NMR-BASED METABONOMICS OF PRE-CLINICAL MODELS OF ISONIAZID AND GENTAMICIN TOXICITY

Thesis submitted by

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ABSTRACT

An individual's response to drug therapy is the result of complex interactions between environmental and genetic factors. The ability to characterise inter-individual variation in response to pharmaceutical intervention is of particular importance in a clinical setting, where variation in response can result in therapeutic failure or adverse effects in individuals or sub-populations of patients. Given the success of NMR-based techniques for metabolic profiling, this thesis focused on characterisation of the systems-wide endogenous metabolic response to isoniazid and gentamicin. Throughout this work, one-dimensional $^1$H and two-dimensional $^1$H NMR of biofluids and tissue was applied to generate metabolic profiles reflective of response to pharmaceutical intervention in the animal model, in conjunction with conventional clinical chemistry and histopathological assessment.

Isoniazid is a widely prescribed anti-tubercular treatment that has toxic side effects: it is widely associated with both hepatotoxicity and peripheral neurotoxicity in the clinical setting. Here, a relationship was established between the post dose profile of drug metabolites and the severity of the adverse effect on the central nervous system. Further, the metabonomic approach resulted in the identification of pre-dose urinary markers of toxic outcome.

The aminoglycoside antibiotic gentamicin is a known nephrotoxin. Clinical chemistry and histopathology identified clear differences in the degree of nephrotoxicity experienced by the rat relative to administration time. Complementary metabolic profiling techniques were then applied to the analysis of urine and kidney tissue, and identified clear metabolic differences in response
to treatment time. A final study then explored whether co-administration of a statin (atorvastatin) could reduce gentamicin-induced nephrotoxicity in the rat. Conventional toxicity assessments indicated that co-administration of gentamicin and atorvastatin was non-toxic, while metabonomic analysis of urine and kidney tissue indicated that there were metabolic differences, likely linked to the antibiotic effect of gentamicin. This work highlights the potential for beneficial drug-drug interactions to decrease a drug's toxic effect, as well as the importance of considering administration time when developing treatment regimens.
STATEMENT OF ORIGINALITY

I certify that this thesis, and the research to which it refers, are the product of my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline.
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And, finally to my family for their continued support. They have never once doubted my sanity in choosing to undertake this PhD, even when I may have.
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ABBREVIATIONS

1D  one dimensional
2D  two dimensional
2-AA 2-amino adipate
2-DOS 2-deoxystreptamine
2-OG 2-oxoglutarate
3-HB 3-D-hydroxybutyrate
3-HPPA 3-hydroxyphenylpropionic acid
3-IS 3-indoxylsulfate
4-HPPA 4-hydroxyphenylpropionic acid
5-NU 5′-nucleotidase
AAC aminoglycoside N-acetyltransferases
AcHz acetylhydrazine
AcINH acetylisoniazid
ADME absorption, distribution, metabolism, and elimination
ADRs adverse drug reactions
AIDS acquired immune deficiency syndrome
AKI acute kidney injury
ALA alanine
ALP alkaline phosphatase
ALT alanine transaminase
AM AM administration, 10.00
ANT aminoglycoside O-nucleotidyltransferases
APH aminoglycoside O-phosphotransferases
AST aspartate transaminase
BUN blood urea nitrogen
CIT citrate
CNS central nervous system
COSY correlation spectroscopy
CPMG Carr-Purcell-Meiboom Gill
CRE creatine
CRN creatinine
Cys-C   cystatin-C  
DiAcHz  diacetylhydrazine  
DMA     dimethylamine  
DMG     dimethylglycine  
EDP-II  energy dependent phase-II  
FID     free induction delay  
FT      Fourier transformation  
GABA    γ-aminobutyric acid  
GABA-T  γ-aminobutyric acid-transaminase  
GAD     glutamic acid decarboxylase  
GC-MS   gas chromatography mass spectrometry  
GGT     γ-glutamyl transferase  
GLC     glucose  
GPC     glycerophosphocholine  
GTP     guanosine-5′-triphosphate  
HET-STOCSY heteronuclear statistical total correlation spectroscopy  
HIP     hippurate  
HIV     human immunodeficiency virus  
HMBC    heteronuclear multiple bond coherence  
HPLC    high performance liquid chromatography  
HMG-CoA 3-hydroxy-3-methylglutaryl CoA  
HSQC    heteronuclear single quantum correlation  
INA     isonicotinic acid  
INA-GLY isonicotinylglycine  
INH     isoniazid  
INH-GLC glucosyl isonicotinylhydrazide  
INH-KA  2-oxoglutarate isonicotinylhydrazone  
INH-PA  pyruvate isonicotinylhydrazone  
INH-PY  isoniazidyl-pyridoxal complex (Schiff base)  
JRES    J-resolved  
KIM-1   kidney injury molecule 1  
LAC     lactate  
LC-MS   liquid chromatography mass spectrometry
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LRP2</td>
<td>lipoprotein related protein-2, megalin</td>
</tr>
<tr>
<td>MA</td>
<td>methylamine</td>
</tr>
<tr>
<td>MAS-NMR</td>
<td>magic angle spinning nuclear magnetic resonance</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>multi-drug resistant tuberculosis</td>
</tr>
<tr>
<td>MI</td>
<td>myo-inositol</td>
</tr>
<tr>
<td>MVLA</td>
<td>microvesicular lipid accumulation</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetyl-β-glucosaminidase</td>
</tr>
<tr>
<td>NAPQI</td>
<td>N-acetyl-p-benzoquinone imine</td>
</tr>
<tr>
<td>NAT1</td>
<td>N-acetyltransferase 1</td>
</tr>
<tr>
<td>NAT2</td>
<td>N-acetyltransferase 2</td>
</tr>
<tr>
<td>NGAL</td>
<td>neutrophil gelatinase-associated lipocalin, or lipocalin-2</td>
</tr>
<tr>
<td>NMNA</td>
<td>N-methylnicotinic acid</td>
</tr>
<tr>
<td>NMND</td>
<td>N-methylNicotinamide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOESY</td>
<td>nuclear Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>OSC</td>
<td>orthogonal signal correction</td>
</tr>
<tr>
<td>O-PLS-DA</td>
<td>orthogonal partial least square discriminant analysis</td>
</tr>
<tr>
<td>P5P</td>
<td>pyridoxal-5-phosphate</td>
</tr>
<tr>
<td>PAG</td>
<td>phenylacetylglycine</td>
</tr>
<tr>
<td>Pb</td>
<td>phenobarbital</td>
</tr>
<tr>
<td>PC</td>
<td>principal component</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PLS</td>
<td>partial least squares</td>
</tr>
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<td>PLS-DA</td>
<td>partial least squares discriminant analysis</td>
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<td>PM administration, 22.00</td>
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<td>pattern recognition</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSPA</td>
<td>recursive segment-wise peak alignment</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley</td>
</tr>
<tr>
<td>STOCSY</td>
<td>statistical total correlation spectroscopy</td>
</tr>
<tr>
<td>STOCSY-E</td>
<td>statistical total correlation spectroscopy-editing</td>
</tr>
<tr>
<td>SUC</td>
<td>succinate</td>
</tr>
<tr>
<td>TAU</td>
<td>taurine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>TCA</td>
<td>tricyclic acid</td>
</tr>
<tr>
<td>THOPC</td>
<td>1,4,5,6-tetrahydro-6-oxo-3-pyridazine carboxylic acid</td>
</tr>
<tr>
<td>TMAO</td>
<td>trimethylamine-N-oxide</td>
</tr>
<tr>
<td>TOCSY</td>
<td>total correlation spectroscopy</td>
</tr>
<tr>
<td>TSP</td>
<td>3-trimethylsilyl-[2,2,3,3-\textsuperscript{2}H\textsubscript{4}]-propionic acid sodium salt</td>
</tr>
<tr>
<td>UPLC-MS</td>
<td>ultra performance liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>XDR-TB</td>
<td>extensively drug resistant tuberculosis</td>
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Chapter 1 INTRODUCTION

The activity of any xenobiotic, such as a drug, can be considered in terms of its action on the body, i.e. pharmacodynamics, ranging from a therapeutic effect to a toxic event, and the action of the body on the xenobiotic, i.e. pharmacokinetics, which includes absorption, distribution, metabolism and elimination (collectively termed ADME). This thesis focuses on the metabolic effects in the animal model of two commonly prescribed therapeutic agents, isoniazid, an anti-tubercular drug, and gentamicin, an aminoglycoside antibiotic.

1.1 DRUG TOXICITY AND ADVERSE DRUG REACTIONS

The development of a new pharmaceutical is a costly and time-consuming process. Figures published in 2003, estimated the cost of getting a new compound from discovery to the point of marketing approval at US$802 million. \(^1\) By 2007, this figure was predicted to rise to US$1.3 billion, in a process that would take an estimated 16 years. \(^2\) Many factors contribute to the high cost of development but a major problem is the rate of attrition of compounds at various stages of the development process: the further a compound gets along this developmental pathway before it is rejected, the higher the cost to the pharmaceutical company.

Once a new compound of interest has been identified, it must undergo rigorous clinical trials before it can be approved for use. This is by far the most costly part of the development process. The trials involve a number of phases and possibly thousands of individuals to ensure the drug is both fit for purpose and safe to use. However, despite employing numerous in vitro experiments prior to these trials, over the ten year period between 1991-2000 almost 90% of all drug
candidates have failed during clinical trials, by which point the pharmaceutical company will already have invested much time and money into the compound. \(^3\) Safety issues with drugs are a significant element of this high level of attrition of development compounds. A more accurate understanding of the mechanisms of toxicity, and the underlying risk factors are necessary to improve both the development process and to reduce the incidents of toxicity, which accounted for more than 100,000 fatalities in the US in 1994, \(^4\) as well as the withdrawal of 121 pharmaceutical products between 1960 and 1999 due to post-marketing toxicity problems. \(^5\) Ultimately, it is hoped that an improved understanding of the mechanisms of toxicity will enable better pre-clinical testing for safety signals, i.e. indications of possible adverse reactions, allowing the elimination of compounds with safety signals early from the development process. It is also possible that improved understanding of toxicity will enable patient stratification, paving the way for the development of personalised healthcare and individualised drug regimes.

A major problem is that individuals vary greatly in their response to pharmaceutical intervention. In a clinical setting, this can result in therapeutic failure or adverse effects in individuals or sub-populations of patients. While many adverse drug reactions (ADRs) can be predicted from the known primary or secondary pharmacology of the drug or known metabolite, many ADRs are not readily predicted. A number of factors contribute to individual variability and may impact on the fate of xenobiotic compounds, including the development of toxicities. These factors include age, sex, disease state, drug interactions, and genetic factors.
1.2 Absorption, Distribution, Metabolism, Elimination (ADME)

Drugs are administered by an enteral (e.g. oral, inhalation) or parenteral (e.g. injection) route. Following administration, the drug is absorbed into the systemic circulation and distributed throughout the body to its target site. The majority of xenobiotics will undergo metabolism, either to produce a pharmacologically active species or as a detoxification mechanism. The primary site of drug metabolism is the liver, although some metabolism may occur in extra-hepatic tissues. All metabolites and unchanged parent compound are then eliminated from the body, with the majority excreted in the urine (kidney) or faeces (biliary).

1.2.1 Metabolism

Once a drug has entered the body, it will likely undergo a biotransformation that will alter both its physical properties and biological effects. In some cases, this is necessary for the therapeutic effect of the drug (e.g. isoniazid is a pro-drug that must be activated by the bacterial catalase katG). In general metabolism leads to loss of therapeutic effect and subsequent excretion, although certain biotransformations can lead to the formation of toxic metabolites. Metabolism of any xenobiotic can be considered to be a complex process involving interactions between mammalian and microbial metabolism. It can be divided into two phases, phase I and phase II, although they are not necessarily sequential in nature.
1.2.1.1  Phase I Metabolism

Phase I reactions are considered to be functionalisation reactions and consist of oxidation and reduction reactions, that alter and create new functional groups, and hydrolysis reactions, that cleave esters and amides to release masked functional groups, thus preparing the compound for subsequent phase II reactions. Most phase I reactions are catalysed by cytochrome P450 enzymes, a family of haem-containing, mono-oxygenase enzymes, embedded in the smooth endoplasmic reticulum that are involved in the metabolism of a range of endogenous and exogenous compounds, including steroids hormones, thyroid hormones, fatty acids and prostaglandins. Different P450 isoenzymes often have distinct, but overlapping, substrate specificity, and genetic polymorphisms are common. In humans, CYP2C9, CYP2D6, and CYP3A4 are most involved in drug metabolism, accounting for 60-70% of all phase I biotransformations. In addition to the cytochrome P450 enzymes, other enzymes may catalyse phase I reactions. For example, ethanol is metabolised by both alcohol dehydrogenase and CYP2E1. Phase I reactions prepare compounds for subsequent phase II reactions. Flavin-containing mono-oxygenases (FMOs) are another important family of enzymes. They catalyse chemical reactions via the bound co-factor flavin and oxygenate sulphur- and nitrogen-containing xenobiotics. To date, five mammalian forms of FMO have been identified (designated as FMO1 to 5).

1.2.1.2  Phase II Metabolism

Phase II reactions can be considered to be conjugation reactions, with the resulting conjugate usually pharmacologically inactive and less lipid soluble than its precursor. Reactions involve the formation of high energy intermediates via the
activation of endogenous metabolites or foreign compounds, and are catalysed by a wide range of enzymes, including sulphotransferase (sulphation), UDP-glucuronyl transferase (glucuronidation) and acetyltransferase (acetylation).

1.2.1.3 Factors Affecting Xenobiotic Metabolism

As previously stated a number of factors impact on an individual’s susceptibility to idiosyncratic ADRs. The gastrointestinal (GI) tract is home to a diverse ecosystem of microbes, collectively known as the ‘microbiota’, \(^9\) that plays a key role in metabolism of both endogenous and xenobiotic compounds. \(^10\) This complex consortium of microbes (approximately 100 trillion microbes, \(^11\) encompassing thousands of bacterial phylotypes) is genetically diverse, with approximately 100-times as many genes as the human genome. \(^12\) The role of the microbiota is diverse, but it is instrumental in energy metabolism and host immunity. In addition, it has been associated with the development of a number of conditions including obesity, \(^13,\) \(^14\) diabetes, \(^15\) inflammatory bowel disease, \(^16\) and cancer. \(^17\) Gut microbiota also play a role in drug metabolism, with numerous drugs identified as substrates. \(^18\) The exact composition of the microbiota varies between individuals, and exerts a strong influence on the individuals metabolic phenotype. \(^19\) This in turn can lead to variations in drug metabolism and toxicity; paracetamol-induced hepatotoxicity has been linked to gut microbiome composition in man. \(^20\) Therefore, differences in microbial composition may be a factor in individual drug responses.

An individual’s response to a pharmaceutical agent will also reflect their genetic make-up. The expression levels of a number of enzymes involved in xenobiotic metabolism are genetically determined, and genetic differences may cause a drug to be detoxified more rapidly or converted to a reactive/toxic
metabolite. A number of enzymes are polymorphic in nature, having two or more active forms. This can lead to marked inter-individual variation in response. Acetylation plays a key role in xenobiotic metabolism. It is catalysed by N-acetyltransferases, and involves the transfer of the acetyl group from acetyl coenzyme A to the acceptor amines, leading to the formation of amides. The N-acetylating capacity of an individual is genetically determined, resulting in ‘slow’, ‘fast’, and ‘intermediate’ acetylators. In humans, slow and fast acetylators differ by a single autosomal gene. Extensive studies have found that acetylator status varies widely between populations, for example, Asian populations have higher levels of fast acetylators than Caucasian populations.

Genetic polymorphism of P450 enzymes is also extensive. The P450 alleles carried by an individual will influence the success, or otherwise, of some drug therapies. For example, individuals that lack functional CYP2D6 genes will metabolise selective substrates at a lower rate, which is likely to be clinically important, given the key role played by CYP2D6 in xenobiotic metabolism in humans. For example, in the case of the anti-anginal agent perhexilene, poor metabolisers will generally have reduced clearance leading to hepatotoxicity and peripheral neuropathy.

While there is clearly a genetic component to many idiosyncratic adverse reactions, the relationship between genotype and clinical phenotype is complex; it is believed that susceptibility to Type B reactions is a multi-factorial process with each predisposing gene neither necessary nor sufficient by itself to produce the ADR, rather each gene increases the risk of experiencing an ADR. Further, there is likely an environmental contribution to the development of ADRs, including concomitant drug use, age, sex, and underlying disease state.
1.3 **Toxins**

The liver, the major site of drug metabolism, and the kidneys, responsible for elimination of the majority of compounds from the body, are both major sites of drug-induced toxicity. This thesis looks specifically at isoniazid, a known hepatotoxin and neurotoxin, and gentamicin, a known nephrotoxin (gentamicin is also ototoxic, i.e. can result in damage to the ear, but this adverse effect was not considered in this study).

1.3.1 **ISONIAZID**

Isoniazid (also known as isonicotinic acid hydrazide, isonicotinyl hydrazine or INH) is a front-line anti-tubercular drug (Figure 1-1). First synthesised in 1912 by Meyer and Mally as part of a doctoral research project, its anti-tubercular properties were not recognised until the early 1950’s. Despite the widespread use of INH, it is associated with a number of adverse effects, ranging from relatively mild reactions, such as skin rash, to more severe incidents of hepatotoxicity, peripheral neuropathy and central nervous system (CNS) effects.

![Figure 1-1 Isoniazid](image)

Figure 1-1 Isoniazid
1.3.1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease, caused by *Mycobacterium tuberculosis*. Pulmonary TB is the most common form, accounting for approximately 75% of cases. Pulmonary TB is characterised by a chronic cough with blood-tinged sputum, fever, night sweats and weight loss. Extra-pulmonary sites of infection include the pleura, skeleton, and central nervous system. There were an estimated 9.27 million incident cases of TB in 2007, increased from 8.3 million in 2000. Of these 9.27 million cases, the majority were in Asia (55%) and Africa (31%), with 2.0 million cases recorded in India in 2007. 33

1.3.1.2 Treatment

Antibiotics, administered over a long period of time (typically 6 to 12 months), are used to treat tuberculosis. In the case of latent TB, i.e. when the subject is infected with *M. tuberculosis* but does not have active TB, the standard treatment is isoniazid alone for a period of 6 to 9 months. For active TB, a combination treatment of rifampicin, ethambutol, pyrazinamide and isoniazid are administered for 2 months, followed by a further 4 to 7 months of isoniazid and rifampicin. Some second-line drugs are also available which may be used where the infection is likely to be resistant to the first-line agents, or where the use of first-line agents has to be abandoned due to unwanted side effects and toxicities. These second-line agents fall into 6 categories: aminoglycosides, polypeptides, fluoroquinolones, thioamides, cycloserines and \(\beta\)-aminosalicylic acid. They are generally less effective and may have more severe side effects. The recommended dose of INH in man for the treatment of active TB is 5 mg/kg per day orally or via intra-muscular injection.
1.3.1.3 Resistance

Mismanagement of anti-TB therapy can lead to the development of resistant forms of the infection, either because of poor compliance (due to side effects of treatment) or failure to complete the treatment program (symptoms may be alleviated within a few weeks, but before the infection has been fully dealt with). Resistant TB is generally classified as multi-drug resistant TB (MDR-TB) or extensively drug-resistant TB (XDR-TB). MDR-TB is considered to be TB that is resistant to at least isoniazid and rifampicin (the two primary drugs for treatment of TB), while XDR-TB displays additional resistance to any fluoroquinolone and at least one of three injectable second-line drugs (kanamycin, capreomycin or amikacin). XDR-TB is therefore resistant to both first- and second-line antibacterial agents, seriously limiting treatment options. The rate of MDR-TB occurrence varies worldwide but countries of the former Soviet Union, as well as some provinces of China, appear to show the highest prevalence of resistance – of all new TB cases reported between 1994 and 2007, more than 6% were MDR-TB. In 2007, there were an estimated 0.5 million cases of MDR-TB, and 26,000 cases of XDR-TB worldwide.

1.3.1.4 Impact of HIV/AIDS

AIDS (Acquired Immune Deficiency Syndrome) is a viral disease that affects the immune system caused by Human Immunodeficiency Virus (HIV). HIV is transmitted through body fluids. The condition is managed through antiretroviral treatments which reduce the morbidity and mortality of the infection, and whilst, as yet, there is no vaccine or cure, the recent introduction of the CCR5 blocker Maraviroc™ from Pfizer has transformed the prognosis of patients with CCR5-tropic virus. HIV/AIDS compromises the immune system and leaves individuals
susceptible to opportunistic infections. One of the most common infections is TB. Of the 9.27 million incident cases in 2007, approximately 15% occurred in individuals that were HIV-positive, equivalent to 1.37 million cases, with 79% of these cases occurring in Africa. According to the World Health Organisation, TB co-infection is the single biggest cause of AIDS morbidity. Co-infection of HIV/AIDS and TB forms a lethal combination, with each increasing the other’s progress. HIV/AIDS weakens the immune system, and consequently an individual who is HIV-positive and infected with TB bacilli is many times more likely to become sick with TB than someone who is infected but HIV-negative. It is a leading cause of death among individuals who are HIV-positive: in Africa, HIV/AIDS is the single most important factor contributing to the increase in TB incidence since 1990. In New York City, the re-emergence of TB in the 1980’s coincided with the emergence of HIV/AIDS, with individuals with AIDS 120 times more likely to develop TB than an immune-competent individual. Co-infection with HIV/AIDS can also impact on the development of resistance, as individuals may not be able to absorb anti-TB drugs as well. In addition, these individuals already have a compromised immune system which is unable to assist in fighting the infection.

1.3.1.5 Mechanism of Action

While the exact mode of action of INH is not fully understood, a number of possible mechanisms have been identified that may act additively or synergistically. INH enters the bacterial cell by passive diffusion. INH itself is not toxic to the bacterial cell, rather it is a pro-drug that is activated by a bacterial catalase, \( katG \), with mutations in the \( katG \) gene being a major cause of INH resistance. \( katG \) activates INH by peroxidation to produce either an isonicotinyl
acyl anion \(^{38}\) or an isonicotinyl acyl radical. \(^{39}\) These INH-derived species couple covalently to a form of Nicotinamide Adenine Dinucleotide (NADH), and it is likely that this activated INH-NADH complex has a number of targets. The most likely target is the disruption of the mycolic acid synthesis pathway, leading to the production of a defective cell wall, resulting in the loss of further material from the cell and ultimately, bacterial cell death. \(^{40}\) Earlier work has established that the INH-NADH complex is a powerful inhibitor of InhA, a \(2\text{-trans}\) enoyl-acyl carrier protein reductase enzyme involved in mycolic acid synthesis. \(^{41}\) Another possible mechanism of action includes the formation of reactive oxygen species resulting in damage to a number of intracellular macromolecules, including nucleic acids, contributing to cell death. \(^{38}\) The role of free radicals is supported by the finding that a free radical scavenger can partially protect mycobacteria against INH. \(^{42}\)

1.3.1.6 Metabolism

The metabolic fate of INH in man has been well described and is presented in Figure 1-2. \(^{43}\) Acetylisoniazid (AcINH) is the major urinary metabolite of INH, generated by hepatic acetylation of INH by the polymorphic enzyme N-acetyltransferase 2 (NAT2). \(^{43-45}\) AcINH is hydrolysed to acetylhydrazine (AcHz) and isonicotinic acid (INA), and AcHz undergoes further acetylation to diacetylhydrazine (DiAcHz). \(^{29, 46}\) INA is also formed, along with hydrazine, via direct hydrolysis of INH. INA undergoes further metabolism, where it is conjugated with glycine by glycine transferase, a mitochondrial enzyme, resulting in the formation of isonicotinylglycine (INA-GLY). Individuals vary in their ability to conjugate glycine which is unrelated to acetylator phenotype. \(^{46, 47}\) Rather, glycine conjugation is affected by variable expression of glycine transferase and availability of glycine, which becomes deficient in low protein diets and is depleted.
by chronic exposure to toxins. INH also undergoes non-enzymatic reactions with the endogenous keto acids pyruvate and 2-oxoglutarate to form pyruvate isonicotinylhydrazone (INH-PA) and 2-oxoglutarate isonicotinylhydrazone (INH-KA), respectively. \(48\) INH-PA is formed more readily than INH-KA, \(49\) but the extent of hydrazone formation is dependent on the availability of INH. \(47\) INH is also believed to form a Schiff base complex (isoniazidyl-pyridoxal complex, INH-PY) with pyridoxal-5-phosphate \(50, 51\) and conjugates with glucose to form glucose isonicotinylhydrazide (INH-GLC). \(52\)
Figure 1-2 Scheme proposed for metabolism of INH in mammals including man. (Adapted from Timbrell et al. 43)

Key: AcHz – acetylhydrazine; AcINH – acetylisoniazid; DiAcHz – diacetylhydrazine; INA – isonicotinic acid; INH – isoniazid; INH-GLC – glucose isonicotinylhydrazide; INA-GLY – isonicotinylglycine; INH-KA – 2-oxoglutarate isonicotinylhydrazone; INH-PA – pyruvate isonicotinylhydrazone; INH-PY – isoniazidyl-pyridoxal complex; NAT2 – N-acetyltransferase 2.

Earlier studies have identified and quantified the major INH metabolites and have shown that in rats the principal metabolites present in urine, 24 hours after administration, are AcINH and INA. 43 Table 1-1 gives details of the metabolites observed in an earlier study 24 hours after the administration of a single low dose of [14C]-INH (100 mg/kg) to an animal cohort (n=3); quantitation
and identification of metabolites was carried out by scintillation counting and confirmed using reverse isotope dilution. 43

Table 1-1 The metabolism and disposition of [14C]-isoniazid in rats.
Results are mean values of three animals ± S.E.; INH (100 mg/kg) was given i.p. (From Timbrell JA et al. 43).

<table>
<thead>
<tr>
<th>Urinary Metabolites</th>
<th>% Urinary Radioactivity (0-24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH-KA</td>
<td>8.5 ± 1.3</td>
</tr>
<tr>
<td>Isonicotinylglycine</td>
<td>8.8 ± 0.9</td>
</tr>
<tr>
<td>Isonicotinic Acid</td>
<td>18.5 ± 0.4</td>
</tr>
<tr>
<td>INH-PA</td>
<td>8.2 ± 1.2</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>Acetylisoniazid</td>
<td>45.6 ± 2.8</td>
</tr>
<tr>
<td>Total</td>
<td>91.8 ± 1.1</td>
</tr>
</tbody>
</table>

Key: INH-KA — 2-oxoglutarate isonicotinylhydrazone; INH-PA — pyruvate isonicotinylhydrazone.

1.3.1.7 Adverse Effects

Though widely used, INH administration in man may be accompanied by hepatic damage and peripheral neuropathy with standard therapeutic doses of INH. The two most likely causes of INH-induced hepatotoxicity are hydrazine and AcHz. Hydrazine, which is formed directly and indirectly following hydrolysis of INH and AcINH respectively, is widely used in industrial applications. It is a well-studied model hepatotoxin and earlier work has shown that it causes fatty liver 53, 54 and, in some cases, liver necrosis. 55 Hydrazine has been directly linked to the hepatotoxic effects of INH, likely due to its metabolism to free radicals capable of causing structural damage to liver cells. 56-59 Hydrazine undergoes mono- and di-acetylation to form AcHz and DiAcHz. AcHz undergoes additional metabolism via
the cytochrome P450 microsomal enzyme system, specifically CYP2E1, to produce a reactive acylating intermediate that covalently binds to hepatic proteins leading to hepatic necrosis in rat models and human populations.

