experience side effects such as euphoria and hallucinations. Deleterious side effects include nausea, coronary arrhythmia and paranoia, subject to the type of drug, dose and frequency of use\(^2\). There are many different psychoactive drugs, many different forms of the same drugs, and many ways of ingesting them to experience maximum effects. The desire for the perceived positive effects means there will always be a demand for such substances.

Novel Psychoactive Substances (NPS) are classified as substances that are synthetic derivatives of psychoactive compounds which are already illegal and controlled\(^3\). The production of such derivatives involves a vast range of chemical modifications. Most often this is the substitution of one functional group for another, or a structural re-arrangement to create an isomer. It therefore follows that this specific compound, of altered structure and chemical composition, is not specifically illegal.

Consequently, they are used recreationally to achieve the same or similar side effects as their illegal forms. This perpetuates the occurrence of what is termed “legal highs”. As the use of NPS increases, so do cases of intoxication and overdose and the risk of long term harm, as with any other drug. The problem with NPS is that the...
appropriate bodies (forensic laboratories and hospitals) cannot accurately test for or identify these drugs. This can hinder toxicological assessment and treatment\(^3\).

Hence, it is important that assays are developed to identify NPS and their metabolites such that reference standards can be developed and quantification achieved for use in forensic, coronial and criminal investigations. Not only does such work provide a means of appropriate treatment to be administered in cases of overdose, it provides the necessary information to enable the relevant bodies to enact legislation to prohibit the drug, reducing its danger to public health\(^4\).

One such NPS which has recently entered the illegal drug market is the NBOMe type. Colloquially known as “N-bomb”, NBOMe-type drugs are potent hallucinogens. The first of these was synthesised in 2003 by the German Chemist Ralf Heim, and was further studied by other chemists such as David Nichols, who determined the structure with the use of radioactive labelling\(^5\). NBOMe can be taken in the form of a tablet, a liquid, powder, or from blotting paper. By 2010, its recreational use had become significant, assisted by its availability online at low cost, as an alternative to the illegal hallucinogen LSD. Worldwide, abusers began to present with symptoms of drug intoxication including convulsions, hyperthermia, tachycardia and renal failure\(^6\). These patients reported use of “N-bomb”, and analysis of urine samples confirmed the presence of a particular form of NBOMe, the same as was found in the early investigative work of Nichols\(^5\).

Nichols found drugs of the NBOMe type to be a synthetic, modified form of the naturally occurring class of organic compound known as phenethylamines, present in some plant species. An example is the hallucinogenic alkaloid mescaline, found in the peyote cactus\(^7\). Modified phenethylamines appear to be the most numerous
among NPS, their structure characterised by substitution of the functional groups at a variety of different positions to confer different properties and potencies \(^{(8)}\). NBOMe is an N-benzyl derivative of the structure and the different substitutions of NBOMe will, most likely, exert effects of different magnitude \(^{(9)}\).

For example, 25B-NBOMe and 25I-NBOMe are both NBOMe-type drugs and share a common basic structure. Both 25B-NBOMe (4-bromo-2,5-dimethoxy-\(\beta\)-phenethyamine), and 25I-NBOMe (4-iodo-2,5-dimethoxy-\(\beta\)-phenethyamine) contain a 2,5-dimethoxy-\(\beta\)-phenethyamine moiety \(^{(9)}\). In 25B-NBOMe and 25I-NBOMe an amino group is replaced by bromine and iodine, respectively \(^{(9)}\).

Figure 1.1 displays the structures of four NBOMe derivatives. The arrows indicate the various functional groups that create the unique derivative. The circle around the carbon on the 25H-NBOMe structure shows the presence of the hydrogen, which may be substituted with a halogen to generate halogenated derivatives \(^{(10)}\).

Figure 1.1 The structures of 25B, 25H, 25C and 25I-NBOMe \(^{(10)}\).
As previously mentioned, drugs of the NBOMe type are taken recreationally for their hallucinogenic effects. Phenethylamines are 5-HT$_{2A}$ (5-hydroxytryptamine, subtype 2A serotonin) receptor agonists, meaning binding of such compounds increases the activity of the neurotransmitter serotonin. Consequently, the mood, behaviour and memory of the user is altered. Additionally, hallucinations are induced when 5-HT$_{2A}$ receptors in the cortex of the brain are stimulated, encouraging the use of the drug for recreational purposes$^{(11)}$. Modifications of phenethylamines to produce an NBOMe derivative can generate a product which exhibits a higher affinity for 5-HT$_{2A}$ receptors. Consequently, NBOMe drugs often display increased potency and more pronounced psychoactive effects at lower doses$^{(12)}$.

Elucidation of drug metabolites is a key process in preliminary investigation of NPS. It is often necessary to identify the metabolites of a drug additionally to the parent compound because, in most cases, time will have elapsed since ingestion and, as a consequence, metabolism will have occurred. Depending on the toxicokinetic properties of the drug in question, and the time elapsed since ingestion, the parent compound may be undetectable in body fluids$^{(13)}$.

**1.2 Legislation surrounding Novel Psychoactive Substances**

It is important to ensure that the legislation surrounding their availability, use and the consequences of NPS use are designed to facilitate the protection of the public. The aim of legislation is to criminalise the manufacture, possession with intent to sell, possession with intent to use, importation, exportation, and use of psychoactive substances$^{(14)}$. Currently, European countries, and in particular, the UK, have enacted the most comprehensive laws pertaining to the control of NPS$^{(15)}$. 
Globally, the use of NPS has increased markedly since 2013. According to the United Nations Office on Drugs and Crime (UNODC), 94 countries reported the use of 348 different NPS between 2008 and 2013. In 2015 alone, 643 new NPS were reported, encompassing 101 countries. These included a diverse range of substances including psychostimulants, opioids and cannabinoids. Different countries, having identified the various NPS via different means, have thus enacted laws prohibiting their use. It follows that with every new psychoactive drug, there may need to be an amendment to the laws prohibiting its possession or use.

The criminalisation of a specific drug requires data collection. Initially, this may be achieved by seizure of the drug itself, or may be derived from its detection in a biological matrix such as blood or urine. It follows that the laboratory performing the analysis must have access to and be capable of performing an assay which accurately identifies the compound. Compound identification and characterisation is essential knowledge because the structure and mechanism of drug action is required to be known before the substance can be rendered illegal and penalties imposed for its possession and use. For laws to be enacted, there must be evidence of the molecular mode of psychoactive action. However, the rapid proliferation of novel drugs, as previously discussed, limits the ability of laboratories to derive assays for their detection in a timely manner because appropriate methods, standards and reagents may not be available. Moreover, once an assay has been developed for a new drug, and laws enacted to make it illegal, it is possible that a different variant has emerged for which the lab has no validated assay for its detection or quantification.
The UNDOC evaluated the efficacy of laws enacted in those countries in which NPS appear to be most prevalent. Laws in some parts of the world are generic; they prohibit substances and any variations or derivatives which have the same core molecular structure\(^{(16)}\). This negates the need to individually and specifically name and outlaw a drug; advantageous because it automatically renders illegal a derivative of the parent drug. However, it can also be argued that this mode of law making is irresponsible because it automatically prohibits the use of a substance which, in comparison to the original form, may be more therapeutically useful\(^{(16)}\).

Alternatively, it is possible to prohibit drugs based on their biochemical activity rather than on the basis of their structure. For example, in the USA the “Synthetic Drug Abuse Prevention Act” of 2012, specifically prohibits any “cannabinimimetic” agent, that functions as a cannabinoid type 1 receptor (CB1) agonist\(^{(16)}\). However, the disadvantage of this method is the potential criminalisation of therapeutic agents with the same biochemical mode of action.

In many countries, drugs are criminalised by being specifically listed in the legislation. For example, in 2011, Brazil specifically listed the synthetic amphetamine “Mephedrone” as illegal under their psychoactive substances act\(^{(16)}\). This approach requires that any new substance be uniquely identified, its structure defined and its mechanism of action elucidated before it can be outlawed. This is not an effective method of criminalisation because it requires constant renewal of legislation. Given the nature of NPS, it is not possible to generate and validate assays rapidly enough to ensure that each new variant is specifically included in the law. This approach, however, may be applicable in countries with a small NPS market where it is more feasible to identify variants in legislation\(^{(16)}\).
To protect the public from “legal highs” in 2015 the Psychoactive Substances Bill in the UK was introduced, in compliance with the Human Rights Act of 1998\(^\text{(17)}\). The bill was drafted in a way to include not only substances already known, but those yet to be identified or yet to be generated\(^\text{(17)}\). This law criminalised the possession of and intent to supply, import or export psychoactive substances to be an offence with a possible maximum penalty of seven years imprisonment. It defined a psychoactive substance as any consumable substance capable of producing effects of a psychoactive nature. Additionally, the bill authorised law-enforcement officers to enter premises, search for, and seize suspicious material. Importantly, it also allows for seized substances to be retained. Consequently, when no longer required as evidence, they could be studied so as to enhance the knowledge of NPS currently circulating in the community\(^\text{(17)}\).

Prior to 2015 laws in the UK banned various phenylethylamine derivatives, however, N-benzyl substitutions were not explicitly included in the legislation. As NBOMes belong in this category, they were not specifically rendered illegal. In 2013, given the increased incidence of use and intoxication by 2C-NBOMe and 2I-NBOMe, swift action was taken by the British government in an attempt to limit the use of NBOMe\(^\text{(18)}\). A temporary class order was declared, immediately prohibiting sale, import and production of N-benzyl substituted phenethylamines, N-benzyl substitution being the chemical modification characteristic of NBOMe-type drugs. However, it did not restrict personal use\(^\text{(18)}\). Temporary class orders are employed when immediate action is required. They take effect within days, and are valid for a maximum of twelve months, during which time a Bill can be drafted to ban the drug permanently.
The lack of legislation specifically pertaining to NBOMe drugs is responsible, in part, for the rapid increase in their production and use in recent years. In the early 2000s the N-benzyl phenethylamine derivatives (NBOMe) became more prevalent at events such as music festivals. In 2013 NBOMe was identified as a major public health concern due to the report of many more derivatives and dangerous intoxications\(^\text{(19)}\).