Long-term exposure to INH has also been shown to result in peripheral neuropathy. The most common symptoms are numbness and tingling of the feet, which may be accompanied by myalgias, weakness, and sensory ataxia. INH-induced peripheral neuropathy is dose related and susceptibility is highest in chronic alcoholics and malnourished individuals, and ‘slow’ inactivators, i.e. slow acetylators (see section 3.1.8 for further details). INH has been shown to form a complex with pyridoxal-5-phosphate, which is renally excreted, with this reaction ultimately depleting the cellular reserve of pyridoxal. Therefore, in the clinic, pyridoxine (Vitamin B₆) is co-administered with INH to counteract the INH depletion of the pyridoxal pool, particularly in the malnourished. Consequently, peripheral neuropathy following INH treatment is now extremely rare.

The primary site of acute INH-induced toxicity in human and animal models is the CNS. Ingestion of 2 to 3 g of INH can cause acute neurotoxicity in adults, while ingestion of 10 to 15 g frequently results in death, if not correctly treated. Severe acute-INH induced neurotoxicity is characterised by a clinical triad of refractory seizures, metabolic acidosis with an elevated anion gap, and coma, with symptoms generally observed within 45 minutes and 2 hours post exposure. Other clinical signs associated with INH-induced neurotoxicity include hyperglycaemia, hypokalaemia, glucosuria, and ketonuria. While it is possible that hydrazine may play a role in the development of INH-induced neurotoxicity (it is known to cause CNS disturbances), the development of INH-induced neurotoxicity is most likely linked to the depletion of GABA levels in the
brain, as a result of depletion of the cellular reserve of pyridoxal. GABA is an inhibitory neurotransmitter and a lack of it can cause seizures. Two enzymes are involved in the synthesis of GABA – glutamic acid decarboxylase (GAD) synthesises GABA from glutamate, and GABA transaminase (GABA-T) degrades GABA into succinate and semi-aldehyde – and both enzymes require the presence of the co-enzyme pyridoxal-5-phosphate (the physiologically active form of pyridoxal). As previously stated, INH sequesters pyridoxal-5-phosphate, reducing the cellular reserve. This in turn reduces the activity of the enzymes GAD and GABA-T. Following administration of INH, GAD activity is rapidly decreased, reducing the body’s ability to synthesise GABA and leading to a reduction in GABA levels. At the same time, there is a fall in GABA-T activity, but as this is a more gradual process, it has the net effect of degrading GABA faster than it is being synthesised. The overall effect is a depletion of GABA in the brain, which leads to excessive CNS stimulation and seizures. The importance of pyridoxal in the prevention of INH-induced neurotoxicity has been demonstrated in a rat model of vitamin B₆-deficient rats that were more susceptible to INH-induced neurotoxicity.

1.3.1.8 Acetylator Status and INH-Induced Toxicity

N-acetylation is an important pathway in the metabolic conversion of a number of compounds, including amines, hydrazine, and sulphonamides. Genetic polymorphisms have been identified in the genes for the two N-acetyltransferase enzymes, NAT1 and NAT2. These enzymes possess differential but overlapping substrate specificities, with INH predominantly acetylated by NAT2. Genetic polymorphism of NAT2 in man has been shown to give rise to slow, intermediate and fast acetylators. Numerous studies have looked at the ratios
of acetylator phenotypes in different populations: for example, the Japanese population was found to have a rapid acetylator ratio of 88% compared to approximately 40% in a study of a German population. In contrast, Sprague-Dawley rats, as used in this study, exhibit a monomorphic acetylation phenotype.

Acetylation of INH is dose related in rat models, and has been shown to be saturable in man and rat models after a therapeutic dose. Therefore, increasing the dose would be expected to result in a decrease in acetylation and an increase in the excretion of unchanged INH and its hydrazones.

Acetylator status has been implicated in the development of INH-induced peripheral neuropathy, with slow acetylators at greatest risk of toxicity. It is also believed that slow acetylators are at greatest risk of developing INH-induced hepatotoxicity due to their relative inability to detoxify AcHz to DiAcHz. This is supported by numerous population studies that have identified a higher incidence of INH-induced hepatotoxicity in slow acetylators. Early work on INH however suggested that rapid acetylators were at a greater risk of developing hepatotoxicity due to rapid formation of AcHz. These studies relied on classifying acetylator status using biochemical techniques – for example, following administration of sulphadimidine, the concentrations of acetylated and free sulphadimidine were determined in urine and blood and if the proportion of free acetylated sulphadimidine was less than 25% in blood or less than 70% in urine, the individual was classified as a slow acetylator rather than genotyping. Acetylation has also been implicated in the development of acute INH-induced neurotoxicity with non-acetylating species, such as dogs, subject to more severe toxicity. No human studies have explicitly linked INH-induced neurotoxicity to acetylator status.
1.3.2 **GENTAMICIN**

Gentamicin is an aminoglycoside antibiotic synthesised by *Micromonospora purpurea*. It was first reported in 1963.\(^8^5\) It is a 4,6-disubstituted 2-deoxystreptamine (2-DOS) that is administered therapeutically as a group of three structurally similar variants, termed C1, C1A and C2 (Figure 1-3). Clinically, gentamicin is used in the treatment of meningitis, pneumonia, and sepsis. While individual risk factors may contribute to the development of gentamicin-induced toxicity, the major factor contributing to organ damage is elevated peak and trough serum drug concentrations. Gentamicin-induced nephrotoxicity is related to the duration for which the trough serum concentration, i.e. the minimum serum concentration, exceeds 2 \(\mu\)g/ml, while the risk of developing either ototoxicity or vestibular toxicity is highly correlated with elevated peak serum concentration (in excess of 10 \(\mu\)g/ml).\(^8^6\) Therapeutically, in patients with normal renal function, gentamicin is administered at a dose of 2 to 5 mg/kg daily.

![Figure 1-3 Structure of gentamicin.](image)

Gentamicin is administered therapeutically as a combination of three structurally similar compounds, C1, C1A and C2.
Aminoglycoside antibiotics are a widely prescribed and highly effective class of drugs. The first example, streptomycin, was isolated from a strain of soil bacteria *Streptomyces griseus* in 1944. Most aminoglycosides are naturally occurring substances produced by actinomycetes of either the genus *Streptomyces* or *Micromonospora*. Those compounds derived from bacteria of the *Streptomyces* genus (i.e. streptomycin, neomycin, kanamycin etc.) are named with the suffix –mycin, whereas those that are derived from *Micromonospora* (i.e. gentamicin, amikacin, netilmicin etc.) are named with the suffix –micin.

### 1.3.2.1 Structure

Aminoglycoside antibiotics consist of an amino-cyclitol ring, saturated with amine and hydroxyl substituents. In most clinically important compounds, this amino-cyclitol moiety is streptamine or 2-DOS; the exception is streptomycin which possesses a streptidine molecule. The amino-cyclitol nucleus is connected to various amino sugars via glycosidic linkages. The position of these glycosidic bonds forms the basis for the structural classification of aminoglycosides. The three structural types are the 4,6-disubstituted 2-DOS derivatives, which include most of the clinically important aminoglycosides (i.e. gentamicin, tobramycin, amikacin and netilmicin), the 4,5-disubstituted 2-DOS derivatives (i.e. neomycin and paromomycin), and others (streptomycin) (Figure 1-4). Aminoglycosides are basic, strongly polar compounds that are cationic. They are highly soluble in water, relatively insoluble in lipids, and have enhanced antimicrobial activity in alkaline environments compared to acidic ones. The cationic nature of aminoglycosides means they are minimally absorbed from the gut and penetrate the blood brain barrier poorly. They cannot be administered orally and must be given parenterally. They are metabolically stable compounds that are excreted
unchanged in the urine. The major limitations of aminoglycosides include a relatively low therapeutic index, lack of oral absorption, and the risk of both nephrotoxicity and ototoxicity.
<table>
<thead>
<tr>
<th></th>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$R^3$</th>
<th>$R^4$</th>
<th>$R^5$</th>
<th>$R^6$</th>
<th>$R^7$</th>
<th>$R^8$</th>
<th>$R^9$</th>
<th>$R^{10}$</th>
</tr>
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<tbody>
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<td>H</td>
<td>OH</td>
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<td>H</td>
<td>H</td>
<td>OH</td>
<td>CH$_2$OH</td>
</tr>
<tr>
<td>Kanamycin B</td>
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<td>H</td>
<td>OH</td>
<td>OH</td>
<td>NH$_2$</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>CH$_2$OH</td>
</tr>
<tr>
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<td>H</td>
<td>OH</td>
<td>H</td>
<td>NH$_2$</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>CH$_2$OH</td>
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<td>CH$_3$</td>
<td>OH</td>
<td>CH$_3$</td>
<td>H</td>
</tr>
<tr>
<td>Gentamicin C2</td>
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<td>H</td>
<td>H</td>
<td>NH$_2$</td>
<td>H</td>
<td>CH$_3$</td>
<td>OH</td>
<td>CH$_3$</td>
<td>H</td>
</tr>
<tr>
<td>Gentamicin C1A</td>
<td>H</td>
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<td>H</td>
<td>H</td>
<td>NH$_2$</td>
<td>H</td>
<td>CH$_3$</td>
<td>OH</td>
<td>CH$_3$</td>
<td>H</td>
</tr>
<tr>
<td>Amikacin</td>
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<td>H</td>
<td>OH</td>
<td>CH$_2$OH</td>
</tr>
</tbody>
</table>

Figure 1-4 Structural families of aminoglycoside antibiotics. The 2-DOS scaffold is highlighted in blue. 

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1.3.2.2 Mode of Action

Aminoglycosides are bactericidal due to inhibition of protein synthesis and subsequent loss of bacterial cell membrane integrity. This binding does not inhibit the formation of the initiation complex of peptide synthesis, rather it impairs the proof-reading process controlling translational fidelity, and hence perturbs elongation of the peptide chain. The impact is compounded by the secondary effects of accumulation of erroneous proteins, i.e. accumulation of these truncated and mis-folded proteins in the bacterial cell membrane leads to increased cellular permeability. This triggers marked acceleration of intracellular accumulation of drug and subsequent cell death. Aminoglycosides are active primarily against aerobic Gram-negative bacilli. Table 1-2 lists the major clinical uses for aminoglycosides. Aminoglycosides display a concentration-dependent bactericidal effect. They also exhibit a post-antibiotic effect, i.e. the persistent suppression of bacterial growth after a brief exposure (1 or 2 h) of bacteria to an antibiotic. Aminoglycosides are able to produce a synergistic bactericidal effect when co-administered with antimicrobial agents that inhibit cell wall biosynthesis, such as β-lactam antibiotics.

Table 1-2 Clinical uses of aminoglycosides

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>Tularaemia, tuberculosis and plague</td>
</tr>
<tr>
<td>Gentamicin, amikacin, netilmicin</td>
<td>Meningitis, pneumonia, sepsis</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>Amoebic dysentery</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>Gonorrhoea</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Burns, wounds, ulcers, dermatitis</td>
</tr>
</tbody>
</table>
1.3.2.3 Resistance

Aminoglycoside resistance is mediated by three distinct mechanisms of resistance: target site alteration; reduced intracellular drug concentration; and deactivation by aminoglycoside-modifying enzymes. These mechanisms may occur in combination.

Modification of the target site can occur through methylation of the aminoglycoside-binding site. Resistance can also be conferred by mutation of the ribosomal target, although this is only clinically relevant for streptomycin treatment of *M. tuberculosis*: *Mycobacterium* is the only genus of eubacteria that contain a single copy of the ribosomal operon. Consequently, a single mutation can confer resistance.

Reduced intracellular drug concentration due to energy-dependent efflux is a major cause of antibiotic resistance, and aminoglycosides have been shown to be substrates for a variety of multidrug efflux pumps. Intracellular concentration can also be affected by mutations in the components of the transmembrane transport systems, leading to membrane impermeability.

The major mechanism of aminoglycoside resistance is via enzymatic modification of the amino or hydroxyl groups, resulting in diminished affinity for the ribosomal A-site target, and reduced uptake via energy-dependent phase II (EDP-II). More than 20 aminoglycoside-modifying enzymes have been identified, falling into three general classes of co-factor dependent enzymes: aminoglycoside *O*-phosphotransferases (APH), aminoglycoside *O*-nucleotidyltransferases (ANT), and aminoglycoside *N*-acetyltransferases (AAC). Each class of enzyme is further sub-divided according to site of enzymatic modification.
1.3.2.4 Toxicity

The most clinically important adverse effects associated with aminoglycoside administration are nephrotoxicity and ototoxicity, observed in man and animal models. Aminoglycoside-induced ototoxicity can affect both the cochlear and vestibular systems, and individual aminoglycosides differ in their ability to cause vestibular versus cochlear damage: streptomycin and gentamicin predominantly cause vestibular damage, whereas neomycin and amikacin mostly affect hearing. Tobramycin affects both equally.\(^{102}\) Cochlear damage manifests as permanent, bi-lateral high-frequency sensorineural hearing loss, while vestibular damage leads to disequilibrium and oscillopsia (i.e. loss of vestibular ocular-reflex). Aminoglycoside-induced ototoxicity is believed to be due to the generation of reactive oxygen species, which promote apoptotic and necrotic cell death. The generation of these species involves the formation of an aminoglycoside-iron complex. Several studies support this theory: gentamicin-induced ototoxicity is inhibited by the use of iron-chelators,\(^{103, 104}\) while the addition of supplemental iron enhances the ototoxic effect of aminoglycosides.\(^{105}\)

Patient susceptibility to aminoglycoside-induced ototoxicity has been linked to genetic predisposition, specifically to an inheritable mutation in the 12S ribosomal RNA. To date, five different homoplasmic mutations in the mitochondrial gene encoding this region have been found to predispose individuals to irreversible aminoglycoside-induced hearing loss.\(^{106-110}\) It has been suggested that mutation increases affinity for aminoglycosides, leading to toxicity in the eukaryotic cell. Ototoxicity is potentiated by the concomitant use of other ototoxic drugs, such as loop diuretics.\(^{111}\)
Aminoglycoside-induced nephrotoxicity presents as non-oliguric renal failure with varying degrees of tubular dysfunction.\textsuperscript{112,113} It is accompanied by a slow rise in serum creatinine and a hyperosmolar urinary output after several days of treatment. Aminoglycosides are excreted almost entirely by glomerular filtration; approximately 80% of the systemic dose is excreted within 24 hours. However, a small proportion (approximately 5-10%) is retained in the tubular epithelium of the S1 and S2 segment of the proximal tubules.\textsuperscript{114} Aminoglycosides are then internalised by endosomes and subsequently transferred to lysosomes, where they accumulate and cause phospholipidosis. This ultimately causes the lysosome to rupture, releasing acid hydrolases and high concentrations of aminoglycosides into the cytoplasm, resulting in disruption to cellular structure and function. As aminoglycoside elimination is almost entirely renal, the nephrotoxic effect of aminoglycosides can further impair their excretion. A number of risk factors have been identified including choice of aminoglycoside and duration of therapy, administration time, hypotension, volume depletion, pre-existing liver disease, and concomitant use of other nephrotoxic agents.\textsuperscript{115}

A number of strategies have been employed in order to reduce aminoglycoside-induced nephrotoxicity. Renal damage is dependent on uptake and accumulation of the drug within the kidney cells, and subsequent phospholipidosis. Therefore, reducing accumulation of aminoglycosides by the kidney, or reducing lysosomal phospholipidosis once the aminoglycoside has been internalised may in turn reduce toxicity.

Megalin plays a key role in aminoglycoside-induced nephrotoxicity. Also known as low-density lipoprotein-related protein 2 or LRP2, megalin is an endocytic receptor expressed in the apical surface of the proximal tubular epithelium and is responsible for the uptake of aminoglycosides into the
endosomes. Therefore, interfering with the activity of megalin should prevent accumulation of aminoglycosides, and the resultant toxicity. It has previously been shown that gentamicin and other polybasic drugs bind to purified megalin, and that a megalin antagonist can inhibit uptake.\textsuperscript{116} This is supported by \textit{in vivo} work that found increased urinary excretion of vitamin D binding protein and calcium following gentamicin administration, both of which are ligands of megalin, suggesting competitive inhibition of megalin binding.\textsuperscript{117} Further, co-administration of gentamicin and megalin substrates, including cytochrome \textit{c}, reduced renal accumulation of gentamicin, whilst also reducing gentamicin-induced nephrotoxicity.\textsuperscript{118} In addition, megalin-deficient mice are protected from renal aminoglycoside accumulation.\textsuperscript{119}

The mevalonate pathway is crucial to megalin function. This pathway is an important cellular metabolic pathway found in all higher eukaryotes and many bacteria. It produces isoprenoids which are vital for a range of diverse cellular functions including growth control and cholesterol synthesis. These isoprenoids, such as geranyl-geranyl-pyrophosphate and farnesyl pyrophosphate, are used for the modification of GTP-binding proteins. Once modified, and therefore activated, these proteins facilitate uptake of aminoglycosides \textit{via} receptor-mediated endocytosis by megalin. The mevalonate pathway is shown in Figure 1-5.
The bulk product of mevalonate metabolism is cholesterol. Mevalonate is also involved in receptor-mediated endocytosis – it is a precursor to geranyl-geranyl-pyrophosphate which is necessary for megalin function. (Adapted from Goldstein and Brown. 120)

It has been proposed that co-administration of gentamicin and statins may offer protection against gentamicin-induced nephrotoxicity, due to interference with the mevalonate pathway. 121 Statins are an important class of drug used to lower plasma cholesterol levels. They act by inhibiting the enzyme 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase, a rate-limiting step in the mevalonate pathway. As well as lowering cholesterol levels, statins also offer a potential strategy to prevent aminoglycoside-induced nephrotoxicity in man by preventing uptake and accumulation of aminoglycosides in tubular cells. A recent in vivo study, showed that gentamicin-induced cell death could be prevented with non-toxic doses of a range of statins. The same study also found that co-incubation with mevalonate reversed this inhibitory effect. 121
Administration time has a significant impact on the severity of renal damage observed following gentamicin administration, with the greatest toxicity in the rat model expected following a morning administration time. The temporal variation in toxicity has been linked to a subject's food consumption: aminoglycosides have been found to be less toxic when administered during the period of food intake (which corresponds to the activity period). For laboratory animals, the activity period occurs at night, therefore they are more protected from gentamicin-induced toxicity when injected in the evening, indicating that the presence of food reduces the absorption of the drug. A possible contributing factor to the temporal variation observed in aminoglycoside-induced toxicity is melatonin. Melatonin (N-acetyl-5-methoxytryptamine) is a naturally occurring compound that controls the circadian rhythm of many biological functions. Produced by the pineal gland, circulating levels vary throughout the day: it is secreted in darkness, and production is inhibited by light. In addition to its role in circadian rhythms, melatonin has also been identified as a potent scavenger of free radicals and a number of studies have reported that administration of melatonin attenuates both the nephrotoxic and ototoxic effects of gentamicin exposure.
1.4 BIOMARKERS OF TOXICITY

Toxicity is assessed using a number of biomarkers, where a biomarker is defined as a ‘characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic agent’. Examples of hepatic biomarkers commonly measured in clinical chemistry screening include alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and total bilirubin. These enzymes are all physiologically important and catalyse a number of reactions, for example, ALT catalyses the reversible inter-conversion of alanine and 2-oxoglutarate (2-OG) to pyruvate and glutamate, while AST catalyses the conversion of aspartate and 2-OG to oxaloacetate and glutamate. Cellular damage and death results in elevated blood levels of these enzymes due to leakage of cellular contents into the bloodstream, for example, elevated levels of ALT and AST may indicate hepatocellular necrosis, while elevated levels of ALP may indicate cholestatic injury. However, it is worth noting that these markers are not mechanism specific, i.e. elevated levels of AST and ALT may be indicative of myocardial or muscle damage, or exercise. Although ALT remains the ‘gold standard’ biomarker for assessing hepatotoxicity, total bilirubin is also used as a marker of hepatic injury, especially cholestasis and biliary effects, though it may also be increased by non-hepatic causes such as haemolysis.

Routinely used measures of renal function include blood urea nitrogen (BUN) and serum creatinine, both of which are affected by glomerular filtration rate. However, they are not region-specific and they increase significantly only after substantial kidney damage has occurred. Therefore, earlier and more sensitive biomarkers of nephrotoxicity are needed. Proteomic and genomic
approaches have lead to the identification of a number of possible biomarkers of renal damage. These include kidney injury molecule-1 (designated as Kim-1 in rodents and KIM-1 in humans), neutrophil gelatinase-associated lipocalin (NGAL or lipocalin-2), N-acetyl-β-glucosaminidase (NAG), clusterin, and cystatin-C. The relative advantage and disadvantages of each are summarised in Table 1-3.

Table 1-3 Potential biomarkers of kidney injury (Adapted from Vaidya et al. 132)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Comments</th>
<th>Species</th>
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<tbody>
<tr>
<td>Kidney injury molecule 1 (KIM-1) 132, 133</td>
<td>Type-1 cell membrane glycoprotein containing a six cysteine immunoglobulin-like domain and a mucin domain in its extracellular domain; up-regulated in dedifferentiated proximal tubule epithelial cells. <strong>Advantages:</strong> Elevated urinary levels are highly sensitive and specific for acute kidney injury (AKI). Earlier diagnostic indicator of kidney injury, compared to conventional markers (30-fold increase reported after 3 daily doses of gentamicin; no significant changes in BUN or serum creatinine 133) Up-regulated following various models of preclinical and clinical AKI, fibrosis, renal cell carcinoma, and polycystic kidney disease.</td>
<td>Zebra-fish, mouse, rat, dog, monkey, human.</td>
</tr>
<tr>
<td>Neutrophil gelatinase-associated lipocalin, lipocalin-2 (NGAL) 132, 134</td>
<td>25kDa protein, initially identified bound to gelatinase in specific granules of the neutrophil. <strong>Advantages:</strong> Sensitive – expression up-regulated following ischemic or cisplatin induced renal damage. Early indicator of AKI following cardiopulmonary bypass. <strong>Disadvantages:</strong> Specificity to sepsis needs to be established.</td>
<td>Mouse, rat, human</td>
</tr>
<tr>
<td><strong>N-Acetyl-β-glucosaminidase (NAG)</strong></td>
<td>Proximal tubule lysosomal enzyme. <strong>Advantages:</strong> Sensitive – elevated following subtle alterations in epithelial cells of brush border of proximal tubules; amount of enzyme can be directly correlated to tubular injury. Extensive preclinical and clinical data available under a variety of conditions. <strong>Disadvantages:</strong> Activity may be inhibited by endogenous urea, some nephrotoxicants and heavy metals. Non-specific – elevated levels reported in absence of clinically significant AKI (e.g. rheumatoid arthritis).</td>
<td>Mouse, rat, human</td>
</tr>
<tr>
<td>Clustering</td>
<td>Multi-faceted glycoprotein. Induced in kidney and urine after various forms of preclinical AKI; expressed in dedifferentiated tubular epithelial cells. <strong>Advantages:</strong> Elevated kidney and urinary levels are very sensitive for AKI in preclinical models. Up-regulated in various rodent models of AKI, fibrosis, renal cell carcinoma, and polycystic kidney disease. <strong>Disadvantages:</strong> No clinical study demonstrating use.</td>
<td>Mouse, rat, dog, monkey, human</td>
</tr>
<tr>
<td>Cystatin-C (Cys-C)</td>
<td>13kDa protein; extracellular inhibitor of cysteine proteinases. <strong>Advantages:</strong> Sensitive serum marker of glomerular filtration rate – strong predictor of death and cardiovascular events in older patients. Serum concentrations appear to be independent of age, sex, muscle mass.</td>
<td>Mouse, rat, human</td>
</tr>
</tbody>
</table>
Despite extensive work, the value of current biomarkers when considered in isolation is limited. Many lack specificity and in terms of detecting changes in the levels of these biomarkers, timing is critical: unless the toxicological insult is very severe (in which case there may be other signs to indicate damage), the enzyme levels may, over time, return to normal. Therefore, a more specific and less invasive technique for identifying biomarkers is needed, and the biomarker itself must be detectable before any gross morphological or functional change takes place. Ideally the biomarker should occur at an early stage in the development of toxicity so it can be predictive, or enable therapy to be terminated before irreversible damage has occurred.
1.5 Metabonomics and Toxicity

Recently the focus has shifted towards identifying potential metabonomic biomarkers of toxicity. Metabonomics is defined as ‘the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification’. Metabolic profiles of biofluids and tissues present a means of visualising the complex relationship between genetic background, environmental factors, such as diet, nutritional status, and lifestyle, and extra-genomic influences such as gut microfloral composition. Metabonomic investigations generally utilise high resolution nuclear magnetic resonance (NMR) spectroscopy, as in this current work, or employ a liquid chromatography mass spectrometry (LC-MS) based analytical strategy. For the purpose of this current work, an NMR-based approach was employed because it facilitates rapid, simultaneous characterisation of endogenous and drug-related metabolites. Metabolic profiling can be applied to a range of biological fluids, intact tissues, and tissue and cell extracts; urine and serum are most commonly used. Interpretation of metabolic profiles is facilitated by the application of an array of multivariate statistical modelling techniques, which reduce complexity and increase ease of interpretation.

Metabonomic methods offer a complementary approach to conventional methods of toxicological assessment. Any change in either the physiological or external conditions to which an individual is subjected will result in a change in their metabolic profile as the organism attempts to compensate. The change may be significant, i.e. the appearance of a previously unseen metabolite, or more subtle, i.e. a change in the relative concentration of metabolites. While a dose-related change in the levels of drug metabolites can in general, be predicted,
perturbation of endogenous metabolite levels may be indicative of both the target and the nature of the toxic insult, for example, renal papillary damage is accompanied by a combination of changes in the urinary levels of trimethylamine-N-oxide, \textit{N,N}-dimethylglycine, dimethylamine and succinate. \textsuperscript{140, 141} Studies involving high resolution spectroscopic techniques combined with multi-variate statistics have been shown to be useful in the elucidation of mechanisms of toxicity, \textsuperscript{142, 143} and the identification of outliers. \textsuperscript{144, 145} In addition, the application of metabonomic techniques enables metabolic changes to be followed across time, showing the onset, progression, and, where applicable, recovery from toxic insult, while identifying differential rates of response to pharmaceutical intervention. \textsuperscript{144, 146}

As the main site of xenobiotic metabolism, much emphasis has been placed on the characterisation and identification of metabonomic biomarkers of hepatotoxicity. Previous metabonomic studies have characterised the metabolic impact of a range of model hepatotoxins, including hydrazine, \textsuperscript{147-149} acetaminophen, \textsuperscript{150} galactosamine, \textsuperscript{141, 142, 145} and 1-naphthylisothiocyanate. \textsuperscript{151} One such study looked at different classes of hepatotoxin including steatotic, cholestatic, and necrotic, and classified then according to their metabolic profiles. A single administration of INH at 110 mg/kg (male Wistar rats, \textit{n}=5) resulted in the detection of decreased urinary levels of succinate, dimethylamine, 2-oxoglutarate, and hippurate via NMR spectroscopy, although there was no significant change in plasma levels of ALT and AST and histopathological examination identified only moderate hepatocellular rarefaction. \textsuperscript{152} Unfortunately a number of other possible biomarkers of INH-induced toxicity were unassigned in this study. Recent work by Sumner \textit{et al}. applied NMR-based metabonomic techniques to the identification of a non-invasive biomarker of microvesicular lipid accumulation (MVLA) in the liver.
following INH administration at 10 and 300 mg/kg/day for up to 8 days. Perturbations in levels of serum enzymes (ALT, AST, sorbitol dehydrogenase) did not correlate with histopathological assessment of MVLA, but NMR analysis of urine and kidney extracts showed significant perturbations in a number of metabolic pathways. MVLA was characterised by changes in amino acid and pyrimidine metabolism and in biochemical energy pathways (TCA cycle, glycolysis). Further work is needed to fully elucidate the metabolic impact of INH.

Besides the liver, the kidney is the major site of drug-induced toxicity, and there have been numerous studies focused on nephrotoxicity. Early efforts have successfully classified renal damage as proximal tubular or medullary in nature for a range of nephrotoxins. In each case, a reproducible and distinct pattern of metabolites was observed using $^1$H NMR urinalysis, although this study did not include any aminoglycosides. Specifically, initial increases in levels of trimethylamine-$N$-oxide (TMAO) and dimethylamine, followed by elevated levels of acetate, succinate, lactate, and alanine were found to be reflective of proximal tubular injury. Following gentamicin exposure at 40 mg/kg over a period of up to 28 days, the earliest metabolic change seen was aminoaciduria, with significant increases in urinary amino acids after a single dose. The same study also detected a significant increase in the excretion of polyamines after a single dose which decreased gradually at later time points. Analysis of kidney tissue using LC-MS revealed reductions in nucleosides, with the effect becoming more severe as dosing continued. Other metabolites identified as potential early biomarkers of gentamicin toxicity were myo-inositol, 3-hydroxybutyrate (both significantly increased after a single dose; the increase persisted for duration of exposure), phosphate (significantly increased after single dose only), and 5-
methyltetrahydrofolate (significantly decreased in kidney tissue up to 5 days post dose). \textsuperscript{155}

A recent study by Sieber \textit{et al.} looked at the relative benefits of renal biomarkers, such as KIM-1 and NGAL, compared with metabolic profiling for the assessment of gentamicin-induced nephrotoxicity. \textsuperscript{156} Although they observed a clear time- and dose-dependent separation of gentamicin treated animals from controls using principal component analysis (PCA; separation was based on reduced levels of citrate, hippurate, \textit{N}-methylnicotinic acid, and 3-Indoxylsulphate and increased lactate, glucose and \textit{N},\textit{N}-dimethylglycine), they highlighted the need for accurate metabolite identification and mechanistic interpretation. On this basis, they favoured the use of KIM-1 and NGAL as biomarkers of renal damage: they were detected prior to significant clinical changes, offered good sensitivity and were mechanistically anchored.

The study protocol used by Sieber \textit{et al.} \textsuperscript{156} employed a combined \textsuperscript{1}H NMR and gas chromatography mass spectrometry (GC-MS) methodology to assess chronic toxicity in the male Wistar rat, and was based on an earlier study conducted by Lenz \textit{et al.}, which utilised \textsuperscript{1}H NMR and LC-MS. \textsuperscript{157} The key metabolic changes noted in the earlier study by Lenz \textit{et al.} were increases in urinary levels of TCA cycle intermediates (i.e. citrate, 2-oxoglutarate), and dimethylglycine, and reduced levels of TMAO and betaine after 3 days. By day 9, the gentamicin treated animals displayed marked glucosuria, increased excretion of \textit{N}-acetyl glycoprotein, citrate and lactate, coupled with reduced urinary excretion of TMAO and betaine. In addition, they noted the presence of elevated levels of an unidentified singlet at δ 1.39 in the day 9 urine sample. \textsuperscript{157} The metabolic changes observed in response to gentamicin administration in these
studies can be considered to reflect both the pharmacological, i.e. antibiotic, and toxicological activity of gentamicin.

1.5.1 PHARMACOMETABONOMICS – PREDICTING TOXICITY

While metabonomics may enable the early detection of a toxic event, the ultimate goal is to avoid toxicity. This offers advantages for both pharmaceutical development and clinical use; those individuals with an intrinsic risk of developing toxicity can be excluded from the testing process or prescribed a more appropriate pharmaceutical agent. Consequently, metabonomics has been applied to the prediction of toxic outcome. Termed ‘pharmacometabonomics’ (the prediction of the outcome of a drug or xenobiotic intervention in an individual based on a mathematical model of pre-intervention metabolite signatures), it takes into account both genetic variation and the environmental factors that impact on an individual’s response to a drug. 20, 158-162 The pharmacometabonomic approach has been successfully applied to the prediction of individual response to paracetamol, in rat and human models. 20, 158, 160

Paracetamol is detoxified in the liver, via sulphation, to paracetamol sulphate, and via glucuronidation, to paracetamol glucuronide. The parent compound also undergoes metabolism to form the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is subsequently detoxified via conjugation with glutathione. Paracetamol toxicity can occur due to overdose of paracetamol which this saturates the detoxification pathways, lack of glutathione as observed in chronic alcoholics, or increased production of NAPQI. Depletion of glutathione combined with direct mitochondrial damage due to NAPQI and covalent binding of NAPQI to hepatic protein, leads to cell necrosis and death. Following administration of a single toxic-threshold dose of paracetamol to an animal cohort (n=65), two pre-
dose models were developed with respect to classification of pre-dose profiles. One showed an association between the pre-dose spectra and the histopathological necrosis severity score after paracetamol exposure, while the second predicted the ratio of post dose paracetamol to paracetamol glucuronide (one of the major metabolites of paracetamol) from pre-dose urinary spectra. \(^{158}\) The pharmacometabonomic approach has also been applied to the investigation of paracetamol metabolism in humans. \(^{20}\) A clear association was identified between an individual's pre-dose urinary metabolic profile and the post-dose profile of paracetamol metabolites, specifically that individuals with high pre-dose levels of \(p\)-cresol sulphate had low post-dose ratios of paracetamol sulphate to paracetamol glucuronide. \(p\)-Cresol sulphate is derived from \(p\)-cresol by sulphation using the same sulphotransferases as paracetamol. \(p\)-Cresol is not a human molecule rather it is derived from gut bacteria, specifically \textit{Clostridium} species. Excess \(p\)-cresol depletes sulphation capacity. Researches concluded that those individuals with high levels of bacterial \(p\)-cresol would have depleted sulphonation capacity and potentially be at greater risk of developing paracetamol-induced toxicity. \(^{20}\) Winnike \textit{et al.} demonstrated a correlation between pre-dose levels of glycine, a component of glutathione, and development of post dose paracetamol-induced hepatotoxicity. \(^{160}\) All three studies suggested that it may be possible to predict an individual's response to paracetamol exposure based on their pre-dose metabolic profile. \(^{20, 158, 160}\)

Recently, pharmacometabonomics has been utilised in the clinical oncology setting. \(^{161, 162}\) Chemotherapy-induced toxicities limit the ability to deliver effective treatment and a recent pharmacometabonomic study has enabled the successful prediction of toxic responses to cancer therapy. Through the
application of high resolution NMR-based methodology, researchers were able to identify a metabolic signature associated with capecitabine-induced toxicity. \(^ {162}\) Furthermore, they identified a gradation of this profile that correlated with the severity of toxicity experienced. \(^ {162}\) Pharmacometabonomics has also been used to predict the efficacy of anti-cancer treatment in breast cancer patients. \(^ {161}\) Stebbing \textit{et al.} identified an association between sera levels of lactate, glucose (both elevated) and alanine (reduced) and disease progression in postmenopausal women undergoing chemotherapy. \(^ {161}\)

Metabonomics can therefore offer a means of gaining a better understanding of the mechanisms of different types of toxicity as well as identifying those individuals at greatest risk of developing these types of toxicity, prior to pharmaceutical intervention. Many drugs are known to have toxic side effects, but are still used as they offer the best, and sometimes only, treatment for particular conditions. Identifying those individuals at greatest risk of experiencing drug-related toxicity could allow judgements to be made based on the ratio of risk to benefit.
Chapter 2 ANALYTICAL STRATEGIES

Throughout this work, an NMR-based metabolic profiling strategy has been applied to interrogate and understand the aetiology of toxic responses to isoniazid and gentamicin, through the analysis of a range of biofluids, including urine, serum and intact tissue. NMR spectroscopic profiling enables simultaneous characterisation of metabolites from many classes. The metabolic changes can be further characterised through the application of pattern recognition (PR) techniques.

2.1 NUCLEAR MAGNETIC RESONANCE

2.1.1 General Principles of NMR

NMR is a powerful analytical tool, which results from the interaction between the intrinsic angular momentum of a nucleus (its spin) and an external magnetic field. The spin quantum number of a nucleus is dependent upon the number of protons and neutrons present, and is the fundamental determinant of whether a particular nuclei will exhibit magnetic resonance phenomena. Nuclei with even numbers of protons and neutrons possess a spin quantum number of 0, so are NMR inactive. Nuclei with odd numbers of protons and/or neutrons possess spin and are therefore detectable in NMR. The rules for determining the spin quantum number are:

- Nuclei with even numbers of protons and even number of neutrons have a spin of zero, e.g. $^{12}\text{C}$, $^{16}\text{O}$ and $^{32}\text{S}$.
- Nuclei with odd numbers of protons and odd numbers of neutrons have spin values of 1, 2, 3 etc. Examples include $^{2}\text{H}$ and $^{14}\text{N}$.
• Nuclei where the number of protons plus the number of neutrons is odd possess half-integral spin values of 1/2, 3/2, 5/2 etc. Examples include $^1$H, $^{13}$C, $^{15}$N and $^{19}$F.

The most commonly studied nuclei in biological systems are $^1$H and $^{13}$C, which have a natural abundance of 99.9% and 1.11%, respectively, and both possess spin of $\frac{1}{2}$. Other nuclei commonly studied by NMR include $^{19}$F and $^{31}$P.

When an external magnetic field ($B_0$) is applied, nuclei that possess spin $\frac{1}{2}$ will adopt one of two spin states: parallel (α-state, lower energy) or anti-parallel (β-state, higher energy) to the applied magnetic field. Therefore a nucleus with a spin of $\frac{1}{2}$, will adopt a spin state of either $+\frac{1}{2}$ or $-\frac{1}{2}$. The population of each spin state is described using the Boltzmann distribution:

$$\frac{N_\alpha}{N_\beta} = e^{\Delta E / KT}$$

where $N_\alpha$ and $N_\beta$ are populations of the α and β states, $K$ is the Boltzmann constant, $T$ is the temperature in Kelvin and $\Delta E$ is the difference in energy between the two spin states α and β. The parallel orientation (α) has the lower energy level and consequently will have slightly more nuclei than the higher energy level. $\Delta E$ is small, and the population difference between the two spin states is correspondingly small. Therefore, NMR is relatively insensitive.

Once aligned with the applied magnetic field, the nuclei precess in the same direction about the axis of the applied field (Figure 2-1), in what is termed the Larmor precession.
In an external magnetic field \((B_o)\), nuclei with a spin of \(1/2\) can precess either parallel or anti-parallel to the field.  

The rate of precession, i.e. the Larmor frequency of a nuclei \(\nu\), is defined as:  
\[
\nu = \frac{\gamma B_0}{2\pi}
\]

where \(\gamma\) is the gyromagnetic ratio (also known as magnetogyric ratio), and \(B_0\) is the applied magnetic field. NMR spectroscopy manipulates the orientation of the nuclei through the application of an additional magnetic field, i.e. the NMR pulse that forces some of the nuclei in the low energy state to adopt a higher energy level. The frequency of this transition energy matches that of the Larmor frequency of a specific nuclei. Therefore the energy required to change spin states is:

\[
\Delta E = h\nu = h \cdot \frac{\gamma B_0}{2\pi}
\]

where \(h\) is Planck's constant, \(\nu\) is the Larmor frequency, \(B_0\) is the applied magnetic field and \(\gamma\) is the gyromagnetic ratio. When the applied radiofrequency energy is removed, the nuclei relax back to their original energy state. It is this period of relaxation, termed the free induction delay or FID that is measured.
during the acquisition of a NMR spectrum. The FID consists of two independent components characteristic of nuclear relaxation: the longitudinal or spin-lattice relaxation time ($T_1$) and the transversal relaxation time ($T_2$). The longitudinal relaxation time is the time constraint for the recovery of the magnetisation along the direction of $B_0$, and the transversal relaxation time is the time constraint for decay of magnetisation in the x-y plane. The decay of $T_2$ occurs approximately 5-10 times more rapidly than $T_1$ recovery, and these relaxation times are dictated by the speed of molecular rotation and its size.

Nuclei in different chemical environments experience different values of $B_0$ due a phenomenon termed chemical shielding, which is mediated by the electrons in a molecule. Chemical shielding is influenced by a number of factors including the inductive effects of electromagnetic groups, the magnetic anisotropy of $\pi$-systems, and hydrogen bonding. Typical chemical shift values for common organic functional groups are presented in Figure 2-2.

![Figure 2-2 Typical $^1$H chemical shift values for some common organic functional groups.](image.png)
Additional spectral information is provided by the splitting pattern of the signals, which occurs due to the interaction between neighbouring groups. Splitting offers a detailed insight into the connectivity of atoms within a molecule. Active nuclei that are close to one another exert an influence on each other's effective magnetic field. If the neighbouring active nuclei are non-equivalent, the effect is observable in the resultant NMR spectrum. This is termed spin-spin coupling or $J$-coupling and is mediated by the electrons in the bonds that link the coupling nuclei. The complexity of the splitting pattern is directly related to the number of neighbouring nuclei. The multiplicity of splitting follows the general rule of $n + 1$, determined by the number of protons ($n$) on the adjacent atoms. For example, a nucleus with two neighbouring chemically equivalent protons gives a resonance signal with a triplet form. The distance between two peaks in a multiplet is termed the $J$-constant, and is independent of the applied magnetic field (i.e. not field strength dependent). This is an example of “First-Order” splitting, and assumes that the spin-coupled nuclei have very different chemical shifts. If the chemical shift difference between the spin-coupled nuclei decreases, then the splitting pattern may be distorted. This is termed “Second-Order” splitting. Second-order effects decrease as the frequency difference between the multiplets increases, therefore NMR spectra display less distortion at higher frequency.

It is also possible to determine the number of $^1$H nuclei contributing to an individual signal (or peaks, in the case of a multiplet), as the signal area is directly proportional to the number of $^1$H nuclei responsible for that signal. Hence NMR is inherently quantitative. $^{153}$ Absolute and relative concentrations can be determined from the intensities of signals relative to the intensity of an internal
standard of known concentration, e.g. TSP, or to other endogenous signals, respectively, providing that all signals of interest are fully or equivalently relaxed.

2.1.2 One and Two Dimensional NMR Experiments

Throughout this study a number of one-dimensional (1D) and two-dimensional (2D) homo- and hetero-nuclear sequences were utilised. The main features of each are summarised below.

1D pulse programs were used extensively in this study. The simplest 1D pulse program consists of a radiofrequency pulse, followed by acquisition of the FID. The radio frequency pulse is repeated multiple times to improve the signal to noise ratio, and subsequent interpretation of the spectra. Throughout this study, 1D spectra were acquired using the first increment of a 2D Nuclear Overhauser Effect Spectroscopy (NOESY) pulse. This pulse sequence with solvent suppression has the form \([\text{-relaxation delay-90° pulse-3 μs delay-90° pulse-} t_m-90° \text{ pulse-acquire FID-}]\), where \(t_m\) is the mixing time. Water is present at high concentrations in biofluids (typically 110 M for \(^1\text{H NMR}\)), and results in an extremely large NMR signal that is likely to obscure other spectral information as analytes of interest are typically present at micromolar concentrations. Therefore, pre-saturation of the water resonance occurs during the relaxation delay of the NOESY sequence.

High molecular weight molecules typically display broader resonances than smaller ones as they possess shorter \(T_2\) spin relaxation times due to the limited rotational and translational motion of large molecules. The Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence enables \(T_2\)-spectral editing. The pulse sequence, \([\text{-relaxation delay-90° pulse-(t-180° pulse-} t\text{-acquire FID-}]\) where \(t\) is the spin echo delay and \(n\) represents the number of loops, reduces the broad
resonances from these macromolecules, facilitating the observation of low molecular weight compounds. This pulse sequence is widely utilised for the analysis of plasma samples, due to their high lipid and protein content.

2D NMR spectroscopic techniques provide useful information for structural elucidation, and have been employed throughout this work. The 2D $^1$H- $^1$H J-resolved experiment (or JRES) provides information on the multiplicity and coupling patterns of resonances. Further examples of 2D homonuclear pulse sequences include correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY), which provide spin-spin coupling connectivities. COSY identifies correlations between directly coupled protons, whilst TOCSY establishes correlations between protons that reside within the same spin system.

In addition to homonuclear 2D experiments, $^{13}$C can be used in heteronuclear correlation experiments such as $^1$H-$^{13}$C heteronuclear single quantum coherence spectroscopy (HSQC) and $^1$H-$^{13}$C heteronuclear multiple bond coherence spectroscopy (HMBC). HSQC identifies directly coupled nuclei while HMBC is used to establish long-range correlations, typically over two or three bonds, thereby providing a wealth of information on the molecular skeleton.

2.1.3 Solid State NMR – Magic Angle Spinning-NMR (MAS-NMR)

The development of high resolution $^1$H magic angle spinning (MAS) NMR enables the acquisition of high resolution NMR data from intact tissues with no pre-treatment. A small sample (typically 10 – 20 mg), is spun rapidly (typically at 5 kHz) at the magic angle ($\theta$) of 54.7° relative to the applied magnetic field. MAS-NMR eliminates many of the line broadening effects due to sample
heterogeneity, dipolar couplings, and chemical shift anisotropy, resulting in sharp, well-resolved spectral lines. All common pulse programs (as described above) can be used with MAS-NMR to study metabolic changes and assist in structural elucidation.

2.1.4 PROCESSING OF NMR SPECTRAL DATA

Prior to the application of suitable PR techniques, data must be pre-processed. The FID is Fourier transformed to convert the time domain signal to the frequency domain, producing the NMR spectrum. The resulting spectrum consists of a series of signals with different multiplicities and chemical shift values ($\delta$), reported relative to a reference value. In this work, deuterated 3-trimethylsilyl-[2,2,3,3-2H$_4$]-propionic acid sodium salt (TSP, $\delta = 0.0$) was used as a reference. Spectra are phased and subject to polynomial baseline correction. Finally, spectral regions that contain noise or offer no diagnostic value are removed. Typically this includes the TSP resonance ($\delta$ -0.2 to 0.2), water (typically from $\delta$ 4.5 to 5.0) and, where present, the urea resonance ($\delta$ 5.5 to 6.1).

Once NMR spectral data has been suitably processed and redundant regions have been removed, spectra must be aligned. A number of factors may potentially impact on chemical shift differences resulting in uncontrollable variation in peak positions between samples. These include instrumental imperfections, such as temperature variation and inhomogeneity in the applied magnetic field, and pH shifts arising from small differences in the amount of either the molecule itself or components of the surrounding matrix. Differences in ionic strength can also impact on peak position; this is especially relevant for the analysis of urine samples where there is likely to be considerable inter-subject variation in both sample dilution and content. Variation in peak position can result
in spurious groupings of samples in multivariate models, therefore a number of strategies exist to address the issue of variable peak position across multiple spectra. Throughout this work the recursive segment-wise peak alignment (RSPA) method was employed,\textsuperscript{174} to account for chemical shift variation between the samples.

Next, the data is normalised. Normalisation is a mathematical correction applied to experimental data to make all samples directly comparable with each other by correcting for variations in volume and concentration. Throughout this study, data was normalised using probabilistic quotient normalisation.\textsuperscript{175} Probabilistic quotient normalisation assumes that changes in the concentrations of single analytes only influence parts of the spectra, whilst changes in the overall concentration of a sample influence the complete spectrum. It has been demonstrated that probabilistic quotient normalisation is not hampered by extreme levels of metabolites, i.e. drug metabolites, and is sensitive to samples with low levels of metabolic variation.\textsuperscript{175} Other commonly applied techniques include normalisation to total area, whereby the total spectral area of each spectrum is set to unity and the intensities of all data points are expressed relative to this, and normalisation relative to a specific peak (such as TSP or creatinine).

Scaling is performed on the columns of data, i.e. on each spectral intensity across all samples.\textsuperscript{176} A number of scaling methods are commonly used. Data is first mean centred to improve interpretability. Mean-centring removes the offset from the data by calculating the average value of each variable, which is then subtracted from the data. This technique is particularly good for identifying large metabolite differences but it may mask discrete metabolite changes. Autoscaling, also called unit or unit variance scaling, is commonly applied to metabonomic data and was employed throughout this study. Each variable is divided by its
standard deviation so all variables have a standard deviation of one, and are 
equally important. A full review of pre-treatment methods has been published by 
van den Berg et al.\textsuperscript{176}
2.2 CHEMOMETRICS

Spectral data sets are complex and a number of PR techniques are used to reduce the dimensionality of complex data sets to allow inherent patterns to be rapidly visualised and enable the identification of discriminatory metabolites. 177 PR techniques are divided into ‘unsupervised’, such as Principal Component Analysis (PCA) and ‘supervised’ such as Partial Least Squares Discriminant Analysis (PLS-DA), PLS-Regression Analysis, Orthogonal Partial Least Squares Discriminant Analysis (O-PLS-DA) and O-PLS-Regression Analysis methods. Both methods were employed extensively in this work.

2.2.1 PRINCIPAL COMPONENT ANALYSIS (PCA)

PCA is a common ‘unsupervised’ technique used to establish if there is any structure in a data set related to the experimental design without the use of any a priori knowledge. PCA involves a mathematical procedure in which a number of variables, in this case spectral data points, are transformed into a smaller number of components called principal components or PC, which are a linear combination of variables. 178 The first PC (PC1) accounts for the direction in the multidimensional space explaining as much of the variance in the data as possible, with each subsequent orthogonal PC accounting for as much of the remaining variance as possible. The PCs are plotted to give a low dimensionality representation of the data set. The plot will show any inherent clustering due to the major sources of variation influencing the samples, e.g. the action of a drug or toxin, and the position of a sample is entirely independent of any knowledge of class membership, hence the model cannot be over fitted. The results of PCA are presented as scores (t) and loadings (p) plots. A scores plot allows visualisation
of the data in, typically two or three PC dimensions, and summarises the similarities and differences between the spectra, with each sample represented by a single data point; two widely spaced data points indicate a high degree of variation between the metabolic profiles. Figure 2-2 details how PCA scores are calculated. The corresponding loadings plot will describe the original variables importance to each PC.
Figure 2-3 PCA Scores (from reference Tsang)\textsuperscript{179}.

(a) Principal Component 1 (PC1) is the vector that best accounts for the variation in the data. (b) The second principal component (PC2) is orthogonal to PC1, to account for the next most amount of variation in the data. Each subsequent component is orthogonal to the previous component. (c) The observations are visualised in a lower dimensional plane, the scores plot. (d) Each observation (i) is projected onto the PC and a new coordinate value is obtained; \(t_{i1}\) is the distance of observation to PC1.\textsuperscript{179}

2.2.2 **Partial Least Squares (PLS)-Based Techniques**

Partial Least Squares (PLS) relates a matrix containing variables from samples (for example, spectral data, the X matrix), to a second matrix containing outcome variables (for example, toxicity scores, the Y matrix). PLS aims to
construct a model that ‘maximises the co-variation between the measured data (X), e.g. the spectral data, and the response variable (Y)’. PLS has been successfully used for multiple regression where Y contains continuous data. PLS can be combined with discriminant analysis, termed PLS-DA, to maximise the discrimination between the two classes by using a dummy Y matrix to enable the data to be modelled according to class, enabling sample classification. PLS-DA is considered to be a supervised pattern recognition technique because it uses prior information about classes to compute its components. PLS-DA can be extended with the use of built-in orthogonal signal correction (OSC) in what is termed orthogonal PLS-DA (O-PLS-DA). O-PLS-DA pre-filters classification irrelevant orthogonal variation from the data and can greatly improve the interpretation of spectral variation between classes. O-PLS-DA gives rise to differential regression coefficients. The differential regression coefficients corresponds to the plot of the covariance upon which the correlation is plotted with a colour code. The size and direction of the peak represents covariance and peaks are colour-coded according to their correlation coefficient value to indicate which peaks are most discriminatory. This plot is often called a differential metabogram in a metabolic profiling context.

2.2.3 Interpretation and Validation

With any multivariate regression model it is important to consider both its fit and its predictive ability. A number of parameters have been used throughout this work to assess the fit of each model and to prevent over-fitting. To test the validity of all discriminant models in this study, 7-fold cross-validation was used, whereby a seventh of the data was left out and then predicted back into the model, repeating the process until all the data have been excluded at least once. A
number of key parameters can also be calculated, including the cross-validated percent of Y variance explained by the model, i.e. the predictive ability of the model, \( Q^2_Y \), and the percent of X variance explained by the model \( R^2_X \). In addition, permutation tests can be used to test the robustness of the model: the Y vector is permutated, typically 1000 times, and the probability of the predictive \( Q^2_Y \) being obtained by chance is calculated.