1.3 Novel Psychoactive substances: A problem faced by forensic laboratories

To identify and quantify a substance, a reference standard and an authentic sample of the substance are required. If the drug is novel, it is highly likely that a reference standard is not available, and established methodology and instrument standardisation procedures are not yet in place.

Analyte biotransformation is another factor that must be taken into consideration. Psychoactive substances are active in biological matrices and are, therefore, subject to metabolic modification. The metabolic transformations that xenobiotics undergo in vivo often direct the analytical methods employed to detect them in body fluids. The parent drug may have been converted into a range of metabolites, some of which may have already been conjugated with other compounds in preparation for excretion. NBOMe drugs are ingested in very small amounts of approximately 50-100μg whereas other psychoactive drugs are consumed in amounts as high as 200μg\(^\text{(20)}\). This is reflected in the fact as little as 50μg of NBOMe can cause psychosis, whereas much higher doses of other drugs, being 100-200μg, is required\(^\text{(18), (20)}\). Therefore, detection of parent NBOMe is already more challenging than the detection of drugs taken at a higher dose. NBOMe is biotransformed into even smaller amounts of a range of metabolites such that the parent drug is often
present merely in trace amounts in blood or urine. In such cases the parent drug may be below the limit of detection.

Given that biotransformation has taken place, it is likely that the concentration of metabolites in the plasma or urine will be higher than that of the parent drug, making it imperative that research is undertaken on such drugs in order to identify their metabolites and to develop sensitive, reliable and validated assays to detect them\(^{(12)}\).

It is often via toxicological analysis of patient samples that new derivatives of novel drugs are discovered. Initially, tests are performed using standards of previously known variants, or with a standard of the parent drug. For example, Laskowski \textit{et al.} (2015), using an NBOMe reference standard, identified a new derivative\(^{(5)}\). Serum and urine samples were taken from a 16-year-old girl presenting with signs of NBOMe intoxication including visual hallucinations, slurred speech and loss of muscle control. The patient admitted to self-administration of a substance called “25I” via blotter paper at a music festival\(^{(5)}\).

Following liquid extraction procedures, the extract was analysed by High Performance Liquid Chromatography and tandem Mass Spectrometry (HPLC/MS/MS). An internal standard 25-NBOMe-d3 was used. Comparison of the resultant chromatograms, that of the reference standard and of the test sample, revealed the presence of chlorine in the test sample\(^{(5)}\). It can be seen from Figure 1.3 that the peaks in the 25-NBOMe-d3 and test chromatograms have different retention times, 9.42 and 7.38 minutes respectively.
Figure 1.3 Chromatographic separation of 25B and 25I-NBOMe-d3 in the patient’s serum sample, displaying the different retention times of each compound, assisting in the identification of a new previously unknown derivative.

The analysis revealed that the substance in the test sample contained a chlorine N-Benzyl substitution, and can thus be termed 25C-NBOMe; a new NBOMe derivative\(^5\). Confirmation of the structure of new derivative resulted from interpretation of the mass spectrum associated with the specific peak identified in the chromatogram.

1.4 Drug metabolism in the human body: an overview

An understanding of the metabolic fate of xenobiotics and the molecular mechanisms via which these biotransformations occur is central to this project. Foreign compounds such as NBOMe are metabolised by liver enzymes in two major phases; termed Phase I and Phase II reactions. Phase I reactions are designed to modify the xenobiotic as a means of reducing its biological activity. These reactions include oxidations, reduction, hydroxylation, deamination, decarboxylation and hydrolysis. These reactions not only (usually) reduce the biological activity of the parent compound, but prepare it for entry into Phase II reactions by providing the
compound with a conjugating site for molecules involved in the Phase II reactions\(^{(21)}\). For example, the hydroxyl group added to the compound in Phase I hydroxylation, provides a site for the enzyme-catalysed addition of a conjugating compound in a Phase II reaction.

Phase II reactions involve the conjugation of the xenobiotic, often, but not always, after Phase I modification, with another compound which renders it soluble in bile or urine so that it can be readily excreted from the body\(^{(21)}\). There are two types of Phase II reactions. The type 1 Phase II reactions involve the activation of the conjugating compound prior to its conjugation with the deactivated xenobiotic. In sulphation for example, sulphate is converted to its active form phospho-adenosine phosphosulphate (PAPS), while glucuronidation is the conversion of glucuronic acid to active UDP glucuronic acid. Similarly, the conversion of acetate to the active thiolester acetyl CoA, allows for the drug to be acetylated in a type 1 Phase II reaction\(^{(22)}\).

Type 2 Phase II reactions, on the other hand, involve activation of the xenobiotic itself, prior to conjugation. For example, the activated xenobiotic may be conjugated with glutathione to generate a urine-soluble mercapturic acid. Following Phase II reactions, the solubilised xenobiotic can then be excreted from the body in either urine or faeces\(^{(22)}\).

1.5 Phase I metabolism

Cytochrome P450 enzymes (CYPs) are the enzymes predominantly expressed in hepatocytes and are principally involved in Phase I reactions. Bound to the surface of the endoplasmic reticulum, CYPs catalyse approximately 75% of the interactions that occur between xenobiotics and human cells\(^{(23)}\). CYPs are the group of enzymes
that catalyse oxidative and hydroxylation reactions. They are feature a haeme group bound to a polypeptide chain and are classified into three “families”; CYP1, CYP2 and CYP3\textsuperscript{(24)}. Additionally, CYPs catalyse demethylation involving sulphur, nitrogen or oxygen atoms; S-, N- or O-demethylation respectively. In the case of O-demethylation, this generates an hydroxyl group which provides a conjugating site for the Phase II reactions. CYPs require both oxidising and reducing power for their catalytic activity, provided by molecular oxygen and NADPH, respectively. For this reason, CYPs are also referred to as “mixed function oxidases”\textsuperscript{(25)}. The “P450” assignment to the name of these cytochromes refers to the wavelength (450nm) at which these cytochromes exhibit maximum spectrophotometric absorption (450nm)\textsuperscript{(26)}.

Caspar et al. (2017) identified some of the CYPs involved in the metabolism of 25B-NBOMe. The authors found that CYPs CYP1A2, CYP2B6, CYP3A4 and CYP3A5 were capable of cleaving an N-demethoxybenzyl group from the parent drug and that CYP1A2, CYP2C9, CYP2C19 and CYP3A4 catalysed O-demethylation of the compound\textsuperscript{(12)}. They concluded from LC-HR-MS/MS analysis that O-demethylation and hydroxylation were the main Phase I biotransformation reactions that occurred. It was reported that 25C and 25I-NBOMe were similarly metabolised and it can be inferred that 25H-NBOMe is likely to undergo similar metabolic transformations catalysed by the same or closely related CYP isoenzymes\textsuperscript{(12)}. 

13
The key Phase I reactions explored in this investigation were demethylation and hydroxylation. Demethylation is the enzyme-mediated removal of CH$_3$ from a compound, shown in Figure 1.5.1 for 25B-NBOMe$^{(27)}$.

![Figure 1.5.1 Example of the Phase I process of demethylation for 25B-NBOMe.](image)

Hydroxylation is the enzyme mediated addition of an OH group to a compound, as shown in Figure 1.5.2 for 25H-NBOMe$^{(27)}$.

![Figure 1.5.2 Example of the Phase I process of hydroxylation for 25B-NBOMe.](image)

1.6 Phase II metabolism

Additionally, Wohlfarth et al. (2016) found that when 25C and 25I-NBOMe were incubated with human hepatocytes, acetylation, sulphation and glucuronidation where the main Phase II reactions to occur. It is speculated that these Phase II reactions will also be characteristic of 25H-NBOMe metabolism$^{(13)}$. Phase II metabolism therefore involves activation of either the conjugating compound or the xenobiotic itself, followed by enzyme-catalysed conjugation of the parent
compound, or a less-biologically active Phase I metabolite. Most commonly, this generates a glucuronide, acetyl or sulphate derivative. Acetylation, glucuronidation and sulphation are all type 1 Phase II reactions, which require activation of the conjugating compound prior to conjugation.

**a. Conjugation with Acetate**

Acetate is activated as shown in Figure 1.6.1\(^{(28)}\):

\[
\text{Acetate} + \text{CoASH} \rightarrow \text{Acetyl CoA (active)}
\]

**Figure 1.6.1** Activation of acetate to Acetyl CoA preceding the process of acetylation.

Through the action of acetyl CoA synthetase, active acetyl CoA is produced which then donates its acetyl group to the xenobiotic in a type 1 Phase II conjugation reaction\(^{(28)}\).

**b. Conjugation with Glucuronic acid**

As illustrated in Figure 1.6.2, UDP glucose pyrophosphorylase is responsible for the synthesis of UDP glucose from glucose-1-phosphate. The UDP glucose generated is oxidised with NADP to form active UDP glucuronic acid and NADPH, the reaction being catalysed by UDP- glucose dehydrogenase\(^{(29)}\).
**Figure 1.6.2** The generation of active UDP glucuronic acid prior to Phase II glucuronidation.

The Phase I metabolite is then conjugated with glucuronic acid to form a glucuronide which is excreted.

**c. Conjugation with Sulphate**

Figure 1.6.3 depicts the formation of “active sulphate” (PAPS), which precedes sulphate conjugation in a type 1 Phase II reaction. Adenosine and phosphate sourced from ATP are joined to sulphate ($\text{SO}_4^{2-}$) to from adenosine phosphosulphate.

Adenosine phosphosulphate is then phosphorylated by ATP to produce phosphoadenosine phosphosulphate, which provides the source of the sulphate for conjugation with the xenobiotic in Phase II metabolism.$^{(30)}$
Phase II biotransformation may further reduce the bioactivity of the Phase I xenobiotic metabolite, and the modification alters its polarity making it more soluble in either urine or bile which aids its excretion from the body.

1.7 NBOMe-type drugs as hallucinogens

It is reasonable to infer the NBOMe drugs are hallucinogens firstly because they are structurally similar to LSD, a known hallucinogen, and secondly, because, phenethylamines are known central nervous system stimulants. Importantly, some users, having experienced hallucinations after reporting LSD use, were actually found to have taken NBOMe\(^{(13)}\). It is reasonable to conclude that NBOMe is psychototropic in some way (affecting the brain and one's mental state). If this is accepted, the next step is to specify the biochemical mechanism by which this occurs.

Rickli et al. (2015) investigated the binding affinities and receptor interaction profiles of a number of a number of 2C (psychedelic phenethylamines with a
methoxy group on the 2 and 5 position of the benzene ring) and NBOMe-type drugs. A number of 2C drugs and their corresponding NBOMe derivatives, along with LSD and mescaline (for corroboration and comparison) were incubated with human cells which had been transfected with various known drug transporters and receptors known to produce psychotropic effects\(^{(31)}\). To evaluate the activity of the serotonin 5-HT\(_{2A}\) receptor in the presence of the various xenobiotics, embryonic fibroblasts (NIH-3T3) cells were incubated with the test substance and the fluorescence subsequently measured, derived as the receptors were tagged with a fluorescent compound\(^{(31)}\).

The activity of the serotonin 5-HT\(_{2B}\) receptor was investigated using HEK 293 (human embryonic kidney cells) in a similar manner\(^{(31)}\). In general, it was found that all of the test substances bound to 5-HT\(_{2A}\) and 5-HT\(_{2C}\) receptors with high affinity. Importantly, the N-2-methoxybenzyl substitution which generates an NBOMe, resulted in a 26 and 14-fold increase in binding affinity for these receptors respectively. This is approximately 8.4 times higher than that resulting from LSD\(^{(31)}\).

It was also found that, like LSD, NBOMe drugs exhibited high affinity for adrenergic \(\alpha_{1A}\) receptors. This correlates with the cardiovascular side effects reported by users including tachycardia and other adrenergic-like effects\(^{(31)}\). The nature of the interaction between NBOMe and the serotonin 5-HT\(_{2A}\) receptors is unknown and its mode of action via such an interaction can only be inferred. It is important to note that though this study concluded that NBOMe drugs bind with increased affinity to 5-HT\(_{2A}\) receptors, studies have not been conducted to confirm whether or not this binding leads to increased receptor antagonism, when compared to LSD\(^{(31)}\).
Serotonin receptors are named because of their capacity to bind 5-hydroxytryptamine (5-HT). Serotonin (5-hydroxytryptamine) is a monoamine neurotransmitter. 5-HT$_{2A}$ receptors are predominantly located on the dendrites of nerve cells in the cortex of the brain$^{(32)}$. Physiologically, serotonin controls functions such as appetite, thermoregulation, mood and various cognitive processes. Additionally, activation of the 5-HT$_{2A}$ receptor has been implicated in stress-mediated pathophysiological responses$^{(33)}$. Release of serotonin is known to have a role in learning and cognition, and increased levels are known to distort perception, creating hallucinations.

The mechanism of 5-HT$_{2A}$ activation involves interaction with a G-protein, the closing of potassium ion channels and consequential depolarisation of the axon$^{(32)}$. The increased excitability of the neuron results in increased serotonin release. This can generate hallucinations due to the wide distribution of the receptors on postsynaptic dendrites in the central nervous system$^{(34)}$.

1.8 The analysis of NPS: Evolution and evaluation of methods over time

The development of sensitive, optimised assays to identify and quantify in body fluids, not only parent drugs of the NPS class, but also their metabolites, is essential from a medical and forensic perspective. One of the early methods used to identify the presence of NPS employed Gas Chromatography-Mass Spectrometry (GCMS). In 2004 Staack and Maurer published the method they employed to detect the amphetamine-derived novel drug 1-(3,4-methylenedioxybenzyl) piperazine (MDBP) and its metabolites in rat urine. They found the drug and its metabolites to be higher in concentration and to persist for longer in the urine than in the plasma. This early study aimed at the detection of NPS was an important advancement$^{(35)}$. 
The components of the incubation mixtures (containing MDBP and its metabolites, both acetylated or methylated) were separated by gas chromatography. The ionisation methods used were EI (electron impact ionisation) followed by PICI (positive ion chemical ionisation). Comparison of the resultant mass spectra with those on the reference database confirmed the identity of the ions in the spectra\(^{(35)}\).

The structure of the various metabolites was inferred from the fragmentation pattern of the ions.

It was concluded that MDBP is metabolised via demethylation followed by glucuronidation and sulfation to render the metabolites urine soluble. This study established protocols for the detection and identification of other novel drugs in urine\(^{(35)}\). Consequently, a similar approach was employed by Caspar et al. (2017) to detect and identify 25B and 25C-NBOMe and their metabolites in rat and human urine\(^{(12)}\).

After the administration of the drugs to male Wistar rats, urine was collected for the next 24 hours. A human urine sample was also obtained antemortem from a suspected case of 2C-B-NBOMe ingestion. Sample preparation involved pH adjustment with acetic acid and incubation with glucuronidase and arylsulfatase to hydrolyse potential conjugates. A series of elution and evaporation steps was performed, and a 5μL aliquot was analysed using LC-HR-MS/MS\(^{(12)}\).

Identification of the metabolites of 25C and 25B-NBOMe metabolites was achieved via interpretation of the high resolution MSMS fragmentation patterns for each drug. The human sample was similarly treated and analysed. The analysis confirmed that fragment ions resulted from cleavage of the NBOMe portion of the drug, rather than
from cleavage of the 2C structure. The fragmentation patterns were found to be similar to that of 25I-NBOMe\(^{(12)}\).

This analytical approach appears to be suitable for the detection of NBOMe metabolites and can provide information on the biotransformations undergone \textit{in vivo}. However, as the majority of xenobiotic metabolism occurs in the liver, more direct information on the Phase I and Phase II reactions involved in the metabolism can be obtained from \textit{ex vivo} studies using hepatocytes or from \textit{in vitro} studies using microsomal preparations.

Logically, the former approach is preferable as it more closely approximates \textit{in vivo} conditions. However, in most laboratories it is not feasible to use hepatocytes directly. This is because the maintenance of hepatocyte cell cultures requires expensive equipment and nutritional media. Additionally, the expertise required to maintain cell culture growth and analysis requires expertise not accessible to every laboratory\(^{(11)}\). Consequently, \textit{in vitro} approaches using liver microsomes are often favoured.

Liver microsomes are fragments of endoplasmic reticulum to which are bound enzymes involved in the Phase I metabolism of xenobiotics. Of significance are the CYPs, Flavin-containing monooxygenases (FMOs) and UDP-glucuronosyltransferases (UGTs)\(^{(4)}\) They are obtained and isolated from hepatocytes in a series of homogenisation and centrifugation steps. Human Liver Microsome preparations (HLMs) do not contain any cytosol, therefore, in incubation mixtures where HLMs are the biological matrix of choice, the mixtures need to be supplemented with cytosolic enzymes known to be involved in the enzymatic processes being investigated. Incubation of these preparations with a xenobiotic of
interest in vitro, followed by analysis of the products via LC or GC and MS, provides a means by which metabolites can be characterised\(^{(4)}\).

In 2016, Nielson studied the metabolism of 25I-NBOMe by incubating the drug with human liver microsomal preparations in the presence of NADPH. Supernatants obtained from the incubations were then analysed by LCMS. Interpretation of the resultant mass spectra allowed identification of metabolites produced by O-demethylation, N-dealkylation and dehydrogenation\(^{(8)}\).

A potentially more accurate and broad-spectrum approach to the investigation of xenobiotic metabolism in the liver is to incubate the drug in question, in vitro, with S9 liver preparations. This preparation is similarly obtained via homogenisation and centrifugation of hepatocytes at 9000g (hence the name “S9”\(^{(4)}\)). These preparations are also referred to as “post-mitochondrial supernatants” because they do not contain mitochondria, these having been lysed and released in the homogenisation process and removed by low speed centrifugation. S9 preparations contain both hepatocyte microsomes and cytosol (unlike microsomes which do not contain cytosolic enzymes). Consequently, S9 preparations not only contain the microsomal monooxygenases associated with Phase I biotransformation, but the soluble, cytoplasmic enzymes involved in Phase II conjugation reactions. S9, therefore, is more representative of the hepatic enzymes in drug metabolism\(^{(4)}\). Figure 1.8 illustrates the difference between S9 and HLMs.
Figure 1.8. How S9 and HLMs are derived from hepatocytes\textsuperscript{(36)}.

Otto \textit{et al.} (2008) designed an assay to investigate the potential hepatotoxicity of the non-steroidal anti-inflammatory drug diclofenac, and the antibiotic, minocycline. S9 liver preparations and an NADPH regenerating system were employed to maximise the \textit{in vitro} yield of Phase I metabolites\textsuperscript{(37)}. Being that microsomal monoxygenases require the supply of a reductant, in the form of NADPH, and an oxidant, in the form of molecular oxygen, the incubation mixtures were supplemented with NADP\textsuperscript{+} and glucose-6-phosphate. During the course of the incubation, glucose-6-phosphate dehydrogenase, present in the cytosolic component of the S9 preparations, converts glucose-6-phosphate dehydrogenase to 6-phosphoglucono-δ-lactone, with the concomitant production of NADPH from NADP\textsuperscript{+}. This NADPH regeneration process provides a constant supply of the reducing power required for Phase I
monooxygenases such as the CYP group of isoenzymes\textsuperscript{(37)}. Following further refinement, the incubation mixtures were analysed by TSQ Quantum Ultra AM quadrupole mass spectrometry to identify the metabolites\textsuperscript{(37)}.

The use of human S9 preparations to investigate xenobiotic metabolism \textit{in vitro} is preferable to \textit{in vivo} studies in rats because S9 preparations are a commercially available product, negating the requirement for animal ethics approval. Moreover, differences in metabolism exist between species, and it is clearly not possible to conduct \textit{in vivo} experiments on humans for ethical reasons.