2.2.4 **Statistical Total Correlation Spectroscopy (STOCSY)**

The development of statistical techniques such as Statistical Total Correlation Spectroscopy (STOCSY) has enhanced information recovery from complex data sets, such as NMR spectral data of biofluids obtained in metabonomic studies.\(^ {183}\) STOCSY utilises the co-linearity that exists between variables of differing intensities in a set of spectra to generate connectivities between resonances from molecules present at different concentrations in each sample. In contrast to traditional 2D spectroscopic techniques, STOCSY is not constrained by parameters such as lack of J couplings and extended distances. Additional information can be extracted from the data set by lowering the correlation coefficient and looking at additional correlations to highlight metabolic pathway linkages, between two or more molecules. The technique can be applied to the construction of virtual 2D heteronuclear correlation spectra to aid in metabolic assignment in what is termed heteronuclear-STOCSY (HET-STOCSY). HET-STOCSY has been successfully applied to correlate \(^1\)H and \(^{31}\)P MAS-NMR spectra obtained from intact liver tissue,\(^ {184}\) and to correlate \(^1\)H and \(^{19}\)F spectra from human urine samples.\(^ {185}\)
In addition to the traditional STOCSY approach, an extended version called STOCSY-editing or STOCSY-E can be used. STOCSY-E enhances information recovery from complex NMR data sets. By identifying strongly correlated resonances, for example drug metabolite resonances, these resonances can be subtracted from the original data set, enabling the construction of a new matrix comprising endogenous metabolites only. This has been found to improve statistical analysis and ultimately enhance the detection of endogenous biomarkers, as the presence of drug metabolites can skew the models being constructed. 186

2.2.5 Metabolite Assignment

Throughout this work, metabolites were assigned using a combination of methods. Those metabolites most commonly encountered in biological samples were assigned using literature values. Drug-associated resonances were assigned, wherever possible, using standard compounds. In the case of INH, a number of drug-related resonances were observed that have never previously been assigned using NMR. The identification and assignment of these resonances formed a significant part of the INH study. For other metabolites, 2D NMR was employed to obtain structural information based on cross-peaks and splitting patterns. STOCSY was also employed to identify structural information, as well as identification of related metabolites. This information was then used to enable a targeted search of metabolite databases. Once potential metabolites had been identified, final assignment was then made where possible through the use of 'spike-in' experiments.
Chapter 3  ISONIAZID

AIMS AND OBJECTIVES

- Assessment of the response to a single dose of isoniazid (INH) using traditional clinical chemistry and histopathological assessments of toxicity, alongside complementary high resolution nuclear magnetic resonance spectroscopic methods.
- Identification and quantification of urinary metabolites of INH in the rat model using high resolution nuclear magnetic resonance spectroscopic methods.
- Exploration of inter-individual variation in response to INH administration through characterisation of the endogenous and xenobiotic metabolic profile.
- Construction of pre-dose classification models of INH-induced adverse CNS effects – application of pharmacometabonomics.
3.1 METHODS

3.1.1 ANIMAL HANDLING AND SAMPLE COLLECTION

All animal manipulations were conducted by Pfizer Global R&D, Amboise France, in accordance with the relevant national requirements and local guidelines. Male Sprague-Dawley (SD) rats (7 weeks of age, approximately 250g, \( n=30 \), from Charles River, France) were allocated randomly to three dose groups (\( n=10 \) per group) and administered via intra-peritoneal (i.p.) injection, a single dose of vehicle (0.9% saline, Control) or INH in vehicle at 200 mg/kg (Low Dose) or 400 mg/kg (High Dose) (dose volume 10 mL/kg). Animals were housed in individual metabolism cages with free access to water and a standardised diet (diet A04C, Usine d’Alimentation Rationnelle, Villemoisson-sur-Orge, France). Temperature (20 ± 2 °C) and relative humidity (60 ± 20%) were maintained, with a 12 hour light/dark cycle throughout the study. The planned protocol was that half of the animals from each group were euthanized via CO\(_2\) anaesthesia at 48 h post-treatment, and the remainder at 168 h post-treatment for removal of target organs for histopathological examination.

Urine samples were collected into ice-cooled vessels containing 0.1 mL sodium azide (100 mg/mL) over the following time periods: -48 to -41 h; -24 to -17 h; 0 to 7 h; 7 to 24 h; 24 to 31 h; 48 to 55 h; 72 to 79 h; 96 to 103 h; 120 to 127 h; 144 to 151 h. All urine samples were centrifuged at 9447 g for 10 minutes at room temperature to remove particulate matter, divided into aliquots and stored at -20°C. Blood was sampled prior to animals being placed in the metabolic cages, and prior to planned termination, i.e. at 24 and 168 h post-dose, into commercially available plastic tubes containing lithium heparin as an anti-coagulant, from which
plasma samples were isolated by centrifugation. Clinical observations were carried out once a day over the period -48 to 0 hours, and 48 to 168 hours, and twice daily over the period 0 to 48 hours. Four animals in the high dose group displayed clinical signs of adverse CNS effects at approximately 2 hours post-treatment and were euthanized. Post-dose urine was collected from 3 of these 4 individuals that developed an adverse response, whilst post-dose plasma samples were not collected from these individuals.

3.1.2 CLINICAL CHEMISTRY AND HISTOPATHOLOGY

Urine parameters (total volume, pH, bilirubin, ketone substances, glucose, haemoglobin and protein concentration) were recorded using an automatic test-strip reader (Clinitek 200, Bayer Diagnostics). Plasma was analysed for urea, creatinine, glucose, cholesterol, triglycerides, albumin, total protein, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), 5'-nucleotidase (5'-NU), γ-glutamyl transferase (GGT) and bile acids using an AU600 multiparametric clinical analyser (Olympus) following the standard operating procedures of the laboratory. Significant changes from control levels were determined using a Student’s two-tailed t-test (with a significance threshold of \( p < 0.05 \)). Tissue from the left lobe of the liver, kidney and brain were sampled at termination (scheduled at 48 hours and 168 hours post-treatment; the CNS responders were sampled within 2 hours of dosing as they were found dead or sacrificed as moribund) and fixed in a 10% formalin solution. Representative samples were processed routinely in an automatic tissue processor, embedded in paraffin, sectioned at 4-6 μm, stained with haematoxylin and eosin (H&E), and examined histologically using light microscopy. Periodic acid Schiff staining was performed on additional samples of the left liver lobe to
further characterise hepatic glycogen content. All clinical chemistry and histopathological toxicity assessments were carried out by Pfizer Global R&D, Amboise France.

3.1.3 NMR SPECTROSCOPIC ANALYSIS OF URINE SAMPLES

Urine samples were thawed, vortexed, and allowed to stand for 10 minutes at room temperature prior to mixing (400 μL) with phosphate buffer (200 μL, 0.2 M in 90:10 H₂O/D₂O, pH 7.4, containing 3 mM 3-trimethylsilyl-[2,2,3,3,3H₄]-propionic acid sodium salt (TSP, Sigma Aldrich) and 3 mM sodium azide (Sigma Aldrich)). The urine-buffer mixtures were centrifuged at 9447 g for 10 minutes at room temperature. Supernatants (550 μL) were transferred to 5 mm NMR tubes (Norell Standard Series, HP507) for NMR analysis. The D₂O provided a field frequency lock and TSP a chemical shift reference (¹H, δ 0.0).

One-dimensional (1D) ¹H NMR spectra were acquired on a Bruker Avance DRX 600 MHz Spectrometer (Bruker BioSpin, Rheinstetten, Germany), operating at 14.1 T (600.13 MHz ¹H frequency) and at a temperature of 300 K, using a standard 1D solvent suppression pulse sequence of the form [relaxation delay-90° pulse-3 μs delay-90° pulse-mixing time-90° pulse-acquire FID].¹⁶⁵ For each sample, 196 transients were collected into 64 K data points, using a sweep width of 12019.2 Hz, with a relaxation delay of 4 s, an acquisition time of 2.72 s and a mixing time of 100 ms. Pre-saturation of the water signal was applied during the relaxation delay and mixing time. A line-broadening factor of 0.3 Hz was applied to all spectra prior to Fourier transformation (FT).
3.1.4 Data Processing

The $^1$H NMR spectra were manually phased, baseline corrected and referenced to TSP ($\delta$ 0.0). Full resolution $^1$H NMR spectra were imported into MATLAB (version 7.6, The MathWorks, Natick, USA) using MetaSpectra 4.1.1 (an in-house routine written by Dr O. Cloarec). The regions corresponding to water ($\delta$ 4.70 to 4.85), TSP ($\delta$ -0.20 to 0.20), and urea ($\delta$ 5.50 to 6.10) were removed. The spectra were aligned using the recursive segment-wise peak alignment (RSPA) method to account for any chemical shift variations induced by differences in pH between samples. The urinary spectral regions containing drug-related resonances ($\delta$ 2.14 to 2.24, 2.37 to 2.39, 2.47 to 2.52, 2.78 to 2.92, 4.24 to 4.29, 7.67 to 7.95, and 8.60 to 8.82) were separated from the remaining spectral regions to form a xenobiotic spectral data set (Figure 3-1a). The remaining urinary spectral regions represented an endogenous metabolic data set (Figure 3-1a). The xenobiotic and endogenous metabolic spectral data sets were modelled separately to reflect the metabolic fate of INH and its endogenous consequences. This approach is summarised in Figure 3-1.
Figure 3-1 A scheme summarising the approach of separating the $^1$H NMR spectral profiles of rat urine into both endogenous and xenobiotic metabolic profiles (From Cunningham et al. $^{187}$).

These metabotypes are treated separately for subsequent normalisation and multivariate statistical modelling.
Both data sets were normalised separately using probabilistic quotient normalisation \(^{175}\) to partially correct for variations in urinary volume and concentration. Multivariate statistical analysis (PCA, PLS-DA and O-PLS-DA) was carried out in MATLAB. \(^{178,180,181}\) PCA models were computed using scaling to unit variance for the separate endogenous and xenobiotic metabolic profiles (Figure 3-1b). Metabolic trajectory plots were calculated using the mean PCA score (PC1 and PC2) values of the control, low and high dose urinary spectra at each time point, for each data set separately (Figure 3-1b). Relative concentrations of key endogenous and xenobiotic metabolites, as identified as discriminatory from loadings plots, were calculated by integration of their NMR spectral resonances, with integrals corrected for the number of protons giving rise to a resonance. Pair-wise O-PLS-DA models were computed between the control and sub-categories of high dose animals (i.e. CNS responders and CNS non-responders) to identify the specific metabolic impact of INH administration in responders and non-responders, where controls were classed as group 1 and treated samples were classed as group 0. To test the validity of all O-PLS-DA models, 7-fold cross-validation was used, whereby a seventh of the data was left out of the model and then predicted back into the model, repeating the process until all of the data have been excluded at least once to calculate the Q\(^2\)Y value (a measure of the cross-validated predictive ability). A permutation test was also performed to test the robustness of the models: the null hypothesis was simulated by 1000 random permutations of the Y vector. The permutation \(p\)-value was then obtained by ranking the actual Q\(^2\)Y within the permuted Q\(^2\)Y calculated by the simulation.
3.1.5 ASSIGNMENT OF INH METABOLITES

The NMR resonance of INH-related metabolites were assigned using 1D and 2D NMR spectra of standard compounds and incubations of standards. 5 mM solutions of INH (Sigma Aldrich), isonicotinic acid (INA; Sigma Aldrich) acetylhydrazine (AcHz; Sigma Aldrich), acetyllisoniazid (AcINH; synthesised by Pfizer Global R & D), and diacetylhydrazine (DiAcHz; Lancaster Synthesis), were prepared in 0.2 M phosphate buffer (as above) and analysed by 1D $^1$H NMR using the standard 1D $^1$H NMR solvent suppression sequence described above. Typically, 128 transients were collected into 64 K data points using a sweep width of 12019.2 Hz, and a relaxation delay of 2 s. A line-broadening factor of 0.3 Hz was applied to all spectra prior to FT.

Additional solutions of pyruvate (Sigma Aldrich), 2-oxoglutarate (Sigma Aldrich), pyridoxal (Sigma Aldrich), pyridoxal-5-phosphate (Sigma Aldrich), glycine (Sigma Aldrich) and glucose (Sigma Aldrich), were prepared. 24 hour incubations at 37°C of INH (500 µL, 5 mM in 0.2 M phosphate buffer, as above) with pyridoxal, pyridoxal-5-phosphate, pyruvate, glycine, glucose, and 2-oxoglutarate (all 500 µL, 5 mM in 0.2 M phosphate buffer, as above) were carried out, and all reaction products (5 mm NMR tube, 600 µL volume) analysed using the standard 1D $^1$H NMR solvent suppression sequence described above. $^1$H Glucosyl isonicotinylhydrazide (INH-GLC) was prepared as described previously by heating equivalent amounts of INH and glucose (both 5 mM solutions in methanol, 5 mL of each) at 90°C degrees for approximately 2 hours, at which point the methanol has evaporated off. The product was reconstituted in phosphate buffer and characterised by 1D $^1$H NMR as described above. Additional time-course incubation reactions were carried out to follow the
formation of INH hydrazone and hydrazide metabolites: an initial spectrum of INH (500 µL, 5 mM) in urine and/or buffer was acquired, the second compound (100 µL, 5 mM in 0.2 M phosphate buffer, as above) added, and a series of spectra acquired over a period of approximately 6 hours (acquisition parameters as above; incubation time subject to variation). Visual comparisons and ‘spike-in’ experiments were carried out to identify and confirm the presence of reaction products in the post-treatment urinary spectra.

Additional 2D homonuclear and heteronuclear spectra were acquired as necessary to confirm peak assignment, using an NMR spectrometer, operating at 14.1 T (600.13 MHz $^1$H frequency) and a temperature of 300 K. For $^1$H-$^1$H correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) experiments, 64 scans (8 dummy scans) per increment were collected into 256 increments at a spectral resolution of 8 K. 2D $^1$H J-resolved (JRES) spectra were acquired using the standard pulse sequence. Typically, 128 transients (8 dummy scans) per increment were acquired into 128 increments at a resolution of 64 K. The sweep width was 12019.2 Hz in F2 and 50 Hz in F1 and water pre-saturation was employed during a relaxation delay of 4 s. Spectra were processed using a SINE window function along the F1 and F2 axes, with SSB = 0 and a resolution of 64 K (in F2) and 512 (F1) data points. The peaks were subsequently tilted by 45$^\circ$ and symmetrised.

Two dimensional heteronuclear multiple bond correlation (HMBC) $^1$H-$^{13}$C spectra were acquired with 512 scans (32 dummy scans) collected into 152 transients at a spectral resolution of 8 K in F2 across a sweep width of 6203.5 Hz and 33523.1 Hz for the $^1$H and $^{12}$C axes respectively, with an acquisition time of 0.568 s and a relaxation delay of 1.5 s. Heteronuclear single quantum coherence
(HSQC) $^{170}$ $^1$H- $^{13}$C echo-anti-echo spectra were acquired using 512 transients (and 32 dummy scans), collected into 8 K data points per increment for 128 increments. A sweep width of 7211.5 Hz in the F1 dimension ($^{13}$C) and 30187.8 Hz in the F2 dimension ($^1$H) were used with a relaxation time of 0.284 s and an acquisition time of 1.5 s.

3.1.6 IDENTIFICATION OF PRE-DOSE METABOLITES OF TOXICITY

O-PLS-based models were constructed to investigate the statistical relationship between the pre-dose NMR spectral data (X) and selected post-dose response variables, i.e. the development of INH-induced adverse CNS effects (Y, with those animals exhibiting overt signs of CNS disturbances classed as group 1; all other animals were classed as group 0), and the ratio of AcINH/pyruvate isonicotinyl hydrazone (INH-PA; Y). The statistical validity and robustness of each model was assessed using 7-fold cross validation and permutation test (details as above).

A series of 2D homo- and heteronuclear NMR spectroscopic techniques were applied to enable the structural elucidation of the discriminatory metabolites highlighted in these models. COSY, $^{168}$ TOSCY, $^{169}$ JRES, $^{167}$ HSQC $^{171}$ and HMBC $^{170}$ spectra were acquired for the pooled pre-dose urine samples, using the acquisition parameters detailed above.
3.2 RESULTS

3.2.1 IDENTIFICATION OF URINARY INH METABOLITES AND ASSIGNMENT OF THEIR NMR SPECTRA

NMR spectroscopic profiling enabled simultaneous characterisation of urinary INH-related metabolites and endogenous INH-induced metabolic changes. A representative 600 MHz $^1$H NMR spectrum of urine collected 0-7 hours after administration of 400 mg/kg INH is shown in Figure 3-2 to highlight the simultaneous assignment of diverse urinary INH-derived metabolites and endogenous metabolites from many chemical classes.
Figure 3-2 Representative 600 MHz 1D $^1$H NMR spectrum of rat urine collected 0-7 hours after administration of 400 mg/kg of isoniazid.

Two areas have been expanded to show the regions of the spectrum containing NMR spectral resonances due to INH-derived metabolites.

Key: DMA – dimethylamine; DMG – dimethylglycine; TMAO – trimethylamine-N-oxide.

The use of authentic compounds enabled the assignment of resonances due to INH, AcINH (the acetylated metabolite of INH), and INA (derived from hydrolysis of INH and AcINH). Further INH-related metabolites were identified through the use of incubation reactions. INH undergoes non-enzymatic reactions with the endogenous keto-acids, pyruvate and 2-oxoglutarate, to form INH-PA and 2-oxoglutarate isonicotinylhydrazone (INH-KA), respectively. Spectroscopic characterisation of the incubation solution of INH with pyruvate (Figure 3-3), and
of INH with 2-oxoglutarate (Figure 3-4) led to the identification of the syn and anti isomers of these metabolites. Both forms were detectable in the post-dose urine.

Figure 3-3 Formation of pyruvate isonicotinylhydrazone (INH-PA) via reaction of INH with pyruvate over time in phosphate buffer at 37°C.

Standard spectra of the parent compounds (INH and pyruvate) are shown, along with the progression of INH-PA formation at approximately 15 and 135 minutes post incubation. Key: INH – isoniazid; INH-PA – pyruvate isonicotinylhydrazone. A and B refer to the two forms of INH-PA (i.e. syn and anti forms).
Figure 3-4 Formation of 2-oxoglutarate isonicotinylhydrazone (INH-KA) via reaction of INH with 2-oxoglutarate over time in phosphate buffer at 37°C.

Standard spectra of the parent compounds (INH and 2-oxoglutarate) are shown, along with the progression of INH-KA formation at approximately 65 and 210 minutes post incubation.

Key: 2-OG – 2-oxoglutarate; INH – isoniazid; INH-KA – 2-oxoglutarate isonicotinylhydrazone. A and B refer to the two forms of INH-KA (i.e. syn and anti forms).

Variable-temperature NMR experiments revealed the coalescence of the resonances from the syn and anti isomers of INH-PA and INH-KA with increasing temperature, indicative of rapid exchange between the isomers (Figure 3-5).
Figure 3-5 Effect of temperature on $^1$H NMR spectra.

(a) 600 MHz 1H NMR spectra of standard solution of INH-KA showing the region $\delta$ 8.85 to 8.62. (b) 600 MHz $^1$H NMR spectra of urine collected 0-7 hours after administration of INH at 400 mg/kg showing the region of $\delta$ 2.13 to 2.24.

Spectra were acquired at 290 K, 300 K, 310 K and 330 K.

Key: AcINH – acetylisoniazid; INH – isoniazid; INH-KA – 2-oxoglutarate isonicotinylhydrazone; INH-PA – pyruvate isonicotinylhydrazone.

The ratio of the syn and anti isomers of both INH-PA and INH-KA was approximately 2:1, as determined from the relative ratio of the integral for the respective NMR resonances in the post-dose urinary spectra, where A referred to the major form and B to the minor form of the compound.

1D and 2D spectroscopic analysis of the reaction products of INH and glucose enabled characterisation of the corresponding urinary metabolites as INH-GLC (Figure 3-6). These resonances were consistent with literature values for INH-GLC. It was not possible to measure accurate $J$-coupling constants for all the protons due to a significant degree of peak overlap.
Figure 3-6 Formation of $\beta$-glucosyl isonicotinylhydrazide (INH-GLC) via reaction of INH with glucose over time in methanol.

Standard spectra of the parent compounds (INH and glucose) are shown, along with the results of the reaction between these compounds at 90°C for 120 minutes.

Key: GLC – glucose; INH – isoniazid; INH-GLC – $\beta$-glucosyl isonicotinylhydrazide.

Spectroscopic analysis of the products of the incubation of INH with glycine detected resonances assigned to isonicotinylglycine (INA-GLY, Figure 3-7), which were also observed in the 0-7 hour post-dose urinary spectra.
Characterisation of the products from the reaction of pyridoxal and pyridoxal-5-phosphate with INH indicated that the isoniazidyl-pyridoxal complex (Schiff base) was not detected in the post-dose urine by NMR spectroscopy; this was consistent with earlier reports on the urinary metabolites of INH in a rat model. As a consequence of the above analysis, INH-related metabolites were assigned as shown in Figure 3-8. Full spectral assignment details for INH and its related metabolites are provided in Table 3-1.
Figure 3-8 Representative 600 MHz $^1$H NMR spectrum of rat urine collected 0-7 hours after administration of 400 mg/kg isoniazid, showing assignment of endogenous and exogenous metabolites.

Full details of INH-related metabolites are given in Table 3-1.

Table 3-1 Full assignment of INH and related metabolites.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$^1$H shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (INH)</td>
<td>8.685 (d, 2H, H2 + H6, $J = 6.2$ Hz)</td>
</tr>
<tr>
<td></td>
<td>7.705 (d, 2H, H3 + H5, $J = 6.2$ Hz)</td>
</tr>
<tr>
<td>Isonicotinic acid (INA)</td>
<td>8.619 (d, 2H, H2 + H6, $J = 6.1$ Hz)</td>
</tr>
<tr>
<td></td>
<td>7.748 (d, 2H, H3 + H5, $J = 6.1$ Hz)</td>
</tr>
<tr>
<td>Acetylisoniazid (AcINH)</td>
<td>8.733 (d, 2H, H2 + H6, $J = 6.3$ Hz)</td>
</tr>
<tr>
<td></td>
<td>7.812 (d, 2H, H3 + H5, $J = 6.3$ Hz)</td>
</tr>
<tr>
<td></td>
<td>2.150 (s, COCH$_3$)</td>
</tr>
<tr>
<td>Pyruvate isonicotinylhydrazone A (INH-PA A)</td>
<td>8.751 (d, 2H, H2 + H6, $J = 6.2$ Hz)</td>
</tr>
<tr>
<td></td>
<td>7.803 (d, 2H, H3 + H5, $J = 6.2$ Hz)</td>
</tr>
<tr>
<td></td>
<td>2.194 (s, CH$_3$)</td>
</tr>
<tr>
<td>Pyruvate isonicotinylhydrazone B (INH-PA B)</td>
<td>8.741 (d, 2H, H2 + H6, $J = 6.1$ Hz)</td>
</tr>
<tr>
<td></td>
<td>7.813 (d, 2H, H3 + H5, $J = 6.1$ Hz)</td>
</tr>
<tr>
<td></td>
<td>2.220 (s, CH$_3$)</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>8.751 (d, 2H, H2 + H6, $J = 5.8$ Hz)</td>
</tr>
<tr>
<td>isonicotinylhydrazone A (INH-KA A)</td>
<td>7.804 (d, 2H, H3 + H5, $J = 5.8$ Hz)</td>
</tr>
<tr>
<td></td>
<td>2.813 (t, 2H, CH$_2$, $J = 7.6$ Hz)</td>
</tr>
<tr>
<td></td>
<td>2.494 (t, 2H, CH$_2$, $J = 7.6$ Hz)</td>
</tr>
</tbody>
</table>
2-Oxoglutarate isonicotinylhydrazone B (INH-KA B)  
8.762 (d, 2H, H2 + H6, J = 5.8 Hz)  
7.908 (d, 2H, H3 + H5, J = 5.8 Hz)  
2.887 (t, 2H, J = 6.2 Hz)  
2.559 (t, 2H, J = 6.2 Hz)

β-Glucosyl isonicotinylhydrazide (INH-GLC)  
8.693 (d, 2H, H2 + H6, J = 6.0 Hz)  
7.723 (d, 2H, H3 + H5, J = 6.0 Hz)  
4.263 (d, 1H, H1’, J = 8.9 Hz)  
3.409 (dd, 1H, H2’)  
3.575 (t, 1H, H3’)  
3.467 (dd, 1H, H4’)  
3.744 (dd, 1H, H5’)  
3.925 (dd, 2H, H6’)  
Accurate J-coupling constants were not available for H2’ – H6’ due to peak overlap.

Isonicotinylglycine (INA-GLY)  
8.689 (d, 2H, H2 + H6, J = 6.1 Hz)  
7.791 (d, 2H, H3 + H5, J = 6.1 Hz)  
3.983 (s, 2H, CH2)

Acetylhydrazine (AcHz)  
1.96 (s, COCH3)

Diacetylhydrazine (DiAcHz)  
2.07 (s, COCH3)

Key: A/B refer to the syn/anti isomers of INH-PA and INH-KA, although the structures shown are for illustrative purposes only. It has not been possible at present to assign syn/anti isomers. AcHz and DiAcHz were not detected in post-dose urine.
3.2.2 **INTER INDIVIDUAL VARIATION IN RESPONSE TO INH EXPOSURE**

Clinical observations of adverse CNS effects following administration of the high dose of INH enabled classification of "CNS responders" (4 of 10 rats). The remainder of the high dose cohort displayed no clinical signs of INH-induced CNS effects (6 of 10 rats) and were therefore classified as "CNS non-responders". Statistically significant reductions in the plasma activity of AST and ALT were reported at 24 hours post-dose in the high dose CNS non-responders (and the low dose cohort), relative to control activity (Figure 3-9). No clinical chemistry data was available for the high dose CNS responders. Histopathological assessment of the liver revealed hepatocellular glycogen depletion in 3 of the 4 CNS responders (representing a sampling point within 2 hours post-dosing) and in one CNS non-responder (later classified as CNS non-responder B; see below for further details). There was no histopathological evidence to suggest the presence of a hepatic necrotic lesion in the CNS non-responders at 48 hours or 168 hours post-treatment. In addition, histopathological assessment of brain and kidney tissues revealed no microscopic abnormalities.
INH-induced changes in plasma levels of clinical chemistry parameters.

A significant change in ALT levels relative to control was observed at (a) 24 hours and (b) 168 hours post-dose. A significant change in AST levels relative to control was observed at (c) 24 hours post-dose. Values are expressed as mean ± S.D. Each point represents an individual animal. Individual values are identified with black dots. The work was conducted by Dr Claude Charuel and the technical staff at Pfizer Global R&D, Amboise France.

Key: Control (n=5; ); low dose (200 mg/kg, n=5; ); high dose CNS non-responders (400 mg/kg, n=3; ); ALT – alanine aminotransferase; AST – aspartate aminotransferase. (*) $p < 0.05$. (***) $p < 0.001$.

The variability in response to INH and the existence of sub-populations within the high dose group was characterised via PCA analysis of post-dose urinary NMR spectra following separation of the NMR spectral profiles into distinct
endogenous and xenobiotic metabolic data sets as illustrated by the scheme in Figure 3-1. PCA analysis of the $^1$H NMR spectra of urine from both low dose and high dose animals, and the computation of a metabolic trajectory from the mean PC1 and PC2 score values enabled visualisation of INH-induced perturbations in the endogenous (Figure 3-10a) and xenobiotic (Figure 3-10b) data sets across time (from 24 hours pre-dose to 55 hours post-dose). The low dose endogenous metabolic trajectory showed clear deviation from the control endogenous metabolic space over the collection period 0-7 hours post-dose, followed by a return towards the control endogenous space over the collection period 7-31 hours post-dose (Figure 3-10a). The endogenous metabolic trajectory was indicative of INH-induced homeostatic perturbation, which was most marked over the period 0-7 hours post-dose, followed by recovery of metabolic homeostasis by 31 hours post-dose. In comparison, the movement observed in the low dose xenobiotic trajectory reflected the presence of INH and its metabolites at 0-7 and 7-24 hours post-treatment, followed by complete clearance of INH-related metabolites from 24 hours onwards (Figure 3-9b).

The high dose CNS non-responders exhibited a similar trajectory to the low dose animals but experienced a greater displacement from the control endogenous metabolic space at 0-7 hours post-dose with recovery over the period 24-31 h post-dose (Figure 3-10a). The PCA trajectory representing the xenobiotic metabolic profile for the high dose CNS non-responders exhibited a more prolonged perturbation relative to the low dose cohort (Figure 3-10b).

The endogenous and xenobiotic metabolic trajectories for the high dose CNS responders exhibited greater displacement compared to the high dose CNS non-responders (Figure 3-10a and -10b)
Figure 3-10 PCA analysis of $^1$H NMR spectra.

PC1 vs PC2 scores trajectory plot showing the mean points of the individual PC scores for control, low and high dose urinary NMR spectra across the time course from 24 hours pre-dose to 55 hours post-dose for (a) endogenous metabolic data set and (b) xenobiotic metabolic data set. The high dose animals were sub-divided according to the severity of the observed CNS response and classified as CNS responders and CNS non-responders. The numbers refer to the sampling time. The error bars indicate the standard deviation.