**1.9 Liquid Chromatography-Quadrupole Time of Flight -Mass Spectrometry (LC-QTOF-MS)**

In this study of the \textit{in vitro} metabolism of 25H-NBOMe, LC-QTOF-MS will be used to separate and identify the metabolic products generated in the incubation mixtures. The schematic shown in Figure 1.9.1 summarises the sequence of events that will be applied to separate the components of the mixture according to their differential affinities for the liquid and stationary phases. In this process, the separation depends on differences in the molecular size and polarity on the compounds present, and is also influenced by the type of column used\textsuperscript{(38)}. To identify the compounds present, each is injected into the ionisation chamber of the mass spectrometer where the chosen ion source bombards it with high energy molecules, such as electrons, fragmenting the sample into high energy ions\textsuperscript{(39)}. 
The quadrupole time of flight mass analyser filters the ions according to velocity. “Time of Flight” refers to the fact that the m/z (mass to charge ratio) of each ion is determined according to the time taken for it to reach the detector. The quadrupole mass filter is one of the most common and accurate mass analysers. It consists of four metal cylinders acting as electrodes. A radio frequency is applied to the rods, and the ions from the samples travel in the space between them. The trajectory of the ions is influenced by the particular current applied. Being that ions of different masses travel through the mass analyser at different velocities, each ion is conveyed through the quadrupole and thus reaches the detector at a unique time, facilitating its identification.

The mass spectrometric analysis is complete once all ions derived from the fragmentation of the compound have reached the detector and the mass spectral
fragmentation pattern has been generated. The mass spectrum is a plot of the abundance of a particular ion (y-axis) versus m/z (x-axis). The mass spectrum, which depicts the unique fragmentation pattern of the analyte, can then be used to deduce the molecular structure of the compound, or can be identified by comparing the spectra obtained with those derived from compounds of known identity.

The use of tandem mass spectrometry aids high resolution analysis and is commonly referred to as “MSMS”. In tandem mass spectrometry, ions are firstly formed by the ion source and separated by m/z (MS1). Precursor ions are then selected and the fragment ions (also referred to as product ions) are created via the collision of ions in the chamber. The resulting ions are then separated and detected (MS2). Three different voltages are applied to the molecule in this process, universally 10V, 20V and 40V. Accordingly, the mass spectra obtained at each voltage will differ, resulting in highly sensitive detection of compounds and an ability to infer the structure of the compound from the mass spectra. This will be illustrated in the context of this study in later chapters\cite{40}.

Additionally, a chromatogram of the components present in the incubation mixture can be obtained. This is a visual representation of the separation of components during the chromatographic process. Each peak corresponds to a particular compound in the mixture\cite{41}. The identification of the compounds present requires interpretation of three different characteristics for each peak. Firstly, each peak displays a retention time, which is the time taken for the compound to reach the detector. Retention time reflects the polarity of the compound and characterises the compound under the chromatographic conditions employed, which is not necessarily unique to the compound. Secondly, the mass of the molecular ion provides information as to what group or groups may have been removed from or added to the
parent compound during the metabolic transformations. Thirdly, interpretation of the MS/MS spectrum allows the location of these metabolic changes on the molecule to be identified\(^{(41)}\). By comparing these characteristics against reference standards or library spectra, compounds can be identified and the metabolic processes involved in generating the compound, ascertained.

Importantly, high resolution LC-QTOF-MS detects the m/z of compound to 4 dp. When comparing an experimentally obtained value, the relative acceptable criterion is that the m/z of the fragment found in the sample must be within 5ppm of that of the reference sample. This is calculated according to the following formula:

\[
\text{ppm} = \frac{(\text{measured mass} - \text{reference mass})}{\text{reference mass}} \times 1000000
\]

Importantly, Wohlfarth et al. (2016) demonstrated that this method was applicable to the identification of 25C and 25I-NBOMe metabolites and the derivation of the structures of these metabolites\(^{(13)}\).

1.10 Aims

The first aim of this study is to develop reliable, reproducible and optimised chromatographic techniques for the identification of 25H-NBOMe in biological matrices. The second aim of the study is to separate and identify, using LC-QTOF-MS, the metabolites of 25H-NBOMe generated by the enzymatic activity of human S9 preparations in incubation mixtures \textit{in vitro}.

1.11 Hypothesis

It is hypothesised that that Phase I metabolism of 25H-NBOMe will parallel that of 25B, 25C and 25I-NBOMe and will be characterised by Phase I demethylation and hydroxylation. It is also hypothesised that, in corroboration with previously studied
NBOMes, acetylation, glucuronidation and sulfation will constitute the major Phase II biotransformations\(^{(13)}\).
Chapter 2: Materials
2.1 Incubation assays

Chemical reagents

Dipotassium hydrogen sulfate $\text{K}_2\text{HPO}_4$ (Sigma Aldrich)

Potassium dihydrogen phosphate $\text{KH}_2\text{PO}_4$ (Sigma Aldrich)

$\beta$-Nicotinamide adenine dinucleotide 2$\prime$-phosphate reduced tetrasodium salt hydrate

NADPH (Sigma Aldrich)

N-(2-methoxybenzyl)-2,5-dimethoxyphenethylamine (25H-NBOMe, ChemCentre)

2-(dimethylaminomethyl)-1-(3-methoxyphenyl) cyclohexanol (Tramadol, ChemCentre)

1-pentyl-3-(1-naphthoyl) Indole (JWH18, ChemCentre)

N-acetyl-para-aminophenol (Paracetamol, ChemCentre)

Codeine (ChemCentre)

Acetyl Coenzyme A sodium salt (Acetyl CoA, Sigma Aldrich)

Uridine 5-diphosphoglucuronic acid trisodium salt (UDP glucuronic acid, Sigma Aldrich)

Adenosine 3$\prime$-phosphate 5$\prime$-phosphosulphate lithium salt hydrate (Phosphoadenosine phosphosulphate, PAPS, Sigma Aldrich)

S9 from pooled human liver microsomes 20mg protein/ml (Sigma Aldrich) divided into 50μl aliquots, stored in Eppendorf tubes at -80°C

2.2 Sample extraction and reconstitution

Chemical reagents
Ethyl acetate

2.3 LC-QTOF-MS

Instrumental details

Agilent technologies Dual AJS ESI ion source MS Q-TOF

Component model G6540A

Column: ACE Excel 3micron Super C18 100 x 3.0 mm id

2.4 Data Analysis

Agilent Technologies Mass Hunter Qualitative Analysis Version B.07.00
Chapter 3: Methods
3.1 Preparation of parent xenobiotic

25H-NBOMe (4.288mg) was dissolved in 1mL of acetonitrile. Tramadol (2.2mg) and JWH18 (2.2mg) were dissolved in 1mL of acetonitrile to generate a 4.4mg/mL standard of tramadol plus JWH18. These two compounds were used as the xenobiotic in initial incubation mixtures because the metabolites they generate are well documented. Consequently, they are appropriate compounds to assess the S9 incubation conditions.

3.2 Preparation of incubation mixtures

$K_2HPO_4$ (1.387g) and $KH_2PO_4$ (0.534g) were dissolved in 100mL of deionised water to generate a 100mM phosphate buffer. The pH was adjusted to 7.4 using KOH (to increase pH) or $H_3PO_4$ (to decrease pH) as required. NADPH (16.5mg) was dissolved in 100μL phosphate buffer to generate a 200mM solution.

3.3 Phase I metabolism: Incubation of 25H-NBOMe with S9

Buffer (169μL), NADPH (10μL) and S9 (20μL) were added. Following pre-incubation at 37°C for 5 minutes, the reaction was commenced by the addition of 1μL of substrate (25H-NBOMe). After mixing, the tubes were incubated for one hour. The incubation concentration of 25H-NBOMe was 71μM and the final volume of the incubation mixture was 0.200mL.

3.4 Phase II metabolism: Incubation of 25H-NBOMe with S9 and phase II conjugating compounds

Buffer (159μL), NADPH (10μL) and S9 (20μL) S9 were added. 10μL of the relevant conjugating compound stock solution was added prior to the addition of 1μL of 25H-NBOMe to commence the reaction. The Conjugating compound stock...
solutions were Acetyl CoA (2.87mg) dissolved in 5mL of buffer, UDP glucuronic acid (2.294mg) dissolved in 5mL of buffer, and PAPS (0.7204mg) dissolved in 2mL of buffer. The concentration of each in the final incubation mixture was 35.5μM.

3.5 Sample extraction and reconstitution

After incubation, the contents of each test tube was transferred to appropriately labeled Eppendorf tubes. Ethyl acetate (600μL) was added to each and vortexed for 1 minute to mix. The sample was then centrifuged for 6 minutes to separate the phases and the organic layer was separated with a glass pipette and transferred into newly labelled test tubes. Following evaporation with nitrogen gas, 100μL of acetonitrile was added to each tube to reconstitute the sample.

3.6 Incubation Mixture Protocol

To confirm that the S9 fraction to be used in the investigation of the metabolic fate of 25H-NBOMe contained active enzymes involved in Phase I metabolism, incubations were carried out, initially, using xenobiotics for which the metabolites were already known. The xenobiotics chosen were JWH18 and tramadol.

The composition of the incubation mixtures (final volume, 0.200mL) used to assess the capacity of the S9 fraction to generate Phase I metabolites from JWH18 and tramadol was adapted from a protocol provided by Sigma Aldrich and is shown in Table 3.6:
Table 3.6 Concentration of reagents in preliminary incubation mixtures.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration in Incubation mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>100mM</td>
</tr>
<tr>
<td>S9</td>
<td>0.5mg/mL protein</td>
</tr>
<tr>
<td>NADPH</td>
<td>200mM</td>
</tr>
<tr>
<td>Xenobiotic</td>
<td>42μM tramadol and 31μM JWH18</td>
</tr>
<tr>
<td>TOTAL</td>
<td>200μL</td>
</tr>
</tbody>
</table>

Analysis of sample extracts was carried out via LC-QTOF-MS.

3.7 Variable 1: Incubation time

Incubations were carried out for 1 and 3 hours and the mixtures were then analysed for metabolites.

3.8 Variable 2: Effect of enzyme concentration

A series of incubations was carried out to evaluate the effect of enzyme concentration on the metabolite production. The protocol is shown in Table 3.8 below.

Table 3.8 Incubation assays carried out to investigate the effect of enzyme concentration.

<table>
<thead>
<tr>
<th>Tube number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer(μL)</td>
<td>184</td>
<td>179</td>
<td>174</td>
<td>169</td>
<td>164</td>
</tr>
<tr>
<td>NADPH (μL)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>25H(μL)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>S9(μL)</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Amount of protein(μg)</td>
<td>100</td>
<td>200</td>
<td>300</td>
<td>400</td>
<td>500</td>
</tr>
</tbody>
</table>
3.9 Variable 3: Extraction efficiency

Extraction efficiency was evaluated using the initial incubation protocol. Extraction was carried out one, two, three and four times. For the sample extracted twice, extraction was carried out with two separate 600μL of ethyl acetate aliquots, which were pooled, evaporated to dryness and reconstituted. A similar protocol was employed three extractions 3 ethyl acetate aliquots were collected, and so on.

The following formula was used to calculate the recovery of the parent drug:

\[
\% \text{ Recovery} = \left( \frac{\text{Response Extracted sample with analyte}}{\text{Response Post extracted spiked sample}} \right) \times 100
\]

The “extracted sample with analyte” was the sample that has been incubated as per the standard incubation protocol. The “post extracted spiked sample” was the sample to which the parent drug was added after the ethyl acetate was added where 100% of the parent drug would remain in the sample because it was not incubated and therefore not metabolised.

3.10 Phase I 25H-NBOMe metabolism

After the experimental variables had been optimised, the parameters were applied as shown Table 3.9 to evaluate the generation of Phase I 25H-NBOMe metabolites at 37°C for 1 hour:

Table 3.10 Contents of the incubation mixtures to assess Phase I 25H-NBOMe metabolism.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Incubation Mixture Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>100mM</td>
</tr>
<tr>
<td>NADPH</td>
<td>200mM</td>
</tr>
<tr>
<td>S9</td>
<td>2mg/mL protein</td>
</tr>
<tr>
<td>25H-NBOMe</td>
<td>71μM</td>
</tr>
<tr>
<td>200μL</td>
<td>TOTAL</td>
</tr>
</tbody>
</table>
3. 11 Phase II 25H-NBOMe metabolism

Incubation mixtures for the investigation of Phase II metabolism were carried out according to Table 3.11 at 37°C for 1 hour.

Table 3.11. Incubations carried out for Phase II metabolism.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Incubation Mixture Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>100mM</td>
</tr>
<tr>
<td>S9</td>
<td>2mg/mL</td>
</tr>
<tr>
<td>NADPH</td>
<td>200mM</td>
</tr>
<tr>
<td>25H-NBOMe</td>
<td>71μL</td>
</tr>
<tr>
<td>Activated Phase II conjugating compound</td>
<td>35 μL</td>
</tr>
</tbody>
</table>
Chapter 4: Results and Discussion
Method development: Identification of parent Tramadol and JWH18 and metabolites to confirm S9 activity

Incubation of S9 preparations with either tramadol or JWH18 using the basic incubation and assay protocols described in the previous chapter demonstrated that Phase I metabolites were generated from Tramadol and JWH18 in vitro. The presence of each compound was confirmed by LC-QTOF-MS analysis via the characteristics of retention time, a corresponding chromatogram and generation of an MSMS spectrum. Incubations were carried out with these two compounds to confirm S9 activity and the ability to detect the metabolites generated.

4.1. Tramadol: Identification of the parent compound

The presence of the parent compound in the incubation mixtures was firstly confirmed via LC-QTOF-MS analysis of an extract of the incubation mixture. The data was analysed for the formula chemical formula C_{16}H_{25}NO_{2}. According to the Agilent Mass Hunter Personal Compound Database and Library (PDCL), the expected mass of this parent compound (M+1) has an m/z of 264.1958. Figure 4.1 is the chromatogram obtained of the compound with an m/z of 264.1651, displaying the elution of parent tramadol at 7.6 minutes.

Figure 4.1.1 Chromatogram derived from incubations containing tramadol.
The library spectra of tramadol (see Appendix) indicates that at 10V the fragment ion of tramadol has an of m/z 58.06512. The unfragmented parent ion has an m/z of 264.1951 (M+1). At both 20V and 40V there is only a singular fragment present, of m/z 58.0651. Figure 4.1.2 displays the mass spectrum obtained from the chromatogram in Figure 4.1.1.

**Figure 4.1.2** Mass spectra obtained from the MSMS of tramadol.

At 10V a fragment of both m/z 264.1950 and m/z 58.0655 is present. The fragment 58.0655 is also present at 20V and 40V. The difference between the actual and theoretical m/z values is less than 5ppm (2.64) and therefore acceptable. Therefore, it can be concluded that this mass spectra was produced by parent Panadol.

### 4.2. Demethylated tramadol

Tramadol is known to be demethylated in metabolism, resulting in a compound with a chemical formula of C₁₅H₂₃NO₂\(^{(42)}\). A high resolution search with this formula returned the chromatogram of a compound with an M+1 value of m/z 250.1790. The chromatogram in Figure 4.2.1 displays two peaks corresponding to this m/z value, with retention times of 4.97 and 5.4 minutes. The presence of two peaks suggests two possible sights of tramadol demethylation.
Figure 4.2.1 Chromatogram of demethylated tramadol obtained from incubation mixtures.

The mass spectrum in Figure 4.2.2 details the fragmentation of this compound at a retention time of 5.4 minutes at 10V, the spectra at 20V and 40V displaying the same fragments.

Figure 4.2.2 Mass spectrum of demethylated tramadol indicating the loss of a methyl group.

The spectrum displays an ion of m/z 250.1790 at 10V (M+1), 14.0161 units less than the parent compound, which consistent with the loss of a CH₃ group. Additionally, the mass spectrum of the compound, obtained at 4.9 minutes, shows a fragment ion
of m/z 44.0498. This fragment is 14.0156 units less than the fragment of m/z 58.0654, suggesting another site of demethylation, as shown in Figure 4.2.3.

![Mass spectrum of demethylated tramadol](image)

**Figure 4.2.3** Mass spectrum of demethylated tramadol for the compound eluting at 4.9 minutes.

Accordingly, interpretation of these spectrum allows for the fragmentation of the tramadol molecule to be concluded. This fragmentation is detailed in Figure 4.2.4

![Fragmentation of tramadol](image)

**Figure 4.2.4** Fragmentation of tramadol and its demethylation sites as deduced from the mass spectra.

Figure 4.2.4 illustrates the two different tramadol metabolites. When the oxygen is demethylated a fragment of m/z 58.0665 is produced, while the demethylation of
nitrogen generates a fragment of m/z 44.0498. Therefore, it can be concluded that the incubation conditions and detection parameters are favourable for the generation of demethylated metabolites including those with the same chemical formula but with different retention times.

4.3. JHW18: Identification of the parent compound

The presence of parent JWH18 (C$_{24}$H$_{23}$NO) was confirmed. LC-QTOF-MS extract of chemical formula returned a result of a compound with an M+1 value of m/z 342.1841. The chromatogram in Figure 4.3.1 shows the elution of a compound at 10.1 minutes.

![Chromatogram](image)

**Figure 4.3.1** Chromatogram obtained of incubation mixtures containing parent JWH18.

The library spectra of parent JWH18, (see Appendix), displays fragment ions of m/z 324.1852, 214.1226 and 155.0491 at 10V. At 20V there is an additional fragment of m/z 127.0542 and at 40V, a fragment of m/z 43.0542. The mass spectra obtained for this sample, Figure 4.3.2, displays a similar fragmentation pattern, again confirming the presence of the compound in this mixture.
Figure 4.3.2 Mass spectra of parent JHW18 from incubation mixtures, displaying the same fragment ions as the library spectra.

The major ions have an m/z of 214.1221, 155.0490, 217.0541 and 43.0545. The parent compound (M+1) had an m/z of 342.1843. The theoretical mass of parent JWH18, according to the PCDL library, is only 3ppm different (342.1852), consistent with this compound.

4.4 Monohydroxylated JWH18

It is known that one of the most readily detectible metabolites of JHW18 is the monohydroxylated form of the drug (C_{24}H_{23}NO_{2})^{43}. A compound with an M+1 of m/z 358.1082 was found for this formula, the chromatogram displayed in Figure 4.4.1.
Importantly, the chromatogram features three partially resolved peaks, suggesting the monohydroxylation process can occur at three sites on the molecule. The mass spectra obtained after MSMS for the compound eluting at 8.9 minutes is displayed in Figure 4.4.2.

At 10V and 20V the spectra indicates the m/z of the parent ion to be 358.1796, 15.9953 mass units greater than the m/z of the parent compound, consistent with hydroxylation. It appears that some parts of the molecule remain unaltered, as fragments of m/z 155.0490 and 127.0522 are retained in the spectrum. Of note is a fragment ion of m/z 230.1123, 15.9902 mass units greater than 214.1121, consistent with hydroxylation. Accordingly, the potential sites of JWH18 hydroxylation which generate these characteristic fragments are inferred according to the Figure 4.4.3. To be noted is the five possible sites on the pentyl chain.
Figure 4.4.3 Proposed fragmentation and monohydroxylation of JWH18 at three suggested sites\(^{(43)}\).

The presence of three peaks in the chromatogram suggests that three MSMS spectra should be obtained for the unique retention time of each peak. Evidentially, only one chromatogram was extracted. Where multiple peaks have the same m/z value (as is the case here), the data needs to be manually extracted because at times MSMS data is only generated for the selected peak. This requires the data to be sorted by retention time, where the MSMS data can then be generated according to retention time after it is confirmed that the mass of the parent ion in the generated MSMS spectra has the correct m/z value. Manual extraction of the data was not carried out due to time constraints.