Key: (■) – control; (■) – low dose; (▲) – high dose CNS non-responders; (▲) – high dose CNS responders.
The differential metabotypes of the high dose CNS responders and non-responders were further explored through PCA analysis of the endogenous and xenobiotic data sets at the 0-7 hours post-treatment time-point. The PC1 vs PC2 scores plot from PCA analysis of the 0-7 hours post-dose urinary xenobiotic metabolic data set showed separation of the high dose CNS responders and non-responders along PC1 (Figure 3-11a). The corresponding PCA loadings plot indicated that elevated levels of INH-PA and INH-GLC, coupled with low levels of AcINH were seen in the urinary spectra of CNS responders in comparison to CNS non-responders (Figure 3-11b). One animal displayed no clinical signs of toxicity, i.e. was classified as a CNS non-responder, but was clustered with the metabolic profiles of the responders on the PCA scores plot (non-responder B). The urinary spectrum from this animal showed high post-dose levels of INH-PA and INH-GLC, but no significant reduction in urinary levels of AcINH as was seen in the remaining animals classified as CNS responders, which partially explains its anomalous position in the PCA scores plot.
PC1 vs PC2 scores plot derived from urinary spectra collected 0-7 hours after administration of high dose INH (400 mg/kg) for drug-related resonances only. (a) PC1 vs PC2 scores plot. (b) PCA loadings plot corresponding to the first principal component for this model. Individuals have been colour-coded on the basis of response to INH.

Key: (■) – CNS non-responders; (■) – CNS responders; (■) – CNS non-responder B*. AcINH – acetylisoniazid; INA – isonicotinic acid; INH – isoniazid; INH-GLC – β-glucosyl isonicotinylhydrazide; INH-KA – 2-oxoglutarate isonicotinylhydrazone; INH-PA – pyruvate isonicotinylhydrazone.

(* This individual exhibited no overt clinical signs of an adverse CNS effect but shared some of the metabolic features of the CNS responders).

To further characterise the CNS responder and CNS non-responder metabolic phenotypes with respect to the metabolic fate of INH, integrals of the INH metabolite spectral resonances were calculated from the ¹H NMR post-dose urinary spectra (Table 7-1, Appendix I). A number of peak ratios were calculated to express the relative urinary excretion of the key INH metabolites (Table 3-2). CNS non-responder B was excluded from the CNS non-responder group and considered separately. Within the high dose group, the CNS responders excreted significantly lower ratios of AcINH relative to the parent INH (a ratio of 0.37 compared to 0.70, \( p < 0.05 \) ) over the period 0-7 hours post-dose, compared to the
CNS non-responders. The post-dose ratio of INH-PA to AcINH was significantly elevated in the CNS responders relative to the CNS non-responders (a ratio of 3.26 compared to 0.65 respectively, $p < 0.01$). In addition, the post-dose ratio of INH-GLC to AcINH was significantly elevated in the CNS responders relative to the CNS non-responders (a ratio of 5.75 compared to 0.31 respectively, $p <0.05$). The increase in the ratio of INH-GLC to AcINH ($p < 0.05$) in the CNS responders was less statistically significant than that of INH-PA to AcINH ($p < 0.01$), due to the high degree of inter-individual variation in urinary levels of INH-GLC. Therefore, the ratio of INH-PA to AcINH was considered a more robust marker of the responder phenotype and the development of an adverse CNS effect. Full integral values for INH and its metabolites for both low and high dose cohorts at 0-7 hours and 7-24 hours post-dose are presented in Appendix I, Table 7-1. It was notable that the urinary levels of AcINH over the period 0-7 hours post-dose were comparable in the low dose cohort (200 mg/kg) and high dose CNS non-responders (400 mg/kg).
Table 3-2 Quantification of INH-related metabolites.

<table>
<thead>
<tr>
<th></th>
<th>AcINH:INH</th>
<th>INH-PA:INH</th>
<th>INH-GLC:INH</th>
<th>INH-PA:AcINH</th>
<th>INH-GLC:AcINH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low dose</td>
<td>0.97 ± 0.74</td>
<td>0.57 ± 0.22</td>
<td>0.31 ± 0.22</td>
<td>0.80 ± 0.35</td>
<td>0.36 ± 0.10</td>
</tr>
<tr>
<td>High dose CNS non-responders</td>
<td>0.70 ± 0.11</td>
<td>0.45 ± 0.10</td>
<td>0.22 ± 0.05</td>
<td>0.65 ± 0.15</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>High dose CNS responders</td>
<td>0.37 ± 0.13 *</td>
<td>1.21 ± 0.44</td>
<td>2.25 ± 1.17</td>
<td>3.26 ± 0.41 **</td>
<td>5.75 ± 1.53 *</td>
</tr>
<tr>
<td>High dose CNS non-responder B</td>
<td>0.55</td>
<td>1.18</td>
<td>1.56</td>
<td>2.15</td>
<td>2.85</td>
</tr>
</tbody>
</table>

Ratios of AcINH, INH-PA and INH-GLC to INH, and of INH-PA and INH-GLC to AcINH present in the spectra of urine collected 0-7 hours after administration of INH at 400 mg/kg. Ratios were calculated for individual animals and the mean ± standard deviation calculated for each group. Significant changes in the ratios observed in the high dose CNS responders relative to CNS non-responders were determined using a Student’s two-tailed t-test. Full integral values are presented in Appendix I Table 7-1.

Key: AcINH – acetylisoniazid; INA – isonicotinic acid; INH – isoniazid; INH-GLC – β-glucosyl isonicotinylhydrazide; INH-KA – 2-oxoglutarate isonicotinylhydrazone; INH-PA – pyruvate isonicotinylhydrazone. (*) p < 0.05. (**) p < 0.01.

The endogenous metabolic phenotype reflective of differential CNS response to INH was assessed through PCA analysis of the endogenous metabolic data set for CNS responders and CNS non-responders at 0-7 hours post-treatment (Figure 3-12). A PCA scores plot of PC1 vs PC2 revealed the separation of CNS responders and CNS non-responders along PC1 (Figure 3-12a) with significant inter-individual variation in INH-induced endogenous metabolic response between the CNS responders. The corresponding loadings plot indicated the presence of elevated levels of glucose and lactate, coupled with low levels of creatinine in the urine of CNS responders relative to CNS non-responders (Figure 3-12b). CNS non-responder B, the individual that displayed a
unique INH metabolite profile i.e. elevated urinary levels of INH-PA and INH-GLC, compared to the remaining non-responders, was positioned between the CNS non-responder cluster and the CNS responders on the basis of the PCA scores plot of the endogenous data set. The corresponding loadings plot showed that this individual had elevated post-dose urinary levels of glucose relative to the CNS non-responders, but no increase in urinary levels of lactate was observed.

Figure 3-12 PCA analysis of $^1$H NMR spectra.

PC1 vs PC2 scores plot derived from urinary spectra collected 0-7 hours after administration of high dose INH (400 mg/kg) for endogenous resonances only. (a) PC1 vs PC2 scores plot. (b) PCA loadings plot corresponding to the first principal component for this model. The metabolites responsible for separation were assigned from standard spectra and literature values. Individuals have been colour-coded on the basis of response to INH.

Key: (■) – CNS non-responders; (□) – CNS responders; (▲) – CNS non-responder B*; CRN – creatinine.

(* This individual exhibited no overt clinical signs of an adverse CNS effect but shared some of the metabolic features of the CNS responders).
To provide an interpretation of the urinary metabolic signature changes reflective of non-toxic exposure to INH, the supervised discriminant analysis technique O-PLS-DA was employed to maximise the discrimination between the control and treated groups. Pair-wise models were computed for the high dose CNS non-responders versus the control animals at 0-7 hours post-dose, and for the CNS responders versus the control animals at the same collection point, for the endogenous metabolic data set alone (Figures 3-13 and 3-14). For the purpose of this analysis, the outlying CNS non-responder identified in the earlier PCA analysis (CNS non-responder B) was excluded as it was established that its endogenous metabolic profile was distinct from the remaining CNS non-responders. There was significant discrimination between the control and treated individuals, as denoted by the robust model statistics (high $Q^2$ and $R^2$ values and significant permutation $p$-values, see individual figures for full details). The O-PLS-DA loadings plot for the urinary collection period 0-7 hours post dose showed that 3-IS and dimethylglycine were present at lower levels in high dose CNS non-responders relative to controls (Figure 3-13). The loadings plot also revealed that high dose CNS non-responders excreted lower levels of the TCA cycle intermediates, 2-oxoglutarate and citrate, over the same period. Further significant discriminators at 0-7 hours post-dose were elevated levels of methylamine, acetate, creatine, and creatinine in the urine collected from high dose CNS non-responders.
Figure 3-13 O-PLS-DA model derived from $^1$H NMR spectra of control ($n=10$) and INH-treated CNS non-responding ($n=5$) animals 0-7 hours following administration of INH at 400 mg/kg.

(a) O-PLS-DA scores plot (cross-validated scores, Tcv, vs class) showing separation between the groups. Control (■); INH-treated (■). (b) O-PLS-DA loadings coefficient plot, using univariance scaling, showing endogenous metabolites responsible for separation between control (top) and treated (bottom) animals. The metabolites responsible for separation were assigned from standard spectra, literature values and 'spike-in' experiments.

Model Statistics: $Q^2 Y$ 0.87; $R^2 Y$ 0.98; $p$ 0.001.

Key: 2-OG – 2-oxoglutarate; 3-IS – 3-indoxylsulfate; CIT – citrate; CRE – creatine; CRN – creatinine; DMG – dimethylglycine; MA – methylamine.
A second O-PLS-DA model was computed to illustrate the endogenous metabolites responsible for discrimination between the control and high dose CNS responders at 0-7 hours post-dose (Figure 3-14). Discrimination between the groups was primarily due to reduced urinary levels of 3-IS, phenylacetylglutamine (PAG), 3-hydroxyphenylpropionate (3-HPPA), 4-hydroxyphenylpropionate (4-HPPA), 2-oxoglutarate, citrate, dimethylglycine, and succinate, along with elevated levels of acetate, lactate, dimethylamine and glucose in the urine of high dose CNS responders relative to control levels.
Figure 3-14 O-PLS-DA model derived from $^1$H NMR spectra of control animals ($n=10$) and INH-treated CNS responders ($n=3$) 0-7 hours following administration of INH at 400 mg/kg.

(a) O-PLS-DA scores plot (cross-validated scores, $Tcv$, vs class) showing separation between the groups. Control ( ); INH-treated CNS responders ( ). (b) O-PLS-DA loadings coefficient plot, using univariance scaling, showing endogenous metabolites responsible for separation between control (top) and treated (bottom) animals. The metabolites responsible for separation were assigned from standard spectra, literature values and 'spike-in' experiments.

Model Statistics: $Q^2Y 0.83; R^2Y 0.96; p 0.004$.

Key: 2-OG – 2-oxoglutarate; 3-HPPA – 3-hydroxyphenylpropionic acid; 3-IS – 3-indoxylsulfate; 4-HPPA – 4-hydroxyphenylpropionic acid; DMA – dimethylamine; DMG – dimethylglycine; PAG – phenylacetylglucose.
The dominant endogenous metabolic changes reflective of high dose (400 mg/kg) INH exposure in CNS responders and non-responders are presented in Table 3-3.

Table 3-3 Metabolic perturbations following INH exposure at 400 mg/kg, relative to control urinary spectra, in CNS responder and non-responders at 0-7 hours post-dose. Metabolites were increased (↑) or decreased (↓) in treated urine, relative to control urinary spectra at each defined time-point. The scale of change (as indicated by the first value in brackets) has been expressed as the median log fold change of the metabolite resonance in the treated urinary cohort relative to the median metabolite resonance in the control cohort. The O-PLS-DA model coefficient of determination values ($r^2$) indicate which metabolites were most discriminatory. The resonance integrated for each metabolite is underlined. Metabolites were assigned via a combined application of literature values, 'spike-in' experiments and STOCSY.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$^1$H Chemical Shift + Multiplicity</th>
<th>CNS Non-responders</th>
<th>CNS Responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Oxoglutarate</td>
<td>2.45 (t), 3.01 (t)</td>
<td>↓ (-0.48; $r^2 = 0.83$)</td>
<td>↓ (-0.47; $r^2 = 0.69$)</td>
</tr>
<tr>
<td>3-Hydroxyphenyl-propionate</td>
<td>2.48 (t), 2.84 (t), 6.76 (d), 6.80 (s), 6.92 (dd), 7.27 (t)</td>
<td>-</td>
<td>↓ (-0.42; $r^2 = 0.86$)</td>
</tr>
<tr>
<td>3-Indoxylsulfate</td>
<td>7.21 (dd), 7.28 (dd), 7.36 (s), 7.51 (d), 7.71 (d)</td>
<td>↓ (-0.23; $r^2 = 0.95$)</td>
<td>↓ (-0.57; $r^2 = 0.85$)</td>
</tr>
<tr>
<td>4-Hydroxyphenyl-propionate</td>
<td>2.45 (t), 2.81 (t), 6.85 (d), 7.18 (d)</td>
<td>-</td>
<td>↓ (-0.29; $r^2 = 0.83$)</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.94 (s)</td>
<td>↑ (0.21; $r^2 = 0.79$)</td>
<td>↑ (0.33; $r^2 = 0.82$)</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.56 (d), 2.72 (d)</td>
<td>↓ (-0.17; $r^2 = 0.97$)</td>
<td>↓ (-0.18; $r^2 = 0.87$)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.05 (s), 4.05 (s)</td>
<td>↑ (0.08; $r^2 = 0.85$)</td>
<td>-</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>2.73 (s)</td>
<td>-</td>
<td>↑ (0.83; $r^2 = 0.88$)</td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td>2.93 (s)</td>
<td>↓ (-0.47; $r^2 = 0.91$)</td>
<td>↓ (-0.46; $r^2 = 0.81$)</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.2 – 3.9, 4.65 (d), 5.22 (d)</td>
<td>-</td>
<td>↑ (1.58; $r^2 = 0.76$)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33 (d), 4.11 (q)</td>
<td>-</td>
<td>↑ (1.26; $r^2 = 0.89$)</td>
</tr>
<tr>
<td>Methylamine</td>
<td>2.61 (s)</td>
<td>↑ (0.30; $r^2 = 0.91$)</td>
<td>-</td>
</tr>
<tr>
<td>Phenylacetyl-glycine</td>
<td>3.69 (s), 3.74 (d), 7.35 (m), 7.38 (m), 7.41 (s)</td>
<td>-</td>
<td>↓ (-0.38; $r^2 = 0.85$)</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.41 (s)</td>
<td>-</td>
<td>↓ (-0.18; $r^2 = 0.81$)</td>
</tr>
</tbody>
</table>
3.2.3 Pre-dose Classification of Post-dose Variability in Response to INH

O-PLS-based pattern recognition methods were used to identify relationships between the pre-dose metabolic profiles and post-dose outcome with respect to an adverse CNS effect following high dose INH exposure (400 mg/kg). An O-PLS-DA model was constructed to explore discrimination of the pre-dose urinary spectral profiles of CNS responders and CNS non-responders (with CNS non-responder B excluded). The cross-validated scores plot for this model, which was characterised by a $Q^2_Y$ of 0.34, and $R^2_Y$ of 0.76, showed clear separation between the pre-dose urinary spectra derived from the high dose CNS responders and CNS non-responders (Figure 3-15a). The corresponding loadings plot indicated that the discrimination was due to elevated levels of a number of urinary spectral resonances in the CNS responders, specifically resonances at $\delta$ 5.11 (d, 7.6 Hz), 6.65 (dd) and 6.72 (s) (Figures 3-15b and 3-15d). Visual comparison confirmed increased intensities of these resonances in the pre-dose urinary spectra of CNS responders (Figures 3-15c and 3-15e).
Figure 3-15 O-PLS-DA model derived from $^1$H NMR spectra of animals that developed INH-induced CNS effects ($n=4$) and those that did not ($n=5$), collected prior to administration of INH at 400 mg/kg.

(a) Cross validated scores versus Y response: CNS non-responder ( ), CNS responder ( ). (b, d) Loadings coefficient plot showing metabolites responsible for discrimination between individuals that developed CNS effects (top) and those that did not (bottom). (c, e) Corresponding $^1$H spectral regions colour coded according to severity of post-dose response (red, CNS responders; blue, CNS non-responders). Animal 206 was excluded. Model Statistics: $Q^2_Y 0.34; R^2_Y 0.76; p 0.14$.

Having established the existence of a relationship between the development of CNS adverse effects and the ratio of post-dose excretion of INH-PA:AcINH, an O-PLS-regression model was constructed using the post-dose ratio
of INH-PA to AcINH (Figure 3-16). The resulting scores plot showed moderate separation between the differential response phenotypes, whilst the loadings plot indicated the same discriminatory spectral resonances. (Figure 3-16)

![Scores Plot](image)

![Loadings Plot](image)

Figure 3-16 O-PLS-regression model derived from $^1$H NMR spectra collected prior to administration of 400 mg/kg INH, comparing pre-dose metabolic profile with post-dose ratio of pyruvate isonicotinylhydrazone (INH-PA) and acetylisoniazid (AcINH).

(a) O-PLS-regression scores plot of cross-validated scores (Tcv) versus ratio of INH-PA:AcINH showing separation between the groups. (b, c) Regions of O-PLS-regression loadings coefficient plot, showing metabolites responsible for discrimination on the basis of post-dose ratio of INH-PA to AcINH. The upper section of the plot represents metabolites that were elevated in pre-dose urine from individuals with low post-dose levels of AcINH relative to INH-PA.

Key: (■) – CNS non-responders; (■) – CNS responders.

Model Statistics: $Q^2$Y 0.71; $R^2$Y 0.95; $p$ 0.02.
Variable intensities of these signals were also observed in the pre-dose urinary spectra of the low dose cohort (Figure 3-17). Interestingly, the highest levels of these pre-dose signals corresponded with those individuals with the lowest levels of AcINH in the 0-7 hour post-dose urine following the low-dose INH treatment.

![Figure 3-17 Expanded regions of 600 MHz 1D 1H NMR spectra of rat urines collected 24 hours prior to administration of 200 mg/kg INH.](image)

Spectra have been colour coded to highlight those spectra with elevated levels of the resonances of interest. (c) Table detailing corresponding post-dose levels of AcINH; values have been colour coded corresponding to pre-dose spectra: everything below $3.0 \times 10^8$, red; everything above $3.0 \times 10^8$, blue. Integrals were calculated for the AcINH resonance at $\delta 2.15$.

Key: AcINH – acetylisoniazid.
3.2.4 NMR Analysis of Unknown Urinary Metabolites in Pre-Dose Urine of CNS Responders.

A range of 2D NMR spectroscopic experiments were applied to the structural identification of the pre-treatment urinary spectral resonances identified as discriminatory in Section 2.3 above. From the statistical models and from observation of the $^1$H NMR spectra, it was determined that the metabolite(s) of interest were present at very low concentrations. Therefore, pre-dose urine was pooled from a number of samples ($n=7$) to increase the concentration of the metabolite(s) of interest and enhance the resultant 2D spectra. $^1$H-$^1$H TOCSY showed the aromatic resonances at $\delta$ 6.65, and 6.72 were coupled to a resonance at $\delta$ 7.26 (Figure 3-18); this pattern is typical of a 1,2,4-trisubstituted phenolic moiety. In addition, the resonance at $\delta$ 5.11 was found to be coupled to a resonance at $\delta$ 3.61 and is typical of an ether glucuronide moiety, or more specifically an axial anomeric proton in a glucuronide (Figure 3-16). These NMR signals have therefore been tentatively assigned to a phenolic ether-glucuronide, although further experiments are necessary to fully elucidate and confirm an exact molecular structure.
Figure 3-18 2D 600 MHz $^1$H-$^1$H TOCSY of pooled urine from 10 rats collected 24 hours prior to administration of 400 mg/kg isoniazid and subsequent development of clinical signs of toxicity.

The regions $\delta$ 6.4 – 7.8 and $\delta$ 3.4 – 5.2 have been expanded. Cross-peaks are highlighted in red and red lines have been added to show intersections.
3.3 DISCUSSION

Although widely used to treat tuberculosis, INH is associated with adverse toxic effects when administered in isolation or in combination therapies, though the reported incidence vary. INH has been shown to induce an increase in plasma ALT levels in up to 2% of patients, with over hepatotoxicity identified in up to 1% of patients, with a variable and often lengthy delay before the onset of hepatotoxicity. In a study with a cohort of 519 subjects, 7% of subjects exhibited severe hepatotoxicity (i.e. necessitating termination of therapy) following a standard dose of 5 mg/kg/day, although this rate varies considerably between studies; Nolan et al. reported an incidence of less than 1%. INH has also been reported to cause peripheral neuropathy and neurotoxicity in humans, although the administration of INH with pyridoxine to combat peripheral neuropathy is recommended as standard clinical practice. Further, while chronic INH-induce toxicity manifests as hepatotoxicity or peripheral neuropathy, neurotoxicity has been widely reported following acute INH exposure.

Here, a dual metabonomic and pharmacometabonomic strategy for exploration of INH-induced toxicity in the rat model was applied by considering the systemic response to INH through analysis of distinct xenobiotic and endogenous metabolic phenotypes, or metabotypes. This led to the classification of CNS responders (40% of cohort) and CNS non-responders (60% of cohort) based on the presence or absence of unexpected adverse neuropathic effects. Histopathological assessment revealed hepatic glycogen depletion in 3 of 4 CNS responders which suggested a rapid utilisation of hepatic glycogen stores in response to INH-induced CNS toxicity. There was no histopathological evidence of hepatic necrosis or a hepatic lesion in CNS non-responders. Reductions in
plasma activity of ALT and AST were observed in the low dose and high dose CNS non-responders at 24 hours post-treatment, which supports previous findings. Both enzymes require the co-factor pyridoxal-5-phosphate, which is known to be sequestered by INH, and by hydrazine, a known metabolite of INH.

\(^1\)H NMR spectroscopic analysis enabled the simultaneous identification and assignment of numerous endogenous metabolites and INH-related urinary metabolites, including AcINH, INA, INH-KA (syn and anti isomers), INH-PA (syn and anti isomers), INH-GLC, INA-GLY and unchanged INH. This represents the first example of the application of \(^1\)H NMR spectroscopy to identify simultaneously multiple INH urinary metabolites, an outcome with clear translational potential to the clinic. PCA analysis of the urinary NMR spectral profiles enabled discrimination of both the endogenous and xenobiotic metabolic profiles of CNS responders and CNS non-responders following INH administration. INH-induced CNS responder effects were characterised by statistically significantly elevated urinary levels of the INH metabolites INH-PA and INH-GLC, accompanied by low levels of AcINH, a metabolite produced via acetylation of INH. Acetylation has been identified as a contributing factor in the development of INH-induced hepatotoxicity and peripheral neuropathy. Further, acetylation of INH has been shown to be saturated at high doses of INH in both human and rat models. In the current study, saturation of acetylation was observed following administration of INH at 200 mg/kg as comparable urinary levels of AcINH were excreted by both the low dose animals and the high dose CNS non-responders. In contrast, the high dose CNS responders excreted significantly lower levels of AcINH than the high dose CNS non-responders suggesting that acetylation capacity was lower in those individuals that experienced INH-induced
effects on the CNS. As a direct consequence of the lower acetylation capacity of CNS responders, a greater proportion of INH would be thus be expected to be metabolised via other routes. Evidence to support this was provided from the observation that significantly elevated levels of the INH hydrazones and hydrazides (i.e. INH-PA, INH-GLC) were excreted by the CNS responders suggesting enhanced metabolism of INH via conjugation with pyruvate and glucose. The application of a metabonomic platform has thus enabled the determination of unique INH metabotypes representing differential metabolic fate of INH of relevance to differential neuropathic outcome.

In humans, severe acute INH-induced neurotoxicity is characterised by a clinical triad of refractory seizures, metabolic acidosis with an elevated anion gap, and coma, with symptoms generally observed between 45 minutes and 2 hours post exposure. This neurotoxicity latency period is consistent with previous animals studies, and with the clinical signs of an adverse CNS effect observed at 2 hours post-treatment in this study. INH-induced neurotoxicity in man has also been associated with hyperglycaemia, hypokalaemia, glucosuria, and ketonuria. Discrimination of high dose CNS responders from CNS non-responders was also determined from modelling of the distinct metabolic profiles representing the endogenous metabolic complement. The CNS responders excreted significantly elevated urinary levels of glucose and lactate. Metabolic acidosis following INH-induced neurotoxicity in man is associated with elevated urinary lactate, which was apparent in those animals that displayed overt clinical signs of CNS effects, i.e. CNS responders. Though the exact mechanism of INH-induced metabolic acidosis is unknown, the most likely causes are tissue hypoxia, induced by seizures, and inhibition of lactate dehydrogenase, responsible for the conversion of lactate to pyruvate. It is worth noting that the primary site of lactate
dehydrogenase activity is the liver, suggesting a degree of hepatic dysfunction in the responders. Glucosuria, as observed in the high dose CNS responders, has also been associated with INH-induced neurotoxicity in man. 69 Reduced urinary levels of creatinine were observed in the CNS responders relative to the CNS non-responders. While creatinine is generally used as a measure of glomerular filtration rate and therefore renal function, here it likely reflects a generalised state of homeostatic perturbation.

The endogenous metabolic effects of a non-neuropathic response to INH was also considered through multivariate modelling of the urinary spectra representing animals that did not display marked effects on the CNS, i.e. the high dose CNS non-responders. O-PLS-DA models indicated that urinary levels of a number of metabolites, specifically 3-IS, dimethylglycine, and methylamine, were perturbed in the high dose CNS non-responders 0-7 hours after INH administration. Perturbation in levels of 3-IS are reflective of impaired bacterial catabolism of tryptophan, 199 while dimethylglycine and methylamine are all implicated in bacterial-mediated choline metabolism 200 and may collectively indicate an impact on the gut microbiota. Although excretion of INH is largely via urine, with up to 95% of INH and its metabolites being renally excreted within 24 hours, 43 a small proportion is reabsorbed into the bile, and subsequently excreted in the faeces, where it may directly alter the gut microbial composition.

Elevated creatinine was observed in the high dose CNS non-responders, relative to control levels at 0-7 hours post-dose. Creatinine is generally considered a marker of renal function. Here it is believed to indicate a generalised state of homeostatic perturbation.

Transient perturbations in 2-oxoglutarate and citrate were also observed in the high dose CNS non-responders at 0-7 hours post-dose suggestive of
mitochondrial metabolic perturbation. Earlier work on INH-induced hepatotoxicity reported similar reductions in urinary levels of 2-oxoglutarate in response to INH administration. Reductions in urinary levels of 2-oxoglutarate at both dose levels were also a likely consequence of the formation of the metabonate INH-KA, via non-enzymatic reaction of INH with 2-oxoglutarate.

Pharmacometabonomics has proved beneficial in the prediction of inter-individual variation in animal and human models of xenobiotic interventions, and was applied here to classify toxic outcome on the basis of pre-dose urinary metabolic profiles. An O-PLS-DA model identified a positive relationship between the pre-dose levels of urinary metabolite(s) and the development of INH-induced adverse effects on the CNS, providing further support for the value of the pharmacometabonomic concept. A second model, albeit a less statistically significant one, also identified a positive correlation between the post-dose ratio of AcINH to INH-PA and the pre-dose metabolic profile. In addition, an elevation in this pre-dose metabolic signature was detected in 3 of 10 individuals administered a low dose of INH, corresponding to those individuals with the lowest post-dose levels of AcINH. It was therefore hypothesised that this pre-dose metabolic signature may be predictive of the capacity for acetylation of INH in this pre-clinical model. On the basis of a range of 2D NMR experiments, this metabolite was tentatively assigned as a phenolic glucuronide moiety.
3.4 Conclusion

Here, the xenobiotic and endogenous metabolic phenotypes associated with inter-individual variability in toxicity outcomes for a pre-clinical model of INH treatment in rats have been characterised. NMR spectroscopic analysis was applied to characterise the urinary metabolic fate of INH, which was seen to differ with respect to acetylation capacity and production of hydrazone and hydrazide metabonates in responders that developed adverse effects on the CNS. The endogenous consequences of INH administration were also identified, highlighting the power of this multivariate approach for the assessment of a systems level response to a toxin. The application of pharmacometabonomics enabled identification of a pre-treatment metabolic signature that was reflective of the differential metabolic fate of INH and the subsequent development of INH-induced adverse CNS effects. Such an approach is widely applicable to the clinical setting to explore the relationship between adverse drug reactions, the metabolic fate of pharmaceuticals and their impact on metabolic homeostasis. This metabonomic strategy may lead to novel means of determining the metabolic basis for adverse drug reactions and ultimately enable the prediction of susceptibility to adverse drug reactions.
Chapter 4 GENTAMICIN

AIMS AND OBJECTIVES

The aim of this work was to employ an integrated approach to characterise the metabolic impact of gentamicin exposure in the rat model through the integration of traditional toxicity assessment (histopathological assessment, measurement of kidney injury molecule 1, N-acetyl-β-glucosaminidase, and total protein) with high resolution NMR spectroscopy of urine, serum, and intact kidney and liver. The relationship between gentamicin-induced nephrotoxicity and administration time was further explored to determine if the severity of gentamicin-induced nephrotoxicity was linked to administration time and whether this relationship was reflected in differential metabolic profiles.
4.1 METHODS

The following study was carried out collaboratively with Dr Antoine and Prof Park at the MRC Centre for Drug Safety Science at the University of Liverpool, who designed the study. All animal manipulations were conducted by the MRC Centre for Drug Safety Science at the University of Liverpool.

4.1.1 ANIMAL HANDLING AND SAMPLE COLLECTION

Male SD rats (approximately 250-330g, \( n=16 \)) were randomly allocated to four dose groups \( (n=4) \): control (AM), control (PM), gentamicin (AM), and gentamicin (PM). Animals were dosed once daily for nine days via intra-peritoneal (i.p.) injection with control vehicle (0.9% saline), or gentamicin (200 mg/kg in 0.9% saline). “AM” dose was administered daily at 10am; “PM” dose was administered daily at 10pm. Full details are given in Table 4-1.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Animal Numbers</th>
<th>Dosing Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>1, 2, 3, 4</td>
<td>PM (22.00)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>5, 6, 7, 8</td>
<td>AM (10.00)</td>
</tr>
<tr>
<td>Control</td>
<td>9, 10 11, 12</td>
<td>AM (10.00)</td>
</tr>
<tr>
<td>Control</td>
<td>13, 14, 15, 16</td>
<td>PM (22.00)</td>
</tr>
</tbody>
</table>

Urine was collected over a period of 24 hours following administration of the final dose, and blood collected at termination via cardiac puncture. Liver (right lateral lobe) and kidney tissue (left kidney section including papilla, cortex and medulla) samples were collected at termination. Three aliquots were snap frozen
pending further analysis, and the remainder frozen in 10% formalin. The experimental timeline is outlined in Figure 4-1.

![Figure 4-1 Experimental timeline.](image)

Gentamicin or control vehicle were administered daily at 10.00 (AM group) or 22.00 (PM group) for nine days, as indicated by the green lines. Urine was collected over a period of 24 hours (hatched black area) and serum and tissue sampled at termination (red line).

Key: Gen – gentamicin.

### 4.1.2 **CLINICAL CHEMISTRY AND HISTOPATHOLOGY**

Urinary N-acetyl-β-glucosaminidase (NAG) was measured spectrophotometrically as described by Horak *et al.*[^202] and urinary kidney injury molecule 1 (KIM-1) was measured using the xMAP Luminex technology.[^203] Other urinary parameters were measured using a Roche Modular Analyser. Serum creatinine was measured using a standard clinical chemistry analyser (Roche-Modular). This work was conducted by Dr Antoine at the Vaidya/Bonventre laboratory, Harvard Medical School. Significant changes in these parameters from control levels were assessed using a Students two-tailed t-test. The liver and kidney were sampled and fixed in a 10% formalin solution. Representative samples were processed routinely in an automatic tissue processor, embedded in paraffin, sectioned at 4-6 μm, stained with haematoxylin and eosin, and examined histologically using light microscopy to determine the extent of macroscopic

[^202]: Horak *et al.*
[^203]: Other
damage. This work was conducted in Professor Park’s laboratory at the University of Liverpool.

4.1.3 $^1$H NMR Spectroscopy of Urine

Urine samples were prepared and 600 MHz $^1$H spectra were acquired using a standard 1D solvent suppression pulse, as described previously (Chapter 3, section 1.3). For each sample, 256 transients were collected into 64 K data points, using a sweep width of 12019.2 Hz with a relaxation delay of 4 s, an acquisition time of 2.72 s, and a mixing time of 100 ms. Pre-saturation of the water resonance occurred during the relaxation delay and the mixing time. A line-broadening factor of 0.3 Hz was applied to all spectra prior to FT.

4.1.4 $^1$H NMR Spectroscopy of Serum

Serum (200 $\mu$L) was mixed with 400 $\mu$L of saline, containing 20% D$_2$O; no sample was available for animal number 4 (gentamicin, PM). Samples were centrifuged at 9447 g for 10 minutes and supernatants (550 $\mu$L) transferred to 5 mm NMR tubes (Norell Standard Series 507) for NMR analysis. 1D $^1$H NMR spectra were acquired on an NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 14.1 T (600.13 MHz $^1$H frequency) at a temperature of 300 K, using the standard 1D solvent suppression pulse sequence, as described above, in which 128 transients were collected into 64 K data points, with a sweep width of 12019.2 Hz, a relaxation delay of 4 s, an acquisition time of 2.72 s and a mixing time of 100 ms. In addition, the Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with a fixed spin-spin relaxation delay ($2n\pi$) of 64 ms was applied to attenuate broad resonances from high molecular weight compounds. For each sample, 512 transients were collected into 64 K data points.
with a sweep width of 12019.2 Hz, a relaxation delay of 4 s, and an acquisition time of 2.73 s. A line-broadening factor of 0.3 Hz was applied to all spectra prior to FT.

4.1.5 $^1$H MAS-NMR Spectroscopy of Intact Kidney and Liver Samples

Kidney (single sample of cortex) and liver tissue samples (approx. sample weight of 10 mg) were rinsed with D$_2$O and placed in a 4 mm zirconium oxide rotor with an insert to ensure a spherical cavity, and spun in air at the magic angle ($\theta = 54.7^\circ$) at a spin rate of 5 kHz using a Bruker DRX-600 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 14.1 T (600.13 MHz $^1$H frequency). The MAS-NMR spectra were acquired at 283 K, using the CPMG $^{166}$ spin-echo pulse sequence described above. Typically, 128 transients were collected into 64 K data points over a sweep width of 12019.2 Hz with a relaxation delay of 4 s and an acquisition time of 2.73 s. A line-broadening factor of 0.3 Hz was applied to all spectra prior to FT.

4.1.6 NMR Spectral Data Processing

The $^1$H NMR spectra were manually phased, baseline corrected and referenced to TSP (δ 0.0) for urinary spectra, α-glucose (δ 5.233) for serum spectra, and lactate (δ 1.33) for tissue samples. Full resolution NMR spectra were imported into MATLAB (version 7.6, The MathWorks, Natick, USA) using MetaSpectra 4.1.1 (an in-house routine written by Dr O. Cloarec). The regions corresponding to water (δ 4.73 to 4.90), and, where present, TSP (δ -0.20 to 0.20) were removed. In addition, the region containing the urea resonance (δ 5.50 to 6.10) was removed from urinary spectra. The spectra were aligned using the
RSPA method,\textsuperscript{174} and the data normalised using probabilistic quotient normalisation.\textsuperscript{175}

Multivariate statistical analysis (PCA, PLS-DA, and O-PLS-DA) was carried out in MATLAB using univariance scaling.\textsuperscript{178,180,181} To test the validity of all PLS-DA and O-PLS-DA models, 7-fold cross-validation was used, and the $Q^2_Y$ value (a measure of the cross-validated predictive ability) calculated. A permutation test was performed to test the robustness of the models. Metabolites were assigned via a combined application of literature values and 'spike-in' experiments. To assist in the assignment of metabolite signals, homonuclear $^1\text{H}-^1\text{H}$ STOCSY analysis was performed as necessary.\textsuperscript{183} Relative concentrations of key endogenous metabolites, as identified from O-PLS-DA loadings plots, were calculated by integration of specific NMR spectral resonances, with integrals corrected for the number of protons giving rise to spectral resonances. The scale of metabolic perturbation was assessed from calculation of the log (base 10) of the median fold change of integrals of resonances of the discriminatory metabolites, relative to median control levels.
4.2 RESULTS

4.2.1 CLINICAL CHEMISTRY AND HISTOPATHOLOGY

The effects of gentamicin were assessed using a range of conventional clinical chemistry analyses, including histopathological examination of the kidneys and urinary levels of KIM-1, NAG and total protein (Figure 4-2). A once daily PM administration of gentamicin induced mild nephrotoxicity in 3 out of 4 rats (severity score of 1), characterised by tubular protein casts and focal cortical interstitial mononuclear infiltrate with mild fibrosis; one individual in this treatment group exhibited no significant microscopic changes. The nephrotoxic effect of gentamicin was more marked following an AM administration, with all rats ($n=4$) experiencing moderate to severe toxicity (severity score of 2 or 3). This was characterised by tubular protein casts, focal interstitial mononuclear infiltrates, dilation of cortical tubuli and evidence of necrotic epithelial cells, and evidence of vascular leukocyte recruitment. No microscopic changes were observed in kidney tissue from control animals.

Urinary levels of KIM-1, NAG and total protein were measured for all animals at termination (Figures 4-2b, 4-2c, and 4-2d). All values relate to relative urinary levels, rather than absolute excretion and are expressed relative to creatinine. NAG and total protein levels were significantly elevated ($p < 0.05$) following gentamicin administration at both administration times, relative to corresponding control levels (Figures 4-2c and 4-2d). KIM-1 levels were significantly elevated ($p < 0.05$) following AM administration of gentamicin, relative to the corresponding control animals and relative to PM administration of gentamicin (Figure 4-2b). However, all three parameters showed a high degree of
variability in response to gentamicin administration. This inter-individual variation was most notable in the AM treatment group.
Clinical chemistry parameters and histopathology scores following treatment with gentamicin for (a) histopathology severity score of nephrotoxic damage; (b) KIM-1 (nanograms); (c) NAG (milligrams); (d) total protein (milligrams).

Individual values are identified with black dots and the bar represents the mean of each group of samples. Error bars represent standard deviation. All kidneys were scored from 0 (= no necrosis) to 4 (= severe necrosis). Urinary protein biomarker results were corrected for urinary creatinine levels. Analysis of KIM-1, NAG and total protein was conducted by Dr Antoine at the Vaidya/Bonventre laboratory, Harvard Medical School, while histopathological assessment was conducted in Prof Park’s laboratory at the University of Liverpool.

Key: KIM-1 – kidney injury molecule 1; NAG – N-acetyl-β-glucosaminidase. (*) $p < 0.05$; (**) $p < 0.01$. 
NMR spectra from biofluids and tissues are comprised of signals from metabolites representing numerous metabolic pathways. Representative urinary and kidney spectra from control and gentamicin-treated rats (AM administration time), specifically median spectra for each group, are presented in Figures 4-3 and 4-4.

Figure 4-3 600 MHz $^1$H NMR spectra of urine from (a) control rats and (b) gentamicin treated rats (AM administration time).

Examination of the urinary and kidney spectra identified a number of outliers. Specifically, the urinary spectrum from subject 6 (gentamicin, AM) contained significantly higher levels of glucose, lactate, alanine, taurine, and trimethylamine-N-oxide (TMAO), coupled with lower levels of citrate and 2-oxoglutarate compared to the other individuals in this treatment group. This individual exhibited only mild histopathological damage (severity score of 2) characterised by tubular protein casts, focal interstitial mononuclear infiltrates, dilation of cortical tubuli, and excreted the lowest levels of urinary total protein, relative to creatinine, compared to the other individuals in this treatment group. The $^1$H MAS-NMR spectrum from subject 1 (gentamicin, PM) had higher levels of...
a number of amino acids, specifically valine, leucine, isoleucine, and phenylalanine, compared to the other individuals in the group. There were no gentamicin-related histopathological changes observed in the kidney tissue from this individual (severity score of 0). The $^1$H MAS-NMR spectrum from subject 12 (control, AM) was found to contain elevated levels of isovalerate, and acetyl tyrosine, compared to the other individuals in this treatment group. These subjects were excluded from subsequent analysis. To aid comparison, all models were therefore computed with three individuals in the gentamicin (AM) group, three in the gentamicin (PM) group, and three in the control (AM) group.

4.2.3 **Metabolic Impact of Gentamicin Exposure – Urine**

The PCA scores plot of PC1 vs PC2 for the urinary spectra of the control and gentamicin-treated animals, for both administration times, is presented in Figure 4-5. PCA analysis of the $^1$H NMR urinary spectra showed clear segregation of the control animals and the gentamicin-treated animals into three separate clusters: control, gentamicin, AM, and gentamicin, PM (Figure 4-6). The PCA scores plot of PC1 vs PC2 showed discrimination between control and treated animals, and between the treated samples according to administration time in the first component, which accounted for 31% of the total variance in the data. The gentamicin, AM, treated individuals were further from control space along PC1, compared to the PM treated individuals. The PM treated group exhibited a high degree of variability along PC2.
The supervised discriminant analysis technique, O-PLS-DA, was then employed to maximise the discrimination between the control and treated groups and provide an interpretation of the metabolic signature changes characteristic of administration of gentamicin at different administration times. Pair-wise models were computed for the treated versus control animals following both administration times. An O-PLS-DA model, using a single orthogonal component,
was computed to illustrate the metabolites responsible for discrimination between control and gentamicin-treated animals following an AM administration of gentamicin (Figure 4-6). The major urinary metabolic changes contributing to the separation between control and AM treated individuals, as identified from the corresponding loadings plots, were reduced levels of 3-IS, phenylacetylglycine (PAG), and hippurate, and higher urinary levels of taurine, alanine and lactate, relative to control levels (Figure 4-6).
Figure 4-6 O-PLS-DA model derived from $^1$H NMR urinary spectra of control ($n=3$) and gentamicin-treated animals ($n=3$), following AM administration.

(a) Cross validated scores (Tcv1) versus Y response. Control (■); gentamicin-treated ( ).

(b) Loadings coefficients plot showing metabolites responsible for discrimination between control (top) and gentamicin treated (bottom) animals. Metabolites were assigned using standard spectra, literature values, and 'spike-in' experiments (3-IS, PAG).

Model Statistics: $Q^2_Y 0.85$; $R^2_Y 0.94$; $p 0.07$.

Key: 3-IS – 3-indoxylsulfate; PAG – phenylacetylglycine.

A second cross-validated O-PLS-DA model for PM administration of gentamicin was computed to compare the treated group to the corresponding
control group and is shown in Figure 4-7. The major urinary metabolic changes contributing to separation between control and PM treated individuals were reduced levels of hippurate and 3-IS, and an increase in urinary taurine, as with AM administration. In addition, PM administration of gentamicin resulted in significant reductions in urinary levels of 2-oxoglutarate, citrate and succinate.
Figure 4-7 O-PLS-DA model derived from $^1$H NMR spectra of control ($n=4$) and gentamicin-treated animals ($n=3$), following PM administration.

(a) Cross validated scores (Tcv1) versus Y response. Control (■); gentamicin-treated (■). (b) Loadings coefficients plot showing metabolites responsible for discrimination between control (top) and gentamicin treated (bottom) animals. Metabolites were assigned using standard spectra, literature values, and ‘spike-in’ experiments (3-IS).

Model Statistics: $Q^2Y$ 0.78; $R^2Y$ 0.99; $p$ 0.04.

Key: 2-OG – 2-oxoglutarate; 3-IS – 3-indoxylsulfate; PAG – phenylacetylglucose.

A further model was computed to explore the impact of administration time on urinary metabolic profiles. An O-PLS-DA pair-wise model was built comparing the gentamicin treated animals at both administration times (Figure 4-8). The
scores plot showed clear separation between the treated animals on the basis of administration time, and the dominant metabolic changes responsible for separation were identified from the corresponding loadings plot as low urinary levels of 3-IS, and PAG, coupled with elevated levels of alanine, valine, leucine, and lactate following an AM administration of gentamicin.
Figure 4-8 O-PLS-DA model derived from $^1$H NMR urinary spectra following AM ($n=3$) and PM administration ($n=3$) of gentamicin.

(a) Cross validated scores (Tcv1) versus Y response. AM (■); PM (▲). (b) Loadings coefficients plot showing metabolites responsible for discrimination between PM (top) and AM (bottom) administration time. Metabolites were assigned using standard spectra, and literature values.

Model Statistics: $Q^2Y 0.78; R^2Y 0.96; p 0.05$.

Key: 3-IS – 3-indoxylsulfate; PAG – phenylacetylglycine.

A summary of the dominant urinary metabolic changes in response to gentamicin administration at AM or PM, as determined by the O-PLS-DA loadings coefficient, is presented in Table 4-2.
Table 4-2 Summary of metabolites responsible for discrimination between control and gentamicin-treated individuals, following administration of gentamicin.

Metabolites are elevated (↑) or reduced (↓) in spectra of treated individuals relative to control. The scale of change (the first value in brackets) has been expressed as the log fold change of the metabolite resonance in the treated urine relative to the median control urine over the same collection period. For each metabolite, the coefficient of determination ($r^2$) was measured at the peak underlined. Incidents of metabolic perturbations is indicated, i.e. occurrence/total number of individuals. Metabolites were assigned using standard spectra, literature values, and 'spike-in' experiments (3-IS. PAG).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$^1$H Chemical Shift + Multiplicity</th>
<th>Administration Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AM</td>
</tr>
<tr>
<td>U1</td>
<td>1.66 (d)</td>
<td>↑ (0.12; $r^2$ = 0.99; 3/3)</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>2.47 (t), 3.03 (t)</td>
<td>↓ (-0.10; $r^2$ = 0.88; 3/3)</td>
</tr>
<tr>
<td>3-Indoxylsulfate</td>
<td>7.21 (dd), 7.28 (dd), 7.36 (s), 7.51 (d), 7.71 (d)</td>
<td>↓ (-0.28; $r^2$ = 0.97; 3/3)</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48 (d), 3.79 (q)</td>
<td>↑ (0.08; $r^2$ = 0.97; 3/3)</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.55 (d), 2.69 (d)</td>
<td>↓ (-0.13; $r^2$ = 0.94; 2/3)</td>
</tr>
<tr>
<td>Hippurate</td>
<td>3.97 (d), 7.55 (t), 7.64 (t), 7.83 (d)</td>
<td>↓ (-0.74; $r^2$ = 0.99; 3/3)</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33 (d), 4.11 (q)</td>
<td>↑ (0.08; $r^2$ = 0.97; 3/3)</td>
</tr>
<tr>
<td>Phenylacetyl-glycine</td>
<td>3.69 (s), 3.74 (d), 7.35 (m), 7.38 (m), 7.41 (s)</td>
<td>↓ (-0.20; $r^2$ = 0.95; 3/3)</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.41 (s)</td>
<td>↓ (-0.14; $r^2$ = 0.97; 3/3)</td>
</tr>
<tr>
<td>Taurine</td>
<td>3.27 (t), 3.43 (t)</td>
<td>↑ (0.29; $r^2$ = 0.98; 3/3)</td>
</tr>
</tbody>
</table>

Key: U1 – unassigned urinary metabolite.

A number of metabolic commonalities were identified from the O-PLS-DA loadings plots in response to both an AM and PM administration of gentamicin,
specifically a reduction in urinary levels of 3-IS and hippurate, and in increase in urinary levels of taurine and an unassigned multiplet at δ 1.66 (U1).

There were also clear differences in the urinary metabolic profiles of the treated individuals at each treatment time relative to the corresponding control animals. An AM administration of gentamicin resulted in an increase in urinary levels of alanine and lactate, while the same treated individuals excreted lower levels of PAG, relative to control levels. In contrast, levels of these metabolites were not significantly perturbed following a PM administration of gentamicin based on the O-PLS-DA loadings plot. The O-PLS-DA model of control versus gentamicin following a PM administration identified a reduction in urinary levels of a number of TCA cycle intermediates (i.e. 2-oxoglutarate, succinate and citrate) in response to gentamicin; these metabolites did not contribute to the separation observed between the AM treated animals and the corresponding control group.

The O-PLS-DA loadings plot of AM versus PM gentamicin treated rats identified a number of additional metabolites that contributed to separation between the treated groups, specifically lactate, valine, leucine and isoleucine (all elevated in the AM treated group), and PAG and 3-IS (all reduced in the AM treated groups).

4.2.4 **Metabolic Impact of Gentamicin Exposure – Kidney**

This analytical approach was replicated for the kidney $^1$H MAS-NMR spectra. PCA analysis of the $^1$H MAS-NMR kidney spectra showed a degree of separation between the treated individuals and the corresponding control animals: the scores plot of PC1 vs PC2 (where PC1 and PC2 explained 35% of the total variance in the data) showed separation in the first component between control
and treated for both AM and PM administration times, and between the treated animals in the second component (Figure 4-9).

Figure 4-9 PCA scores plot of PC1 vs PC2 obtained from $^1$H MAS-NMR kidney spectra from control or gentamicin-treated animals.

Key: (■) – control, AM; (○) – control, PM; (■) – gentamicin, AM; (▲) – gentamicin, PM.

To provide a clearer interpretation of the metabolic signature changes reflective of gentamicin exposure in the kidney, pair-wise O-PLS-DA models were constructed for the treated animals versus the control group, at each administration time. O-PLS-DA models were computed to illustrate the
metabolites responsible for discrimination between control and gentamicin-treated animals, following an AM administration (Figure 4-10) and a PM administration (Figure 4-11). In both instances, the O-PLS-DA scores plots showed clear separation between the control and treated spectra. The corresponding O-PLS-DA loading coefficient plots indicated that discrimination between AM control and gentamicin treated individuals was primarily due to low levels of amino acids (i.e. alanine, leucine, isoleucine, phenylalanine), tyrosine, and betaine, coupled with high levels of choline, phosphocholine, lactate, myo-inositol (MI), glycine, and creatine, in kidney tissue collected from gentamicin-treated individuals (Figure 4-10).
Figure 4-10 O-PLS-DA model derived from $^1$H MAS-NMR kidney spectra of control ($n=3$) and gentamicin-treated animals ($n=3$), following AM administration.

(a) Cross validated scores ($Tcv1$) versus $Y$ response. Control (■); gentamicin-treated (■). (b) Loadings coefficients plot showing metabolites responsible for discrimination between control (top) and gentamicin treated (bottom) animals. Metabolites were assigned using standard spectra, and literature values, while STOCSY was employed to identify correlated peaks.

Model Statistics: $Q^2Y \text{ 0.75;  } R^2Y \text{ 0.97;  } p \text{ 0.09.}$

Key: CRE – creatine; MI – myo-inositol; PC – phosphocholine.
Figure 4-11 identified a reduction in kidney levels of leucine, and alanine and an increase in creatine, choline and phosphocholine following a PM administration of gentamicin relative to control levels.

Figure 4-11 O-PLS-DA model derived from $^1$H MAS-NMR kidney spectra of control ($n=4$) and gentamicin-treated animals ($n=3$), following PM administration.

(a) Cross validated scores (Tcv1) versus Y response. Control (■); gentamicin-treated (▲). (b) Loadings coefficients plot showing metabolites responsible for discrimination between control (top) and gentamicin treated (bottom) animals. Metabolites were assigned using standard spectra, and literature values, while STOCSY was employed to identify correlated peaks.

Model Statistics: $Q^2_Y 0.71; R^2_Y 0.99; p 0.03$.

Key: CRE – creatine; PC – phosphocholine.

A further O-PLS-DA model was computed to specifically investigate the impact of administration time on gentamicin metabolism in the kidney (Figure 4-
12). This pair-wise model, constructed using univariance scaling, compared the kidney $^1$H MAS-NMR spectra obtained following daily AM and PM administration of gentamicin. The O-PLS-DA scores plot showed clear separation of the gentamicin-treated animals on the basis of administration time. The corresponding loadings coefficient plot indicated that the major metabolites contributing to this separation were lactate, creatine, choline, and MI (all elevated), and amino acids (i.e. leucine, valine, alanine, etc.), tyrosine, histidine, and phenylalanine (all reduced) following daily AM administration of gentamicin.
Figure 4-12 O-PLS-DA model derived from $^1$H MAS-NMR kidney spectra following AM ($n=3$) and PM administration ($n=3$) of gentamicin.

(a) Cross validated scores (Tcv1) versus Y response. AM (■); PM (■). (b) Loadings coefficients plot showing metabolites responsible for discrimination between PM (top) and AM (bottom) administration time. Metabolites were assigned using standard spectra, and literature values, while STOCSY was employed to identify correlated peaks.

Model Statistics: $Q^2 Y 0.73; R^2 Y 0.95; p 0.07$.

Key: CRE – creatine; MI – myo-inositol.

A summary of the dominant endogenous metabolic changes observed in the kidney reflective of gentamicin administration at both administration times, as
determined from the O-PLS-DA loadings coefficient plots, is presented in Table 4-3.

Table 4-3 Summary of metabolites responsible for discrimination between control and gentamicin-treated individuals, following administration of gentamicin.