The detection of monohydroxylated metabolites of JWH18 in post-incubation mixtures confirms the capacity of the S9 preparation to generate hydroxylated
metabolites *in vitro*, and suggests that it is a suitable source of monooxygenases for the potential Phase I hydroxylation of 25H-NBOMe, if such metabolites are characteristic of its biotransformation. Furthermore, the demonstrated capacity of the S9 preparation to catalyse Phase I demethylation and dehydrogenation reactions, indicates that a suite of Phase I enzymes is active within the preparation\(^{(43)}\).

### 4.5 Incubation time

The peak areas of tramadol and JWH18 and their relevant metabolites were obtained after separate incubations of both one and three hours. Table 4.5 illustrates the average peak area of each metabolite obtained in each test after two replicates.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>1h</th>
<th>3h</th>
<th>Fold increase in metabolites from 1h to 3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demethylated tramadol</td>
<td>621156</td>
<td>2348954</td>
<td>3</td>
</tr>
<tr>
<td>C(<em>{15})H(</em>{23})NO(_2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monohydroxylated JWH18</td>
<td>883808</td>
<td>4348977</td>
<td>4</td>
</tr>
<tr>
<td>C(<em>{24})H(</em>{23})NO(_2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrogenated JWH18</td>
<td>161489</td>
<td>656673</td>
<td>3</td>
</tr>
<tr>
<td>C(<em>{24})H(</em>{21})NO(_2)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The table illustrates that for each metabolite, the peak area of the compound was greater after 3h than 1h of incubation. A 3-fold increase from 1h to 3h was observed in demethylated tramadol. For monohydroxylated and dehydrogenated JWH18 the longer incubation time produced a 4 and 3-fold increase in metabolites, respectively.
As expected, with increasing incubation time more metabolite was generated. Taking account of the limited number of replicates, the peak areas of the metabolites generated were approximately three times greater after 3h than 1h. There was reasonable linearity in the production of metabolites over the 3h incubation time. Short incubation times, such as one hour, are predominantly used in metabolic studies due to increasing enzyme instability with increased incubation time\(^{(44)}\). In most systems used for \textit{in vitro} metabolic studies, such as those conducted with hepatocytes or liver microsomes, a reaction rate plateau is reached within one hour of incubation time. Furthermore, as the objective of the investigations described in this thesis was to achieve a qualitative assessment of the metabolites produced, rather than a quantitative one, it was deemed that sufficient product was generated within a 1h incubation and, as a consequence, this incubation time was adopted for all future experiments.

\textbf{4.6 Effect of enzyme concentration}

To optimise the amount of enzyme present to ensure maximum production of the metabolite in the 1h incubation, the effect of enzyme concentration on metabolite production was investigated. The graph constructed after the peak areas of each compound was obtained (via average of two replicates) is displayed in the Figure 4.6.
Figure 4.6 Effect of enzyme concentration on the rate of metabolite production from 25H-NBOMe over a 1h incubation time.

The production of both compounds appears to be linear up to 200 μg until somewhat of a plateau is reached. Consequently, to ensure that the rate of metabolite production was not limited by enzyme concentration, 400μg of protein (20μL S9) was chosen for all future experiments.

4.7 Extraction efficiency

To maximise the extraction of the parent drug and its metabolites from the incubation mixtures, the effect of multiple extractions with ethyl acetate was investigated. The results are shown in Table 4.7.
Table 4.7 Extraction efficiency: Percentage recovery of parent 25H-NBOMe following one, two, three and four extractions

<table>
<thead>
<tr>
<th>Number of extractions</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>55</td>
</tr>
</tbody>
</table>

Maximum recovery was obtained after 2 sequential extractions, each with 600µL of ethyl acetate. Anomalous results were obtained for those incubation mixtures subjected to three or four extractions (Table 4.7). This illustrates that variability appears to be inherent in extraction efficiency, between individual incubation mixtures.

In general, it is desirable for extractions to recover 80% of compounds produced. It is evident in this case that approximately 20% less than this ideal efficiency was achieved. As using two pooled extractions only provided a 7% increase in recovery, a single extraction was used in all future experiments to save time and reagents. It is also assumed that the 25H-NBOMe metabolites are extracted with similar efficiency to that of the parent compound. Given that ethyl acetate functions as a generically useful extraction solvent for compounds of this type, and given that the purpose of this investigation was qualitative rather than quantitative, this assumption was considered acceptable. The recovery of the hydroxylated and demethylated metabolites will differ slightly because they differ slightly in polarity, and in turn this recovery will also differ slightly from the parent compound. Small variations in the recovery of the metabolites is considered acceptable due to the qualitative nature of this investigation.
For future investigations, especially if one of the experimental aims was to quantify the amount of metabolite produced, an alternative extraction method should be investigated. This may include the use of an alternative solvent, such as acetonitrile\textsuperscript{(41)}.\textit{In vitro} metabolism studies such as that conducted by Gandhi \textit{et al}. (2014) involved the use of acetonitrile to extract metabolites after incubation of synthetic cannabinoids with human liver microsomes.\textsuperscript{(41)} Additionally, there were two variables the experiment in this thesis, the first being multiple extractions, and the second being separate extraction. There should have been one variable only.

Alternatively to the use of a separate incubation for each of the numbers of extractions being investigated, simply use one incubation mixture; extract it once with 600 $\mu$L of ethyl acetate, separate the organic and inorganic phases and remove the organic phase for analysis. Then re-extract the same aqueous phase a second time with another 600 $\mu$L of ethyl acetate, remove the organic phase place it in a second tube and extract for a third time and then a fourth time. The amount of drug in each of the four ethyl acetate extracts should then be analysed. Adding the ethyl acetate together from 2, then 3 and then 4 extractions gives the complete information on the recovery efficiency. The experiment should be repeated in triplicate to show variability.

\textbf{25H-NBOMe metabolism}

\textbf{4.8 25H-NBOMe: Identification of parent compound}

In assays of the incubation mixtures, the unmetabolised 25H-NBOMe (C\textsubscript{18}H\textsubscript{23}NO\textsubscript{3}) was identified in chromatograms by searching for the m/z value corresponding to its expected M+1 (302.1756, see appendix). The chromatogram in Figure 4.8 confirms the presence of the compound, eluting at approximately 8 minutes.
The library mass spectrum of 25H-NBOMe shows that mass spectrometry of the compound produces fragments with m/z values of 91.0544, 121.0642. The latter ion is the parent ion of m/z 302.1756 (M+1) of the compound. The same fragments where found to be associated with a compound extracted from the incubation mixtures is shown in Figure 4.8.2.
Figure 4.8.2 illustrates the presence of the key 25H-NBOMe fragments. The M+1 of the parent ion is 302.1740 at 10V. The key fragment ions are also present, being 121.0651 and 91.0546 in the sample. Therefore, the fragmentation of the parent compound can be inferred, according to Figure 4.8.3.

![Figure 4.8.2](image)

**Figure 4.8.2** Predicted fragmentation of 25H-NBOMe derived from interpretation of its mass spectra.

### 4.9 Demethylated 25H-NBOMe

Previous studies of NBOMe variants have identified one of the major Phase I metabolites to be demethylated forms of the parent drug\(^{(13)}\). Consequently, data was analysed for the formula $\text{C}_{17}\text{H}_{21}\text{NO}_3$. This compound was identified chromatographically in the mixture with three peaks, shown to have a retention time of 6.8, 8.2 and 8.8 mins.

![Figure 4.9.1](image)

**Figure 4.9.1** Chromatogram of demethylated 25H-NBOMe obtained from incubation mixtures.
The three peaks in the chromatogram suggest three possible sites of demethylation.

Figure 4.9.2 displays the MSMS spectrum obtained after 6.9 minutes.

![Mass spectra of demethylated 25H-NBOMe correlating to the peak obtained at 6.9 minutes.](image_url)

**Figure 4.9.2.** Mass spectra of demethylated 25H-NBOMe correlating to the peak obtained at 6.9 minutes.

Figure 4.9.4 shows that the M+1 value of the compound is m/z 288.1584. This value is 14.0156, a mass difference consistent with the demethylation process. As seen in the spectrum, the fragments 121.0651 and 91.0546 remain unchanged from that of the parent compound. Accordingly, the sites of demethylation can be hypothesised according to Figure 4.9.3.
Figure 4.9.3. Proposed demethylation sites of 25H-NBOMe.

Figure 4.9.3 shows that demethylation on the second or fifth oxygen gives rise to two possible isomers of demethylated 25H-NBOMe, accounting for two of the peaks in the chromatogram, and the unchanged m/z of the 121.0651 and 91.0546 ions in the spectrum from the demethylated sample, compared to that of the parent.

According to the inferred site of demethylation, these metabolites are named 2-demethyl NBOMe and 5-demethyl NBOMe. Synthesis and LC-QTOF-MS analysis of each isomer and comparison of their retention times and mass spectrum would confirm which isomer(s) is present and thus the exact location of demethylation.

Previous studies of other NBOMe variants, such as that on 25C and 25I-NBOMe, have found that the fragment ion of m/z 91 predominately remains unchanged, meaning it is most likely that the left side (as it has been drawn in Figure 4.9.5; the benzene ring) of the molecule is the site of demethylation\(^{(13)}\). Additionally for this data set, the QTOF data is once again required to be manually extracted to obtain the
mass spectra for each peak to completely analyse the sample and the potential sites of demethylation.

4.10 25H-NBOMe hydroxylation

Similarly, monohydroxylation 25H-NBOMe (C_{18}H_{23}NO_4) was expected to generate an M+1 value corresponding to the addition of 16 mass units compared to that of the parent. The chromatogram in Figure 4.10.1 illustrates the chromatogram obtained for a compound of this chemical formula with an M+1 value of 318.1701.

![Figure 4.10.1 Chromatogram of suspected hydroxylated metabolites of 25H-NBOMe obtained from incubation mixtures.](image)

The chromatogram features five peaks at retention times of 6.5, 6.9, 7.2, 7.5 and 8.8 minutes, suggesting that there are four isomers of this compound, and four possible sites of 25H-NBOMe hydroxylation. The mass spectra in Figure 4.10.2 was obtained following MSMS of the compound at a retention times of 7.1 minutes.
The m/z of the parent ion is 318.1701 at 10V, 15.9959 mass units greater than that of the parent compound, consistent with the addition of an oxygen associated with hydroxylation. A site of hydroxylation is suggested by the m/z (137.0596) of the major ion visible at both 10 and 20V. A further potential site of hydroxylation is suggested by the m/z (107.0495) of the dominant ion at 40V. These ions are 15.9945 and 15.9940 mass units greater than their respective non-hydroxylated fragments (121.0651 and 91.0546).