Metabolites are elevated (↑) or reduced (↓) in spectra of treated individuals relative to control. The scale of change (as indicated by the first value in brackets) has been expressed as the median log fold change of the metabolite resonance in the treated urine relative to the median control urine over the same collection period. For each metabolite, the coefficient of determination ($r^2$) was measured at the peak underlined. Incidents of metabolic perturbations is indicated, i.e. occurrence/total number of individuals.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$^1$H Chemical Shift + Multiplicity</th>
<th>Administration Time</th>
<th>AM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.48 (d), 3.79 (q)</td>
<td>↓ (-0.32; $r^2 = 0.98$; 3/3)</td>
<td>↓ (-0.10; $r^2 = 0.91$; 3/3)</td>
<td></td>
</tr>
<tr>
<td>Betaine</td>
<td>3.27 (s), 3.90 (s)</td>
<td>↓ (-0.14; $r^2 = 0.90$; 2/3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline</td>
<td>3.20 (s), 3.51 (t), 3.51 (t)</td>
<td>↑ (0.08; $r^2 = 0.80$; 3/3)</td>
<td>↑ (0.02; $r^2 = 0.96$; 2/3)</td>
<td></td>
</tr>
<tr>
<td>Creatine</td>
<td>3.04 (s), 3.94 (s)</td>
<td>↑ (0.14; $r^2 = 0.95$; 3/3)</td>
<td>↑ (0.05; $r^2 = 0.88$; 2/3)</td>
<td></td>
</tr>
<tr>
<td>Fumarate</td>
<td>6.53 (s)</td>
<td>↑ (0.96; $r^2 = 0.97$; 3/3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>3.57 (s)</td>
<td>↑ (0.10; $r^2 = 0.82$; 2/3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.94 (t), 1.01 (d), 1.26 (m), 1.48 (m), 1.96 (m), 3.61 (d)</td>
<td>↓ ($r^2 = 0.96$; 3/3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33 (d), 4.11 (q)</td>
<td>↑ (0.27; $r^2 = 0.91$; 3/3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.96 (t), 1.71 (m), 3.73 (t)</td>
<td>↓ (-0.27; $r^2 = 0.95$; 3/3)</td>
<td>↓ (-0.10; $r^2 = 0.92$; 3/3)</td>
<td></td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>3.27 (t), 3.53 (dd), 3.62 (t), 4.05 (t)</td>
<td>↑ (0.07; $r^2 = 0.98$; 3/3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Phenylalanine 3.13 (d), 3.28 (dd), 4.00 (dd), 7.33 (m), 7.39 (t), 7.43 (m) ↓ (-0.27; r² = 0.98; 3/3)

Phosphocholine 3.22 (s), 3.62 (t) 4.18 (dd) ↑ (0.25; r² = 0.94; 3/3) ↑ (0.19; r² = 0.92; 3/3)

Tyrosine 3.06 (dd), 3.20 (dd), 3.94 (dd), 6.91 (d), 7.20 (d) ↓ (-0.25; r² = 0.98; 3/3)

Valine 0.99 (d), 1.04 (d), 2.28 (d), 3.62 (d) ↓ (-0.43; r² = 0.97; 3/3)

It was not possible to determine the scale of change of isoleucine due to peak overlap.

Administration of gentamicin, at either administration time, was characterised by lowered urinary levels of alanine and leucine, and elevated urinary levels of choline, creatine, and phosphocholine relative to control levels. In addition, an AM administration of gentamicin was characterised by a number of unique metabolic changes, as described previously.
4.3 DISCUSSION

An NMR-based metabolic profiling strategy in conjunction with measurement of urinary biomarkers and histopathology was applied to interrogate and understand the metabolic basis of gentamicin-induced nephrotoxicity. Metabolic perturbations following gentamicin administration were detected using solution and solid state NMR spectroscopy of urine, serum, kidney and liver from rats. There were no significant changes noted in either the serum or liver metabolic profiles following gentamicin administration at either treatment time, relative to the corresponding controls. Unlike many drugs, gentamicin is not metabolised in the liver but is excreted unchanged in the urine, accounting for the lack of metabolic impact on the liver samples. Analysis therefore focused on the metabolic changes observed in the urine and kidney. Pattern recognition methods were applied to the identification of discriminatory metabolites in response to differential treatments.

4.3.1 METABOLIC IMPACT OF GENTAMICIN

Conventional histopathological analysis was performed on kidney tissue collected from all animals at termination. No vehicle-related microscopic changes were observed in the kidney tissue. Gentamicin administration resulted in clear microscopic damage, reflective of the nephrotoxic effect of gentamicin. In this study, the microscopic damage observed was most severe in the AM treated group, indicating a greater degree of toxicity, consistent with earlier studies.\textsuperscript{122, 123} It was noted that the PM administration time resulted in a high degree of inter-individual variation on the basis of histopathology severity scores.
Kidney damage proceeds via a number of reversible steps, depending on the degree of toxic insult. The conventional biomarkers of kidney injury (i.e. serum creatinine, blood urea nitrogen) are insensitive, non-specific, and only detectable once severe, often irreversible, damage has occurred. Extensive research has therefore led to the identification of NAG and KIM-1 as potential biomarkers of acute kidney injury (AKI) that could facilitate earlier diagnosis.\textsuperscript{132, 133} NAG and total protein were significantly elevated following gentamicin administration at either dosing time, relative to control levels, indicating that both are potentially useful markers of gentamicin-induced toxicity. Levels of KIM-1, NAG and total protein were elevated in the AM group relative to PM group, though of these only KIM-1 showed a statistically significant increase relative to PM levels. Therefore, NAG and total protein may be considered to lack sensitivity to differential gentamicin-induced nephrotoxicity in response to administration time. However, it should be noted that levels of all markers were highly variable in the AM group, the impact of which was exaggerated by the low animal number.

The application of metabonomic techniques identified a number of metabolic consequences following gentamicin administration on the basis of O-PLS-DA loadings coefficient plots, specifically perturbations in levels of amino acids, gut microbial co-metabolites, renal osmolytes and TCA cycle intermediates. AM administration of gentamicin was characterised by an increase in urinary levels of alanine, lactate and taurine, accompanied by a decrease in urinary levels of 3-IS, hippurate and PAG. A PM administration of gentamicin was characterised by elevated urinary levels of taurine, and a reduction in urinary levels of 3-IS, hippurate, citrate and succinate.

The metabolic impact of gentamicin administration on kidney tissue was more marked following an AM administration with levels of choline, creatine,
fumarate, phosphocholine, glycine, and lactate all elevated in kidney tissue collected post treatment. Further, an AM administration of gentamicin resulted in lower tissue levels of a number of amino acids (alanine, leucine, isoleucine, phenylalanine, and valine), betaine and tyrosine in kidney tissue collected from treated animals. In the PM treatment group, elevated levels of choline, creatine and phosphocholine, and reduced levels of alanine and leucine were detected.

Changes in urinary levels of compounds such as hippurate, 3-IS and PAG are suggestive of perturbations in gut microbial co-metabolism due to the antibiotic activity of gentamicin. Gut microbiota metabolise polyphenols to benzoic acid, which is detoxified via conjugation with glycine to form hippurate, which is secreted by renal tubular cells and subsequently excreted in the urine. Reductions in levels of hippurate are implicated in a number of pathophysiological states, including perturbation of the glycine pathway. However, $^1$H MAS-NMR of liver indicated that gentamicin administration had no significant impact on hepatic levels of glycine (results not shown). Therefore, reduced urinary levels of hippurate are likely due to disturbance in benzoic acid synthesis. Alternatively, a reduction in urinary levels of hippurate may indicate insufficient proximal secretion. Numerous studies have reported reduced urinary levels of hippurate following aminoglycoside administration. Sieber et al. also noted a reduction in urinary levels of another microbial co-metabolite 3-IS. 3-IS is produced via bacterial mediated catabolism of tryptophan. Dietary tryptophan is metabolised to indole by the bacterial enzyme, tryptophanase. Indole is then hydroxylated in the liver by CYP2E1 to form indoxyl, which is subsequently sulphated by sulphotransferases forming 3-IS. Therefore a reduction in urinary 3-IS following gentamicin exposure is likely reflective of the antibiotic activity of gentamicin. Sieber et al. did however express concern about the
relative sensitivity of 3-IS as a potential biomarker of gentamicin exposure: a significant reduction in urinary levels of 3-IS was only observed following high dose administration (120 mg/kg). In comparison, 3-IS was significantly reduced following daily administration of gentamicin at 200 mg/kg in this current study.

Sieber et al. also reported changes in urinary levels of PAG: a significant increase was noted on day 6. In the current study, urinary levels of PAG were decreased following an AM administration of gentamicin, and unchanged in the PM treatment group. It should be noted however that the doses differ (200 mg/kg in present study compared to 120 mg/kg), as well as the dosing schedule (Sieber et al. dosed animals twice daily as opposed to once). PAG is derived from metabolism of phenylacetate as part of the putrefactive processes mediated by colonic bacteria. First, bacteria generate phenylacetate from phenylalanine, which is subsequently detoxified by the host via conjugation with glycine. Reduced levels of PAG indicate disturbance to this pathway. PAG has previously been suggested as a potential biomarker of phospholipidosis, i.e. the accumulation of phospholipids within animal cells characterised by the presence of ‘onion bodies’. Again, this was inconsistent with the current study, though it should be noted that no microscopic signs of phospholipidosis were observed in the current work.

An increase in urinary amino acids (specifically alanine following AM administration), concomitant with a decrease in amino acids levels in kidney tissue (at both administration times, but more marked in the AM group) was observed following gentamicin administration, consistent with earlier work. Approximately 99% of amino acids are reabsorbed in the proximal tubule. Under nephrotoxic conditions, amino acid excretion is increased due to either increased membrane permeability, or impaired reabsorption. Boudonck et
al. reported a significant increase in urinary amino acid levels prior to the development of histopathological kidney damage, following exposure to a number of known nephrotoxins, including gentamicin. Therefore, measurement of increased urinary amino acids may be a biomarker for early renal injury, though it lacks specificity for gentamicin-induced damage. In addition, reductions in levels of amino acids were observed in the kidneys at both administration times. While aminoaciduria can be explained by the known mechanism of gentamicin toxicity, i.e. impaired tubular re-absorption, the reasons for these apparently complementary observations in the kidney tissue are likely to be different, at least in part. Rather, this reduction in amino acid levels in the kidney of gentamicin-treated rats is suggestive of perturbations in energy metabolism, a theory supported by perturbations in lactate levels. Further, tissue levels of leucine and isoleucine were reduced following gentamicin exposure suggestive of catabolism of branched-chain amino acids to produce acetyl CoA and glucose via gluconeogenesis. An increase in urinary levels of 2-oxoglutarate, citrate and succinate was seen in the PM treatment group. All are considered non-specific markers, rather they indicate a generalised perturbation of mitochondrial energy metabolism.

Kidneys respond to hypertonic stress, such as occurs with gentamicin toxicity, by accumulating osmolytes, such as MI, betaine and phosphocholine. Following gentamicin administration, levels of all three metabolites were significantly altered relative to control levels, reflective of gentamicin-induced nephrotoxicity. Taurine is also implicated in homeostatic control of renal osmolarity and was elevated in urine following gentamicin exposure. Minor changes in urinary levels of a number of renal osmolytes were reported by Sieber et al. (i.e. TMAO, betaine, methylamine, dimethylglycine, dimethylamine).
Given that no urinary changes in levels of these metabolites were observed in this current study or in other studies of drug-induced renal stress, \(^{155}\) combined with the knowledge that these metabolites are also implicated in choline metabolism \(^{200, 216}\) many of the changes may in fact be reflective of perturbations of gut microbial activity. Choline metabolism is the result of a complex host-symbiotic relationship, involving mammalian and gut microbial metabolism. \(^{200}\) The first step in the metabolism of dietary choline involves conversion to trimethylamine (TMA), mediated by gut microbiota. \(^{218}\) TMA is subsequently detoxified by oxidation to TMAO. In addition, the methylamines are subject to bacterial-mediated interconversions; perturbations in urinary levels of these metabolites likely indicate disruption to one or other of the pathways.

Many of the urinary metabolic perturbations observed following gentamicin exposure mirror those associated with Fanconi syndrome, specifically elevated urinary levels of lactate and amino acids. Associated with a wide variety of inherited and acquired conditions, Fanconi syndrome is a generalised dysfunction of the renal proximal tubules, characterised by a defect in transport mechanisms, resulting in renal loss of glucose, amino acids, uric acids, phosphate, and bicarbonate. \(^{113}\) Primary Fanconi syndrome is associated with a number of inborn errors of amino acid and carbohydrate metabolism, all of which are inherited in an autosomal recessive pattern, including cystinosis (accumulation of cystine in lysosomes), \(^{219}\) galactosaemia (deficiency in activity of galactose-1-phosphate uridyl transferase) and Wilson’s disease. \(^{220}\) Primary Fanconi syndrome is generally observed in childhood. Fanconi syndrome can also be acquired (i.e. secondary) and a number of drugs, including aminoglycoside antibiotics, have been shown to cause renal tubular defects resulting in Fanconi syndrome. \(^{221}\) The urinary metabolic profiles observed following gentamicin exposure in this current
study were consistent with the development of gentamicin-induced Fanconi syndrome.

4.3.2 **Time-Dependent Metabolic Response to Gentamicin**

The extent of aminoglycoside-induced toxicity is dependent on a number of factors, including dose, route of administration, duration of treatment, age and sex. Administration time has also been identified as a contributing factor to the severity of toxic response, with an administration time corresponding to the rest period identified as resulting in a more toxic outcome. 122, 123

NMR spectroscopic analysis identified clear urinary metabolic differences in response to administration of gentamicin at different times. An AM administration had a more marked impact on urinary levels of gut microbial co-metabolites (hippurate, 3-IS, PAG). As previously stated, this is suggestive of the impact of gentamicin on gut microflora and is believed to be a marker of the pharmacological (antibiotic) activity of gentamicin, suggesting the presence of a differential impact on gut microbiota according to treatment time. Wang et al. noted a circadian variation in urinary levels of 3-IS in healthy male subjects, while diurnal variation in urinary levels of hippurate have been reported in the mouse model. Therefore the observed temporal difference may be due to differences in gut microbiota activities during the day or differences in liver activity. This is further supported by the differences identified in kidney levels of phenylalanine and tyrosine: both metabolites were significantly elevated in the PM group, relative to the AM, based on the O-PLS-DA loadings plot.

Gentamicin-induced nephrotoxicity is associated with an increase in urinary levels of alanine and lactate due to impaired reabsorption in the proximal
tubule. Both metabolites were identified as discriminatory between the treatment groups from the O-PLS-DA loadings plot, suggestive of increased toxicity in these individuals. This is consistent with both the microscopic observations made in this study and with earlier findings. Further, those individuals administered an AM dose of gentamicin experienced a significant increase in measured levels of renal osmolytes and a decrease in levels of amino acids in the kidney tissue, all considered biomarkers of nephrotoxicity.

PM administration of gentamicin resulted in significant reductions in urinary levels of succinate, citrate and 2-oxoglutarate; urinary levels of these metabolites were not significantly perturbed following an AM dose. These metabolites are TCA cycle intermediates, and are suggestive of mitochondrial metabolic perturbation. Perturbation of energy pathways was also identified in the kidney tissue following an AM dose of gentamicin (reduced levels of branched-chain amino acids), therefore the metabolic signature observed in the PM animals may be reflective of a less severe toxic response.

Aminoglycoside-induced nephrotoxicity is dependent on accumulation of the drug within the renal cortex: urinary clearance of the drug is lower during the rest period, leading to increased accumulation and toxicity. However, temporal variation in glomerular filtration rate is unlikely to account for all the variation observed and a number of other mechanisms have been proposed, though the exact mechanism remain unclear.

4.3.3 LIMITATIONS AND COMPLICATIONS

The conclusions drawn in this study are limited due to the low animal numbers and the high degree of inter-individual variation in response to treatment observed in the clinical chemistry and histopathological analysis. This has proved
to be a limiting factor for both conventional toxicological assessment and metabonomic analysis. Therefore, caution is urged when considering these results.

A multivariate statistical approach was employed throughout this work to aid in the interpretation of the metabolic changes in response to gentamicin exposure. Multivariate methods, such as O-PLS-DA, can discover significant patterns of variables that contribute to separation between classes, none of which has any statistical significance when considered in isolation. In contrast, a univariate approach, which may be helpful in order to quantify the degree of metabolic perturbation in response to treatment, is not ideal as it involves univariate analysis of isolated metabolites identified from multivariate profiles. Further, the univariate approach will most likely obscure some of these significant changes as observed here.
4.4 CONCLUSION

Based on metabonomic analysis, histopathological analysis and levels of KIM-1, administration of gentamicin was shown to be more toxic in the rat model following an AM administration. Gentamicin administration was characterised by an increase in urinary excretion of amino acids and lactate and reduced levels of gut microbial co-metabolites, including hippurate and 3-IS. \(^1\)H MAS-NMR analysis of intact kidney tissue detected a reduction in levels of amino acids and an increase in levels of lactate, M1 and choline-related metabolites following gentamicin administration. The study identified a high degree of inter-individual variation in response to treatment, limiting the strength of these results.
Chapter 5 STATINS

AIMS AND OBJECTIVES

The primary purpose of this work was to investigate the potential to reduce gentamicin-induced nephrotoxicity through co-administration with a statin. This was done using a combination of traditional toxicity assessments and metabonomics, specifically high resolution (MAS) NMR spectroscopy of urine and intact kidney. Further, the metabolic impact of co-administration of gentamicin, statin and mevalonate on gentamicin-induced nephrotoxicity was investigated through the integration of traditional toxicity assessments with (MAS) NMR of urine and kidney.
5.1 METHODS

The following study was carried out collaboratively with Dr Antoine and Prof Park at the MRC Centre for Drug Safety Science at the University of Liverpool, who designed the study. All animal manipulations were conducted by the MRC Centre for Drug Safety Science at the University of Liverpool.

5.1.1 ANIMAL HANDLING AND SAMPLE COLLECTION

All animal manipulations were conducted by the MRC Centre for Drug Safety Science at the University of Liverpool in full accordance with UK ethical legislation on animal experimentation. Male Sprague-Dawley (SD) rats (approximately 250-330g, n=24) were randomly allocated to six dose groups (n=4) as outlined in Table 5-1. Animals were dosed once daily for nine days via intra-peritoneal (i.p.) injection with control vehicle (0.9% saline), gentamicin (200 mg/kg in 0.9% saline), mevalonate (100 mg/kg in 0.9% saline), atorvastatin (30 mg/kg in 0.9% saline) or in combination, as described below. All injections were carried out at 10.00 a.m.

Table 5-1 Dosing procedure and group assignments.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Animal Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td>Mevalonate</td>
<td>5, 6, 7, 8</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>9, 10, 11, 12</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>13, 14, 15, 16</td>
</tr>
<tr>
<td>Gentamicin + Atorvastatin</td>
<td>17, 18, 19, 20</td>
</tr>
<tr>
<td>Gentamicin + Atorvastatin + Mevalonate</td>
<td>21, 22, 23, 24</td>
</tr>
</tbody>
</table>
Urine was collected over a period of 24 hours following administration of the final dose, and blood collected at termination via cardiac puncture. Kidney tissue samples were collected at termination. Three aliquots were snap frozen pending further analysis, and the remainder frozen in 10% formalin.

5.1.2 **Clinical Chemistry and Histopathology**

Urine parameters (total volume, creatinine, kidney injury molecule 1 (KIM1), and N-acetyl-β-glucosaminidase (NAG)) were recorded using standard methods, as outlined in Chapter 4. This work was conducted by Dr Antoine at the Vaidya/Bonventre laboratory, Harvard Medical School, Significant changes in these parameters from control levels were assessed using a Students two-tailed t-test. The kidney was sampled and fixed in a 10% formalin solution. Representative samples were processed routinely in an automatic tissue processor, embedded in paraffin, sectioned at 4-6 μm, stained with haematoxylin and eosin, and examined histologically using light microscopy. This work was conducted in Professor Park’s laboratory at the University of Liverpool.

5.1.3 **NMR Spectroscopic Analysis of Urine Samples**

Urine samples were prepared and analysed by $^1$H NMR as described in Chapter 4, Section 4.1.3.

5.1.4 **$^1$H MAS-NMR Spectroscopy of Intact Kidney Samples**

Kidney samples were analysed by $^1$H MAS-NMR as described in Chapter 4, Section 4.1.5.
5.1.5 **NMR Spectra Data Processing**

The $^1$H NMR spectra were processed as previously described (Chapter 4, Section 4.1.6) and subject to multivariate statistical analysis (PCA, PLS-DA, O-PLS-DA). For further details, please refer to Chapter 4, Section 4.1.6. Relative concentrations of key metabolites (as identified using multivariate statistical methods) were calculated by integration of specific peaks. Correlation coefficient (R) heat maps were constructed from O-PLS-DA pair-wise models to summarise the metabolic variation observed in the urinary and kidney models in response to different treatment.
5.2 RESULTS

5.2.1 CLINICAL CHEMISTRY AND HISTOPATHOLOGY

Kidney tissue was examined microscopically and scored on a scale of 1 to 4 according to severity of nephrotoxic damage (Figure 5-1c). There were no microscopic changes observed in the control or mevalonate treated group, while administration of atorvastatin resulted in mild changes in 2 out of 4 individuals (severity score of 1), characterised by the presence of tubular casts and cellular changes in the cortical tubules. Administration of gentamicin alone resulted in moderate damage in 3 out of 4 individuals (severity score of 2 or 3), characterised by the presence of necrotic cells in the cortical tubule and multi-focal mono-nuclear interstitial infiltration. Co-administration of gentamicin + atorvastatin resulted in mild changes in 2 out of 4 individuals (severity score of 1), characterised as above. The remaining animals in this treatment group (2 out of 4) exhibited no histopathological changes. Co-administration of gentamicin + atorvastatin + mevalonate resulted in a high degree of inter-individual variation across the treatment group with 2 individuals subject to moderate damage (severity score of 3), and 2 individuals exhibiting no macroscopic changes post treatment. Moderate damage was characterised as before.

Urinary levels of KIM-1 and NAG were measured for all individuals at termination (Figure 5-1a, 1b). All levels were measured relative to creatinine, and are not absolute excretion values. There were no significant changes in levels of KIM-1 or NAG following administration of mevalonate or atorvastatin only, relative to control levels. There were no significant changes in measured levels of KIM-1 and NAG in the gentamicin + atorvastatin co-administered group relative to the control individuals. KIM-1 and NAG were significantly elevated in the gentamicin
only and the gentamicin + atorvastatin + mevalonate co-administered animals relative to control animal levels, though levels of both parameters exhibited a high degree of inter-animal variation in both of these treatment groups.
Figure 5-1 Clinical chemistry parameters and histopathology scores following treatment with gentamicin, mevalonate, and atorvastatin for (a) KIM-1 (ng/uCr/mg), (b) NAG (IU/uCr/mg), and (c) histopathology severity score of nephrotoxic damage.

Individual values are identified with black dots and the bar represents the mean each group of samples. Error bars represent standard deviation. All kidneys were scored from 0 (= no necrosis) to 4 (= severe necrosis). Urinary protein biomarker results were corrected for urinary creatinine levels. Analysis of KIM-1, and NAG was conducted by Dr Antoine at the Vaidya/Bonventre laboratory, Harvard Medical School, while histopathological assessment was conducted in Prof Park’s laboratory at the University of Liverpool. (**) $p < 0.01$. (***) $p < 0.001$.

Key: KIM-1 – kidney injury molecule 1; NAG – $N$-acetyl-$\beta$-glucosaminidase.
5.2.2 Metabolic Impact of Statin Administration

The impact of statin (atorvastatin) administration on urinary and kidney metabolic profiles was assessed through the application of high resolution NMR spectroscopy. Representative $^1$H (MAS) NMR urinary and kidney spectra from control and atorvastatin treated individuals are shown in Figures 5-2 and 5-3. There were no statistically significant differences detected in metabolite levels in the urine or kidney tissue in response to atorvastatin administration, relative to control levels based on O-PLS-DA models (not shown).

Figure 5-2 Representative 600 MHz $^1$H NMR spectra of urine from (a) control and (b) atorvastatin treated rats.

Key: 2-OG – 2-oxoglutarate; 3-IS – 3-indoxylsulfate; DMG – dimethylglycine; NMNA – $N$-methylnicotinic acid; NMND – $N$-methylnicotinamide; PAG – phenylacetylglycine; TMAO – trimethylamine-$N$-oxide.
5.2.3 Metabolic Impact of Mevalonate Administration

The metabolic impact of mevalonate administration was explored through the use of high resolution NMR spectroscopy of urine, and intact kidney. Mevalonate was identified in urinary spectra by the presence of resonances at $\delta$ 1.26 (s), 1.84 (t), 2.38 (d), and 3.87 (t), which were removed using STOCSYE prior to further analysis. STOCSYE offers a highly efficient means of removing highly correlated peaks, i.e. peaks from the same compound such as mevalonate, thereby facilitating analysis using pattern recognition techniques without interference from drug metabolites. Representative 600 MHz $^1$H NMR urinary spectra from control and mevalonate treated individuals, before and after STOCSYE, are shown in Figure 5-5.

Figure 5-3 Representative 600 MHz $^1$H MAS-NMR spectra of kidney from (a) control and (b) atorvastatin treated rats.

Key: PC – phosphocholine; TMAO – trimethylamine-$N$-oxide.
Figure 5-4 Representative 600 MHz $^1$H NMR spectra of urine from (a) mevalonate treated rats, (b) STOCSE spectra showing mevalonate resonance only, (c) mevalonate treated rats post STOCSE and (d) control rats.

STOCSE was applied to remove resonances associated with mevalonate at $\delta$ 1.26 (s), 1.84 (t), 2.38 (d), and 3.87 (t).

After the removal of the mevalonate-associated resonances, there was no statistically significant separation observed between the control and mevalonate-treated urinary or kidney spectra (not shown).

5.2.4 **Co-Administration of Atorvastatin Reduces Gentamicin-Induced Nephrotoxicity**

The metabolic impact of co-administration of atorvastatin and gentamicin was assessed through the application of high resolution NMR spectroscopy to urine and kidney samples, following treatment. Representative $^1$H NMR urinary spectra from control, gentamicin and gentamicin + atorvastatin treated animals are shown in Figure 5-5. Gentamicin was detected in post-dose urine following administration of gentamicin + atorvastatin. Therefore, STOCSYE was again employed to remove the resonances due to gentamicin prior to statistical analysis.¹⁸⁶
Figure 5-5 Representative 600 MHz $^1$H NMR spectra of urine from (a) control (b) gentamicin, (c) STOCSE spectra showing gentamicin resonances only and (d) gentamicin + atorvastatin treated animals.

Key: 2-OG – 2-oxoglutarate; 3-IS – 3-indoxylsulfate; DMG – dimethylglycine; NMNA – N-methylnicotinic acid; NMND – N-methylnicotinamide; PAG – phenylacetylglycine; TMAO – trimethylamine-N-oxide.
Representative $^1$H MAS-NMR spectra of kidney tissue from control, gentamicin and gentamicin + atorvastatin treated animals are presented in Figure 5-6.

Figure 5-6 Representative 600 MHz $^1$H MAS-NMR spectra of kidney from (a) control, (b) gentamicin, and (c) gentamicin + atorvastatin treated animals.

Key: PC – phosphocholine; TMAO – trimethylamine-N-oxide.

The metabolic impact of co-administration of atorvastatin on gentamicin activity was interrogated through multi-variate statistical modelling of urinary and
kidney spectra. The PCA scores plot (PC1 vs PC2 explaining 47% of the total variability) derived from the urinary spectra of control, gentamicin and gentamicin + atorvastatin animals is presented in Figure 5-7. The gentamicin + atorvastatin individuals had an intermediate metabolic profile relative to the other groups. The PCA scores of gentamicin treated individuals were highly variable.

Figure 5-7 PCA scores plot of PC1 vs PC2 obtained from \(^1\)H NMR urinary spectra of control and treated animals.

Key: (■) – control; (■) – gentamicin; (■) – gentamicin + atorvastatin.
PCA was insufficient to separate the kidney spectra of control, gentamicin and gentamicin + atorvastatin treated individuals (data not shown). The urinary spectra from subject 2 (control) contained high levels of betaine, compared to the other individuals in this treatment group. This subject was excluded from subsequent analysis; all control models (urinary and kidney) were therefore constructed with three individuals only.