Once again, due to time constraints, the expected number of mass spectra (four) was not obtained because the data needs to be manually extracted for each retention time. Interpretation of the spectrum in Figure 4.10.2 allows the fragmentation pattern and the sites of hydroxylation to be inferred (Figure 4.10.3).
Figure 4.10.3 Proposed sites of hydroxylation of 25H-NBOMe.

Figure 4.10.3 shows that it is possible for any carbon in the NBOMe ring to be hydroxylated, thus giving rise to the characteristic ions of 137.0596 and 107.0495 for the compound. Therefore, the hydroxylated metabolites may be 3-hydroxyNBOMe, 4-hydroxyNBOMe, 5-hydroxyNBOMe or 6-hydroxyNBOMe.

Investigation of Phase II metabolism of 25H-NBOMe

4.11 Acetylation

Having confirmed the presence of the demethylated metabolite in incubation mixtures supplemented with acetyl CoA, a search was performed for an acetylated derivative (C_{19}H_{23}NO_4), potentially generated by the Phase II enzymatic addition of an acetyl group (CH_3CO) to the hydroxyl moiety generated by demethylation. Such a product would be expected to produce an M+1 with an m/z of 329, approximately 42 mass units greater than the unacetylated product. A search for this compound by formula yielded no result, and no compound with the relevant M+1 value was found. This suggested that acetylation of the demethylated metabolite did not occur.
Similarly, a search for an acetylated hydroxylated compound failed to yield a result. In this case, the acetylated derivative (C\textsubscript{20}H\textsubscript{25}NO\textsubscript{5}) would be expected to generate an M+1 with an m/z value of approximately 360. This compound was not found by either a search according to chemical formula or by m/z value, and it was concluded that acetylation of the hydroxylated metabolite did not occur.

4.12 25H-NBOMe Glucuronidation

Glucuronidated forms of the Phase I metabolites would be expected to generate a product of 176 mass units greater than the corresponding Phase I metabolite due to the enzymatic addition of a C\textsubscript{6}H\textsubscript{8}O\textsubscript{6} group. A glucuronide derived from a demethylated metabolite would, therefore, have the formula of C\textsubscript{23}H\textsubscript{29}NO\textsubscript{9}. This compound would have an M+1 m/z value of 464. As no compound with this formula or m/z value was found, it can be concluded that glucuronidation of the demethylated metabolite did not occur.

For the hydroxylated metabolite, the glucuronide derivative would have the formula C\textsubscript{24}H\textsubscript{31}NO\textsubscript{10}. Accordingly, this compound would generate an M+1 with an m/z of 494, which, again, was not found.

4.13 25H-NBOMe Sulfation

The presence of both demethylated and hydroxylated metabolites was confirmed in incubation mixtures containing PAPS, and a search for Phase II sulfate conjugates was conducted. These products will have an m/z 64 mass units greater than the relevant Phase I metabolite due to the enzymatic addition of SO\textsubscript{3}-. The sulfate conjugate of the demethylated metabolite (C\textsubscript{17}H\textsubscript{21}NO\textsubscript{6}S) would generate an M+1 with an m/z of 368. Again, a search via chemical formula and m/z value returned no result. Similarly, the sulfate conjugate of the hydroxylated metabolite
(C\textsubscript{18}H\textsubscript{23}NO\textsubscript{7}S) with an M+1 m/z value of 398 was not detected and no evidence of the process of sulfation was found.

4.14 Search for Phase II conjugating enzymes

As glucuronides of the 25H-NBOMe metabolites where not detected in incubation mixtures supplemented with UDP glucuronic acid, experiments were conducted to ascertain whether the appropriate conjugating enzyme, UDP glucuronyl transferase, was present in the S9 preparation.

To investigate this possibility, paracetamol (C\textsubscript{8}H\textsubscript{9}NO\textsubscript{2}), which is known to undergo glucuronidation, was incubated with UDP glucuronic acid and S9 at 37° for 1 hour. The incubation mixture was extracted with ethyl acetate according to the usual protocol and a search was carried out for paracetamol glucuronide (C\textsubscript{14}H\textsubscript{17}NO\textsubscript{8}; M+1 m/z 328) in the extract.

Unmetabolised paracetamol (M+1 m/z 152.1680) was detected in the extract eluting at a retention time of 1.759 minutes (Figure 4.14).

![Figure 4.14](image)

Figure 4.14 Chromatogram of parent paracetamol displaying a well resolved, bell-curved shaped peak, confirming the presence of parent paracetamol.

However, a search for paracetamol glucuronide (C\textsubscript{14}H\textsubscript{17}NO\textsubscript{8}) did not return a result. No compound was detected with an m/z of 328.1680, the expected mass of the M+1 indicating that either glucuronidation of paracetamol did not occur, or the paracetamol glucuronide was not detected. The issue of detection is further discussed in 4.15.
4.15. Failure to produce or detect Phase II metabolites of 25H-NBOMe

Phase II metabolism of 25H-NBOMe was not demonstrated in the preliminary investigations described in this thesis. This may be due to the absence of soluble enzymes required to catalyse the conjugation of the activated compound with the xenobiotic.

The activation of the conjugating compounds Acetyl CoA, UDP-glucuronic acid and PAPS has been explained in detail in Chapter 1. These were compounds added to the incubation mixtures, and are thus already activated. This being the case, the requirement for the activating enzymes of Acetyl CoA Synthetase, UDP glucose pyrophosphorylase, UDP glucose dehydrogenase and ATP is bypassed.

4.16 Absence of soluble enzymes required to catalyse the conjugation of the activated compound with the xenobiotic

As activated conjugating compounds (Acetyl CoA, UDP-glucuronic acid and PAPS) were added to the incubation mixtures in an attempt to identify Phase II conjugates, it is most likely that failure to detect them was due to the absence of the conjugating enzymes in the S9 preparation. Alternatively, but unlikely given the known metabolism of other NBOMe variants, it is possible that the parent drug may not be receptive to conjugation.

(a) Phase II Acetylation

The enzymes responsible for catalysing the conjugation of the activated acetate to the xenobiotic are termed “acetyltransferases”\(^\text{45}\). These soluble enzymes are located in the cytosol. Both O-Acetylation and N-Acetylation are common processes in phase II metabolism\(^\text{45}\). N- acetyltransferases (NATs) have been shown to be involved in the Phase II metabolism and identified as enzymes
active in the phase II metabolism of 2C compounds (psychoactive phenethylamines which feature methoxy groups on positions 2 and 5 of the benzene ring) such as 25H-NBOMe. There are two isoforms of NATs, NAT1 and NAT2. Being expressed in the liver and intestinal epithelium, NAT2 enzymes are of relevance here\(^{(45)}\). However, though acetyl CoA was added to the incubation mixtures in an attempt to identify potential Phase II metabolites, neither O nor N-acetylated conjugates were detected. This may be due to the absence of acetyltransferases in the S9 fraction, preventing the transfer of the acetyl group to either the demethylated or hydroxylated metabolites of 25H-NBOMe (Figure 4.16a).

![Figure 4.16a](image)

**Figure 4.16a** Potential acetylation site of the demethylated metabolite of 25H-NBOMe. This conjugate was not detected.

(b) **Phase II Glucuronidation**

Similarly, despite UDP glucuronic acid being added to the incubation mixtures in an attempt to identify Phase II metabolites, glucuronide conjugates where not detected. This may also have been due to compromised incubation conditions due to the absence of conjugating enzymes.
Predominantly expressed in the liver, UDP-glucuronosyltransferases (UGTs) catalyse a process which transfers a glucuronate moiety from UDP glucuronic acid to an oxygen, carbon or nitrogen atom in the xenobiotic or its Phase I metabolites. The addition of the polar glucuronic acid produces a hydrophilic conjugate which can then readily be excreted (Figure 4.16b)\(^{(46)}\).

**Figure 4.16b** Potential glucuronidation site of the demethylated metabolite of 25H-NBOMe. The conjugate was not detected.

It was expected that UDP-glucuronosyltransferases would be expressed in the S9 preparation. Being transmembrane proteins, expressed particularly in the nucleus and smooth endoplasmic reticulum of hepatocytes, it is logical to expect that in the homogenisation of hepatocytes to generate the S9, these enzymes would be present\(^{(46)}\).

(c) **Paracetamol glucuronidation**

The apparent failure of paracetamol metabolism introduces the idea that, instead of an absence in the system to carry out Phase II metabolism, it may be that the instrumental parameters or incubation conditions are not favourable for the detection of these metabolites, making it appear as though they have not been formed. Paracetamol is known to undergo glucuronidation and that it enzymatically reacts with UDP glucuronic acid to produce a paracetamol glucuronide, unlike 25H-
NBOMe\(^{(42)}\). If the enzymes and substrates required to facilitate glucuronidation where present, the paracetamol glucuronide will have been detected. Failure to detect may been due to the very early elution time, of less than one minute and therefore outside the ability of the QTOF to detect such an early eluting compound.

The addition of a glucuronide group increases the polarity of any compound (the same can be said relating to the processes of acetylation and sulfation). The highly polar nature of the compound reduces its affinity for the chromatography column, reducing its retention time\(^{(46)}\). This may in turn compromise the way in which the compound reaches the mass analyser and is detected, giving the illusion of failed glucuronidation. Time and budgeting did not allow for further investigation into changing the incubation conditions or instrumental parameters to improve glucuronide detection.

**d) Phase II sulphation**

Although PAPS was added to the incubation mixtures in order to identify a third possible Phase II conjugate, sulfate conjugates where not detected. It can be inferred that the transfer of a sulphate group from PAPS to demethylated and hydroxylated 25H-NBOMe is not facilitated by the enzymes present in the S9. One would expect the action of sulphotransferases (SULTS) to carry out the process of sulphation illustrated in Figure 6.16d below.
Sulphotransferases, being cytosolic enzymes of the liver, are expected to be present in S9 preparations. However, no evidence for the generation of sulphate conjugates was found in these incubation mixtures containing 25H-NBOMe and PAPS\(^{(47)}\).

SULTs are known to be susceptible to substrate inhibition \textit{in vitro}. This arises from the conformational change that occurs when the substrate binds to the active site of the enzyme, reducing the ability of the enzyme to catalyse sulphation\(^{(48)}\). This may have been a compromising factor.

Previously discussed in the introduction, the CYP450 group of enzymes is another group of enzymes whose expression and action in dominant in drug metabolism. One may consider the possibility of reduced CYP450 activity the reason behind why the activated compounds where not conjugated to the Phase I metabolite, meaning that the amount of demethylated and hydroxylated metabolite may be too small to facilitate the production of sufficiently detectable conjugate\(^{(21)}\).
Chapter 5: Recommendations and Conclusions
5.1 Enzyme profiling studies

It is known that S9 contains both microsomal and cytosolic cellular components, and as a result should yield a greater metabolic profile than other in vitro means of studying hepatic metabolism, given that it contains the entire spectrum of hepatic enzymes. This is in comparison to the other methods of in vitro hepatic study by the use of human liver microsomes, in which incubation mixtures are required to be supplemented with both conjugating enzymes and reaction substrates\(^{(46)}\). Thus far, the cause of the absence of Phase II metabolism has been speculated, the focal point being the potential lack of enzyme presence or activity. However, it would be pertinent to profile the enzymes of S9 to find out exactly what is required to be added to the system in order to induce metabolism, rather than speculating on what, in theory, should be present and adding reagents accordingly.

There have been multiple studies carried out to profile the exact identity of the enzymes present in HLMs and other hepatic study methods. It being evident that the CYP450 enzymes active in Phase I metabolism are present in the S9, it would be pertinent to investigate the presence of enzymes relevant to Phase II metabolism, by an alternative method than drug incubation, which assumes the presence of the enzymes.

Terai \textit{et al.} (2012) developed a luminescent probe for the detection of NATs and its activity within cells. A probe specific to the genetic sequence of NAT1 and NAT2 type enzymes containing a Tb\(^{3+}\) group and an aniline moiety was synthesised and incubated separately with both pigeon liver and human liver cytosol. When the probe binds to the substrate, photoinduced electron transfer occurs between the alanine
moiety and the Tb atom, inducing luminescence detectable in the near Infra-Red region\(^{(49)}\). This luminescence was detected in assay supplemented with Acetyl CoA, thus leading to the conclusion that the enzymes required for acetylation were present. A similar method could be employed to detect the level of activity of NATs in S9. Increased luminescence inferences increased enzyme, as an increased amount of probe is able to bind to the increased amount of enzyme, inducing increased electron transfer and therefore emission of light\(^{(49)}\). The luminescence produced for the S9 could be compared to that in hepatocytes, liver cytosol, and microsomal preparation, to gain an understanding of the difference in enzyme content between them.

It is recommended that profiling with the same method be carried out in a similar manner to test for the presence of UGTs and SULTs. If the amount of enzyme activity in S9 can be estimated, this information can be used to derive what is required to be added into the S9 incubation regarding the amounts of substrate and enzyme, to increase the chances of inducing Phase II metabolism.

### 5.2 Improving the detection of Phase II conjugates

It is possible that the enzymes are present and the detectability of the Phase II conjugates are compromised. A means of confirming if this is the case is to use a method similar to that implemented by Wohlfarth et al. (2016). Studying the metabolism of 25C and 25I-NBOMe, possible metabolites of each compound where synthesised to create reference standards to which the metabolites detected in human and mouse urine, as well as incubation with human hepatocytes, could be compared\(^{(46)}\). Demethylated, hydroxylated, acetylated, glucuronidated and sulfated potential metabolites where synthesised and diluted in methanol to produce a reference standard of 10μg/ml. These were subject to LC-QTOF-MS in the same
way as the extracts from the urine and hepatocyte samples, with the same mobile phase and flow rate. The conditions for the gradient elution where slightly different as the percentage of each mobile phase and the time at which each was held was altered to favour detection of the synthesised compounds\(^{(46)}\).

These metabolites, both the reference standards and naturally produced metabolites, eluted between six and eleven minutes. Evidentially, the parameters used in this study facilitated the detection of metabolites, and the identity of the metabolites formed in hepatocyte incubations could be confirmed via reference to these standards. Therefore, it is recommended that a similar method be employed for future studies of 25H-NBOMe. The same could be said for paracetamol in its use as a means of investigating the presence of Phase II enzymes. In future work it would be recommended to obtain a synthesised paracetamol glucuronide, such as Paracetamol β-D glucuronide, available from Sigma Aldrich. If this can be detected using the same LC-QTOF-MS conditions as those used for the incubation assays, it can be concluded that detection of the Phase II metabolites is possible. If not, changes can be made to improve such detection.

5.3 Comparison of this investigation to other studies

There have been multiple studies relating to the metabolism of different NBOMe type drugs, which corroborate the findings found in this investigation, particularly regarding Phase I metabolism. Wohlfarth et al. (2016) found, from incubation of hepatocytes with human hepatocytes, demethylated, hydroxylated, glucuronidated and sulfated 25C-NBOMe metabolites. It was found that the methoxy groups where demethylated, as with 25H-NBOMe. Similarly, hydroxylation was confined to the NBOMe ring in both studies\(^{(13)}\).
The same metabolites where found following the incubation of hepatocytes with 25I-NBOMe. The demethylated and hydroxylated Phase I metabolites where dealkylated, glucuronidated and sulfated as part of Phase II metabolism\(^{(13)}\). Thus, it can be stated that the Phase I metabolite profiling of this investigation was conducted with success, as it produced similar results to those found in studies of other NBOMe variants. However, questions pertaining to absence of Phase II metabolism in this investigation remain. Phase II metabolites where detected by Wohlfarth \textit{et al.}, but where not detected in this investigation.

It is possible that use of S9 is not the most efficient means of studying metabolism in the liver. Given the success of other studies, it is recommended that further qualitative studies of 25H-NBOMe metabolism utilises an alternative means of \textit{in vitro} liver preparation. This may be through the use of human liver microsomes, where the conjugating enzymes and necessary substrates are added to the incubation mixtures, ensuring each is present in sufficient concentration to form Phase II conjugates\(^{(21)}\). Additionally, administration to Wistar rats is also recommended, as an \textit{in vivo} model. This involves administration of the drug to the animals, and consequently examining their faeces and urine for the presence of metabolites. For years the administration of xenobiotics to rats has been utilised, however, ethical and financial considerations exist due to the cost of obtaining the rats, ethical approval, and the extra resources required with keeping live animals\(^{(13)}\).

Moreover, manual extraction of MSMS data may reveal the presence of the required data to more accurately infer every possible site of biotransformation on the molecule.
5.4 Significance of this study

This investigation of the metabolism of 25H-NBOMe is two-pronged. Firstly, valuable information has been obtained relating to the Phase I metabolism of 25H-NBOMe. Not only have two major Phase I metabolites been identified; this being the demethylated and hydroxylated forms of the parent compound, but the characteristic fragmentation pattern of each was obtained. In forensic toxicology, where chemical analysis such as LC-QTOF-MS is used, it is very important for the relevant m/z values, to 4dp, of compounds to be identified, so they can be used for reference against samples of unknown identity. Here, the m/z of 288.1584 was identified for the demethylated metabolite, and 318.1707 for the hydroxylated metabolite. As previously discussed, there were conclusions reached regarding the location on the compound of these particular metabolic processes.

It is important for forensic science laboratories to obtain this information pertaining to any kind of drug, particularly regarding novel drugs. With new variants of novel drugs being constantly developed, the practise of identifying their metabolites is important to facilitate their identification in toxicological screening. In the future, this can lead the way for studies to be carried out which corroborate the findings of this investigation and synthesis of reference standards for novel drugs and their metabolites to facilitate their identification with greater precision and certainty.

Perhaps of even greater importance than metabolite identification, is what has been learned regarding the methods used to study drug metabolism. The use of S9 is relatively recent in comparison to the use of HLMs, cryopreserved hepatocytes, urine studies and the use of administration to rats. Much has been speculated relating to, in theory, what should be present in the S9 fraction after processing. However, it
appears that it would be pertinent to carry out work to identify the presence of required enzymes in S9 to confirm whether or not it would be likely to facilitate Phase II metabolism. From here the relevant reagents can be added to produce a successful metabolism profile. The work from this study can be further investigation to produce a sound procedure for the use of S9 in metabolism studies, which would be advantageous due to the fact that S9 is easily obtained and stored, and does not require processing with multiple other reagents, as does the use of HLMs and hepatocytes.

Additionally, this study highlights the difference between qualitative and quantitative studies. Altered incubation times and extraction procedures and well as changes in software analysis would improve the recovery of the compounds and also knowledge pertaining to its activity over an extended period of time, indicative of what may occur after an extended time post ingestion by a user. The simple identity of Phase I metabolites and suggested alterations to the S9 method, pave the way for improvements to be made and the use of reference standards which can be used in quantitative studies. This being a preliminary study, the results focus on metabolite generation. Further studies can take this method and apply it to reference standards to identify the retention times and particular fragmentation of isomers, as well as application of 25H-NBOMe in a quantitative manner.

In conclusion, the identity of Phase I 25H-NBOMe metabolites, and the foundations of the investigation into the use of S9 to study xenobiotic metabolism, has been successfully carried out. The future of such studies will be a refining of the techniques used to create successful Phase II metabolism, and to extend knowledge of 25H-NBOMe in quantitative studies to increase knowledge of its metabolism in vivo.
Chapter 6: References
Reference List


Appendices
A. PCDL spectrum of Tramadol

B. PCDL spectrum of JWH18

C. PCDL spectrum of 25H-NBOMe