To obtain a greater insight into the metabolic impact of co-administration of atorvastatin on gentamicin activity, pair-wise O-PLS-DA models were constructed. An O-PLS-DA model showed clear separation between the gentamicin only and gentamicin + atorvastatin treated individuals (Figure 5-8). Interrogation of the corresponding loadings indicated that this separation was primarily due to low levels of gut microbial co-metabolites (i.e. hippurate, 3-indoxylsulfate (3-IS), phenylacetylglycine (PAG), 3-hydroxyphenylpropionate (3-HPPA), dimethylglycine, dimethylamine) and TCA cycle intermediates (i.e. citrate, 2-oxoglutarate) in the urinary spectra of individuals administered gentamicin only.
Figure 5-8 O-PLS-DA model derived from $^1$H NMR urinary spectra of gentamicin ($n=4$) and gentamicin + atorvastatin ($n=4$) treated animals.

(a) Cross validated scores ($T_{cv1}$) versus $Y$ response. Gentamicin + atorvastatin ( ), gentamicin only ( ). (b) Loadings coefficients plot showing metabolites responsible for discrimination between gentamicin + atorvastatin (top) and gentamicin only (bottom) treated animals. Metabolites were assigned using standard spectra, literature values, and 'spike-in' experiments (3-IS, PAG, 3-HPPA), while STOCSY was employed to identify correlated peaks.

Model Statistics: $Q^2_Y$ 0.68; $R^2_Y$ 0.91; $R^2_X$ 0.29; $p = 0.03$.

Key: 2-OG – 2-oxoglutarate; 3-HPPA – 3-hydroxyphenylpropionate; 3-IS – 3-indoxylsulfate; DMA – dimethylamine; DMG – dimethylglycine; PAG – phenylacetylglycine.
The metabolites responsible for discrimination between the gentamicin only and gentamicin + atorvastatin urinary spectra were comparable to those metabolites responsible for separation between the control and gentamicin animals in Chapter 4, i.e. hippurate, 3-IS, PAG, citrate, 2-oxoglutarate. Therefore, theorising that co-administration of gentamicin + atorvastatin had the potential to return the animals to their base metabolic state, the metabolic differences between the control and gentamicin + atorvastatin co-administered animals was explored. The PCA plot (Figure 4-8) showed metabolic separation between these groups, and the resulting O-PLS-DA model confirmed this (Figure 5-9). The statistically robust model identified the major metabolites contributing to the separation as hippurate, TCA cycle intermediates (i.e. 2-oxoglutarate, citrate, succinate), adipate and 2-amino adipate which were all present at reduced levels in the gentamicin + atorvastatin urinary spectra, compared to levels in the control urine. In addition, the gentamicin + atorvastatin co-treated individuals also excreted higher urinary levels of glucose, dimethylglycine, 3-HPPA, lactate, and various amino acids (i.e. alanine, valine, leucine, isoleucine) compared to the control individuals.
Figure 5-9 O-PLS-DA model derived from $^1$H NMR urinary spectra of control ($n=3$) and gentamicin + atorvastatin ($n=4$) treated animals.

(a) Cross validated scores (Tcv1) versus Y response. Control (■); gentamicin + atorvastatin (■). (b) Loadings coefficients plot showing metabolites responsible for discrimination between control (top) and gentamicin + atorvastatin (bottom) treated animals. Metabolites were assigned using standard spectra, and literature values, while STOCSY was employed to identify correlated peaks.

Model Statistics: $Q^2_Y$ 0.82; $R^2_Y$ 0.97; $p$ 0.02.

Key: 2-AA – 2-aminoadipate; 2-OG – 2-oxoglutarate; 3-HPPA – 3-hydroxyphenylpropionate; CIT – citrate; DMG – dimethylglycine; NMNA – N-methylnicotinic acid.
O-PLS-DA pair-wise models were then constructed to investigate the impact of co-administration of gentamicin + atorvastatin on the kidney metabolic profile. A model was computed to assess the metabolic impact of gentamicin + atorvastatin co-administration relative to administration of gentamicin alone (Figure 5-10). Co-administration of gentamicin + atorvastatin resulted in higher levels of amino acids (i.e. tyrosine, phenylalanine, histidine, valine, leucine, isoleucine) in kidney tissue, relative to administration of gentamicin alone. The individuals exposed to gentamicin only had elevated levels of ethanol and glycine post dose.
Figure 5-10 O-PLS-DA model derived from $^1$H MAS-NMR kidney spectra of gentamicin ($n=4$) and gentamicin + atorvastatin ($n=4$) treated animals.

(a) Cross validated scores (Tcv1) versus Y response. Gentamicin + atorvastatin (■); gentamicin only ( ). (b) Loadings coefficients plot showing metabolites responsible for discrimination between gentamicin + atorvastatin (top) and gentamicin only (bottom) treated animals. Metabolites were assigned using standard spectra, and literature values, while STOCSY was employed to identify correlated peaks.

Model Statistics: $Q^2_Y$ 0.44; $R^2_Y$ 0.86; $p$ 0.07.

The O-PLS-DA model constructed to compare the kidney metabolic profiles of the control and gentamicin + atorvastatin treated animals was statistically valid, but identified only minor metabolic differences between these
groups, specifically lower levels of betaine and glycine in the kidney spectra from the gentamicin + atorvastatin treated individuals (Figure 5-11).

Figure 5-11 O-PLS-DA model derived from $^1$H MAS-NMR kidney spectra of control ($n=3$) and gentamicin + atorvastatin ($n=4$) treated animals.

(a) Cross validated scores (Tcv1) versus Y response. Control ( ■ ); gentamicin + atorvastatin ( □ ). (b) Loadings coefficients plot showing metabolites responsible for discrimination between control (top) and gentamicin + atorvastatin (bottom) treated animals. Metabolites were assigned using standard spectra and literature values.

Model Statistics: $Q^2_Y$ 0.61; $R^2_Y$ 0.99; $p$ 0.05.

5.2.5 MEVALONATE ADMINISTRATION REVERSES THE PROTECTIVE EFFECT OF ATORVASTATIN

Finally, the metabolic impact of mevalonate on the urinary and kidney metabolic profiles was explored. While PCA analysis was insufficient to separate the urinary spectra of gentamicin and gentamicin + atorvastatin + mevalonate
treated individuals, it did show separation of these individuals from the control and gentamicin + atorvastatin co-administered animals (Figure 5-12).

![Figure 5-12 PCA scores plot of PC1 vs PC2 vs PC3 obtained from $^1$H NMR urinary spectra of control and treated animals. Key: (●) – control; (■) – gentamicin; (▲) – gentamicin + atorvastatin; (△) – gentamicin + atorvastatin + mevalonate.]

Figure 5-13 shows the first three components (PC1 and PC2 and PC3 accounting for 40% of the total variance) of the PCA analysis of kidney samples from control, gentamicin, gentamicin + atorvastatin, and gentamicin + atorvastatin + mevalonate treated individuals. PCA analysis was unable to separate the kidney NMR spectra collected from the control and gentamicin + atorvastatin treated individuals, and from the gentamicin and gentamicin + atorvastatin + mevalonate treated individuals.
Figure 5-13 PCA scores plot of PC1 vs PC2 vs PC3 obtained from $^1$H MAS-NMR spectra of control and treated animals.

Key: (■) – control; (■) – gentamicin; (■) – gentamicin + atorvastatin; (■) – gentamicin + atorvastatin + mevalonate.

An O-PLS-DA model was constructed to assess the metabolic consequences of gentamicin + atorvastatin co-administration relative to co-administration of gentamicin + atorvastatin + mevalonate (Figure 5-14). Again, the key metabolites contributing to separation were gut microbial co-metabolites, specifically hippurate, 3-IS, PAG, 3-HPPA and dimethylglycine which were all excreted at lower levels following co-administration of gentamicin + atorvastatin + mevalonate.
Figure 5-14 O-PLS-DA model derived from $^1$H NMR urinary spectra of gentamicin + atorvastatin ($n=4$) and gentamicin + atorvastatin + mevalonate ($n=4$) treated animals.

(a) Cross validated scores (Tcv1) versus Y response. Gentamicin + atorvastatin ( ); gentamicin + atorvastatin + mevalonate ( ). (b) Loadings coefficients plot showing metabolites responsible for discrimination between gentamicin + atorvastatin (top) and gentamicin + atorvastatin + mevalonate (bottom) treated animals. Metabolites were assigned using standard spectra, literature values, and 'spike-in' experiments (3-IS, PAG, 3-HPPA) while STOCSY was employed to identify correlated peaks.

Model Statistics: $Q^2 Y 0.72$; $R^2 Y 0.99$; $p 0.02$.

Key: 3-HPPA – 3-hydroxyphenylpropionate; 3-IS – 3-indoxylsulfate; DMG – dimethylglycine; PAG – phenylacetylglucine.
O-PLS-DA showed clear discrimination between the kidney metabolic profiles of the gentamicin + atorvastatin and the gentamicin + atorvastatin + mevalonate treated individuals due to elevated levels of phenylalanine, tyrosine, histidine and betaine in the kidney spectra of those animals co-administered gentamicin + atorvastatin (Figure 5-15). Ethanol levels were higher in the kidney tissue from the gentamicin + atorvastatin + mevalonate co-administered individuals.
Figure 5-15 O-PLS-DA model derived from $^1$H MAS-NMR kidney spectra of gentamicin + atorvastatin (n=4) and gentamicin + atorvastatin + mevalonate (n=4) treated animals.

(a) Cross validated scores (Tcv1) versus Y response. Gentamicin + atorvastatin (■); gentamicin + atorvastatin + mevalonate (■). (b) Loadings coefficients plot showing metabolites responsible for discrimination between gentamicin + atorvastatin (top) and gentamicin + atorvastatin + mevalonate (bottom) treated animals. Metabolites were assigned using standard spectra, and literature values, while STOCSY was employed to identify correlated peaks.

Model Statistics: $Q^2_Y$ 0.42; $R^2_Y$ 0.93; $p$ 0.07.
An O-PLS-DA model was constructed to identify urinary metabolic differences between the gentamicin only and gentamicin + atorvastatin + mevalonate treated animals: the resulting model had a negative $Q^2_Y$ value (data not shown). Therefore, there was no statistically significant metabolic difference between these groups. Further, O-PLS-DA was unable to separate the MAS-NMR spectra of kidney obtained from those individuals treated with gentamicin alone and those co-administered gentamicin + atorvastatin + mevalonate: again, the O-PLS-DA model constructed comparing these individuals had a low $Q^2_Y$ and a high $R^2_X$, indicating the model was not reliable.

Heat maps were constructed to summarise the results of the O-PLS-DA models computed from urinary and kidney NMR spectra, and identify similarities and differences in the metabolic response to difference treatment regimens (Figures 5-16 and 17). In columns 1 and 3, correlations were expressed relative to the control models, and in columns 2 and 4, to gentamicin + atorvastatin models. In all cases, blue indicated a positive correlation (i.e. elevated levels of this metabolite), while red represented a negative correlation (i.e. levels of the metabolite were decreased).

The urinary and kidney metabolic perturbations in response to gentamicin administration, relative to control levels, were comparable to those identified following treatment with gentamicin + atorvastatin versus gentamicin + atorvastatin + mevalonate. Administration of gentamicin alone resulted in a decrease in urinary levels of TCA cycle intermediates (2-OG, citrate, succinate), gut microbial co-metabolites (3-IS, 3-HPPA, PAG and hippurate), and this metabolic pattern was replicated in the gentamicin treated animals, relative to the gentamicin + atorvastatin animals. Further, a comparable metabolic response was also noted in the kidney models, with gentamicin (relative to control) and
gentamicin (relative to gentamicin + atorvastatin) resulting in a significant decrease in levels of amino acids (alanine, valine, leucine), an increase in renal osmolytes (MI, phosphocholine). In addition, these heat maps also highlight the similarities between the metabolites responsible for separation between the gentamicin + atorvastatin animals relative to the gentamicin + atorvastatin + mevalonate, and those metabolites contributing to separation between the gentamicin only animals, relative to control (and to gentamicin + atorvastatin).

Administration of gentamicin + atorvastatin relative to control resulted in unique metabolic signature in both the urine and kidney models. Urinary levels of amino acids were significantly increased following treatment, while levels of 2-aminoadipate and adipate were elevated. Urinary levels of hippurate and TCA cycle intermediates (2-OG, citrate, succinate) were decreased in treated animals relative to control levels. The heat map derived from the kidney model of control versus gentamicin + atorvastatin showed less significant metabolites changes in response to treatment, although there was mild reductions in levels of kidney amino acids and mild elevations in levels of renal osmolytes in treated kidney relative to control.
Figure 5-16 Correlation coefficient heat map generated from four O-PLS-DA pairwise models, summarising urinary metabolic variation in response to therapeutic intervention.

Metabolites coloured blue are increased relative to control levels (in columns 1 and 2), gentamicin levels (column 3) and gentamicin + atorvastatin (column 4); metabolites in red are decreased relative to these treatment groups.

Key: 3-HPPA – 3-hydroxyphenylpropionic acid; NMNA – N-methylnicotinamide.
Figure 5-17 Correlation coefficient heat map generated from four O-PLS-DA pairwise models, summarising metabolic variation in kidney tissue in response to therapeutic intervention.

Metabolites coloured blue are increased relative to control levels (in columns 1 and 2), gentamicin levels (column 3) and gentamicin + atorvastatin (column 4); metabolites in red are decreased relative to these treatment groups.
5.3 DISCUSSION

Statins are among the most widely prescribed drugs worldwide. They are well-tolerated with limited adverse effects, though they can cause myopathy.\(^\text{228}\) Also known as HMG-CoA reductase inhibitors, statins lower cholesterol levels by competitively inhibiting the rate-limiting step in cholesterol synthesis. The pathway responsible for synthesising cholesterol is called the mevalonate pathway, and it is also implicated in a number of other endogenous functions, including terpenoid synthesis, protein prenylation, cell membrane maintenance, hormone synthesis, protein anchoring and N-glycosylation. Further, the mevalonate pathway plays a key role in the internalisation of gentamicin into the kidney cell resulting in nephrotoxicity. It was therefore theorised by Professor Park of the University of Liverpool that statins have the potential to reduce gentamicin toxicity through the disruption of the mevalonate pathway.\(^\text{121}\) This was assessed through the measurement of urinary biomarkers and high resolution NMR analysis of urine and intact kidney.

5.3.1 ATORVASTATIN IS WELL-TOLERATED WITH LIMITED METABOLIC IMPACT

It has been previously shown that statin exposure in rats, specifically simvastatin, results in elevated urinary levels of 2-oxoglutarate, allantoin and TMAO following daily administration at 80 mg/kg.\(^\text{230}\) It was theorised that statin administration had the potential to induce toxicity via oxidative stress related inflammation. The same study did however observe a high degree of inter-individual variation with the statin-treated group. While these metabolic differences were reflective of differential levels of toxicity observed by Yang \textit{et al.},
based on serum enzyme levels, the differential metabotypes reflective of the two populations were not explored in detail. Kumar et al. looked specifically at the impact of atorvastatin, as used in this current study, and noted urinary perturbations in a number of amino acids through UPLC-MS metabolic profiling, though this study was carried out in hyperlipidaemic rats. In this current study, administration of atorvastatin alone had no significant impact on urinary or kidney metabolic profiles. This was likely due to the differences in administered dose: the present study used 30 mg/kg compared to 80 mg/kg by Yang et al, and 70 and 250 mg/kg by Kumar et al. Statins are well-tolerated and no atorvastatin-induced changes were detected in urinary levels of KIM1, NAG or in the microscopic appearance of the kidney tissue.

5.3.2 ATORVASTATIN REDUCES GENTAMICIN-INDUCED NEPHROTOXICITY

It was proposed that by disrupting the mevalonate pathway co-administration of atorvastatin would prevent the accumulation of gentamicin and the subsequent development of drug-induced nephrotoxicity. This theory was supported by the results of conventional toxicity assessments (i.e. histopathological severity scores, KIM-1 and NAG) which indicated that gentamicin + atorvastatin co-administration was non-toxic in the rat model.

It was established that administration of gentamicin resulted in elevated urinary levels of amino acids, lactate and glucose, due to impaired re-absorption, and reduced levels of a range of gut microbial co-metabolites, linked to the antibiotic action of gentamicin, relative to control levels (Chapter 4, 155, 156, 206, 232). In the present study, a number of the urinary metabolites responsible for separation between the gentamicin alone and gentamicin + atorvastatin animals
were all linked to gut microbial co-metabolism, specifically hippurate, 3-IS, 3-HPPA and PAG \(204, 208-210\), which were all reduced following administration of gentamicin alone. Perturbations in these metabolites are likely reflective of the pharmacological, i.e. antibiotic, action of gentamicin. Analysis of the kidney spectra also identified perturbations in levels of phenylalanine and tyrosine, which were both elevated in the kidney collected from the animals co-administered gentamicin + atorvastatin. Again, both metabolites are implicated in bacterial mediated catabolism \(233\) and an increase in levels of these metabolites was considered suggestive of differential bacterial metabolism between these groups. Therefore, the impact observed on gut microbial co-metabolites in both the urinary and kidney spectra is likely a reflection of the systemic effect of gentamicin administration.

There was no significant increase in urinary levels of amino acids, glucose and lactate following administration of gentamicin alone, relative to co-administration of gentamicin + atorvastatin. If it is assumed that these metabolites are markers of gentamicin-induced nephrotoxicity due to impaired reabsorption, then it appears there was comparable levels of toxicity in both groups. However, it should be remembered that excretion rates are not known and all metabolic changes are expressed relative to control levels. Further, an increase in urinary levels of amino acids, lactate and glucose contributed to the separation observed between the control and the gentamicin + atorvastatin treated animals, also indicating that gentamicin retained a degree of nephrotoxicity when co-administered with atorvastatin. While this suggested that co-administration of atorvastatin did not block gentamicin toxicity, analysis of the individual animal integral values identified a high degree of variability within the treatment groups, therefore reducing the significance of any observed changes. Although it is worth
noting that this variability in metabolic response was also reflected in the results of the conventional toxicity assessments, and is therefore not reflective of any shortcoming in the metabonomic approach. Reduced urinary levels of hippurate, as seen following administration of gentamicin alone and co-administration of gentamicin and atorvastatin, may also reflect gentamicin-induced toxicity. Hippurate is synthesised in renal and hepatic mitochondria from glycine and benzoic acid and secreted by renal tubular cells into the urine.\textsuperscript{205} A decrease in urinary hippurate may therefore indicate perturbation in tubular excretion as a consequence of gentamicin-induced nephrotoxicity, in addition to its importance as a microbial co-metabolite.

Analysis of the kidney spectra suggested that the toxicity experienced by the gentamicin + atorvastatin co-administered animals was less severe than that following administration of gentamicin alone: there were elevated levels of amino acids present in the tissue of those animals co-administered gentamicin + atorvastatin relative to gentamicin alone. Gentamicin administration alone results in a decrease in kidney amino acid levels due to impaired re-absorption and perturbation of energy metabolism (Chapter 4, 155). Therefore, the relative increase in levels of amino acids suggested that this renal impairment was significantly reduced following co-administration of atorvastatin.

Given the small animal numbers and the high degree of variability in response to treatment, the true significance of the metabolic changes observed in this study in response to gentamicin are likely being masked. Allowing for these apparent shortcomings in experimental design, the metabonomic approach proved useful in the detection of subtle changes following gentamicin exposure. Therefore, when considered in conjunction with the conventional markers of
toxicity, the application of metabonomics may serve to offer a more complete picture than either technique in isolation.

Co-administration of atorvastatin with gentamicin was found to reduce the toxicity of gentamicin. Of the animals treated with gentamicin alone or in combination, gentamicin was detected in the post dose urine collected from the gentamicin + atorvastatin individuals only. This suggests that atorvastatin resulted in increased excretion of gentamicin by blocking uptake and accumulation in the kidney, and reducing toxicity. However, metabonomic analysis suggested that co-administration of atorvastatin impacted on the antibiotic action of gentamicin, albeit on a systemic level, likely due to the dose of atorvastatin administered: the dose used was 30 mg/kg compared to a therapeutic dose of between 10 and 80 mg per day. While increasing the dose may result in a greater reduction in toxicity, it may also result in further undesired loss of antibiotic activity, though the extent is unclear based on current work. Clearly, from a clinical viewpoint, any impairment of the antibiotic action of gentamicin is unwanted. In addition, while statins are generally well-tolerated at therapeutic doses, administration of high doses can result in a broad range of adverse effects in a variety of tissue types, due to the importance of the mevalonate pathway and its products in many important endogenous functions. Therefore, complete blockage of the mevalonate pathway is undesirable.

5.3.3 Administration of Mevalonate Induces Minor Metabolic Changes

Mevalonate plays a key role in the internalisation of gentamicin into kidney cells and the subsequent development of gentamicin-induced nephrotoxicity, and administration of mevalonate alone was found to have no significant impact on
the urinary and kidney metabolic profiles or on any of the conventional markers of renal toxicity.

5.3.4 **CO-ADMINISTRATION OF MEVALONATE REVERSES THE PROTECTIVE EFFECT OF ATORVASTATIN**

It has been established that atorvastatin co-administration can reduce the nephrotoxic effect of gentamicin, due to interference with the mevalonate pathway via inhibition of HMG-CoA reductase. Mevalonate is the immediate product of HMG-CoA reductase, therefore it was further theorised that the combined administration of gentamicin, mevalonate and atorvastatin would have a toxic and metabolic impact comparable to that seen following administration of gentamicin alone. Further, co-administration of gentamicin, mevalonate and atorvastatin would provide proof of the importance of the mevalonate pathway in the development of gentamicin-induced nephrotoxicity.

The ability of mevalonate to reverse the impact of statin administration was explored previously. 235, 236 Although this research focused on the potential for statin-induced hepatotoxicity, they observed a reduction in toxicity in the rat, on the basis of serum enzyme levels, following co-administration of lovastatin and mevalonate, relative to lovastatin alone. 235 Similar observations were made in the rabbit. 236

Here, both KIM-1 and NAG were significantly elevated in the gentamicin + atorvastatin + mevalonate animals relative to control levels, and there was no significant difference between this group and the gentamicin only animals. Having established that atorvastatin could negate the toxic effect of gentamicin, this supported the theory that mevalonate could overcome this protective effect, and offered proof of the importance of the mevalonate pathway in the development of
gentamicin-induced nephrotoxicity, thereby supporting the hypothesis originally proposed by Antoine et al. The histopathology scores for the co-administered group offered additional support for this hypothesis: moderate damage was observed in the gentamicin + atorvastatin + mevalonate group, comparable to that observed in the gentamicin only group. It was noted however that all three markers of toxicity showed a high degree of inter-individual variability in response to gentamicin + atorvastatin + mevalonate.

Additional support was obtained through the application of metabolic profiling techniques: the urinary and kidney metabolic profiles of gentamicin only and gentamicin + atorvastatin + mevalonate treated individuals were indistinguishable. There were however clear metabolic differences between the gentamicin + atorvastatin + mevalonate and gentamicin + atorvastatin co-administered individuals. The major urinary metabolites contributing to this separation were linked to gut microbial co-metabolism, specifically hippurate, 3-IS and PAG, which were all reduced in the gentamicin + atorvastatin + mevalonate animals. This was reflective of impaired antibiotic activity following administration of gentamicin + atorvastatin, an effect which was reversed with the addition of mevalonate. There were no significant differences in urinary levels of amino acids, lactate or glucose; given that elevated urinary levels of these metabolites are considered biomarkers of proximal tubule damage it was expected that the gentamicin + atorvastatin + mevalonate animals would excrete elevated levels of these metabolites. It was noted that levels of these metabolites showed a high degree of inter-individual variation in the gentamicin + atorvastatin + mevalonate group which may be obscuring the complete metabolic picture.

Again, the interpretation of the metabolic impact of co-administration of gentamicin + atorvastatin + mevalonate was complicated by the experimental
design and the high degree of inter-individual variation within the treatment groups. Despite this, the combination of conventional urinary markers of toxicity and NMR-based metabonomic analysis of urine and kidney, indicated that the combined administration of gentamicin + atorvastatin + mevalonate resulted in a outcome comparable to administration of gentamicin alone. This further demonstrates the critical role of the mevalonate pathway in the development of gentamicin-induced nephrotoxicity.
5.4 CONCLUSION

Co-administration of a statin was shown to reduce gentamicin-induced nephrotoxicity in the rat model, as measured using urinary levels of KIM-1 and NAG, histopathological examination and high resolution NMR of urine and intact kidney. However, the addition of a statin may potentially impact on the antibiotic effect of gentamicin to a degree, as indicated by perturbations in a number of gut microbial co-metabolites. Further exploration is necessary to quantify this effect. The protective effect of the statin was reversed via co-administration of mevalonate, thereby demonstrating the key role that the mevalonate pathway plays in the development of gentamicin-induced toxicity. Disruption to this pathway can prevent gentamicin uptake and subsequent nephrotoxicity. It is however important to note that inter-individual variation and low animal numbers limit the strength of any conclusions drawn from this study. Further, the experiments were designed to provide the maximum amount of information with the minimum number of animals, an aim that was clearly achieved.
Chapter 6 GENERAL DISCUSSION

The purpose of this work was to increase the understanding of two widely prescribed therapeutic agents through the application of a high resolution NMR-based metabonomic approach, enabling simultaneous identification of endogenous and exogenous metabolites in a range of biological matrices.

A major problem with any treatment regimen is inter-individual variation in response, which can potentially result in pharmacological failure or adverse reactions. The INH study in Chapter 3 concluded that acetylator capacity was key to the development of INH-induced neurotoxicity, and that acetylator capacity could be predicted on the basis of pre-dose urinary metabolic profiles in rats. Further areas of research would include complete characterisation of these pre-dose urinary metabolites and the investigation of the impact of the presence or absence of these metabolites on acetylation of other compounds. Identification of these metabolites has so far been unsuccessful despite the application of a range of 2D NMR experiments and the application of hyphenated techniques. Identification would hopefully enable a greater understanding of the role these metabolites play in INH-induced neurotoxicity. Acetylation is key to drug metabolism and detoxification, therefore, these metabolites may have implications for the efficacy or toxicity of other compounds. Ideally, this would involve a larger cohort of animals and would require urinary profiles to be obtained prior to dosing to enable classification of animals as strong, weak and intermediate responders, to ensure adequate numbers in each group.

Ultimately, the hope is that this work would have a role in the provision of personalised or stratified health care. For this to happen, the findings need to be translatable to humans. Therefore, it would be necessary to analyse human urine
samples for the presence of these metabolites. If it is established as being present, further studies would be necessary to assess the relationship between this metabolite and patient acetylation capacity.

Chapters 4 and 5 focused on the toxic impact of gentamicin and the ability to enhance or reduce its toxicity. This was a pilot study and therefore the animal numbers were small. The studies suggest that gentamicin-induced toxicity could be reduced via co-administration with atorvastatin, and while the results were promising it is clear that further work with a larger cohort is necessary to confirm the findings and account for the high degree of inter-individual variation observed. It would therefore be premature to talk about the future implications of this work on human healthcare, though the findings are potentially of great benefit. Gentamicin is widely prescribed due to its relative cost and high efficacy but development of nephrotoxicity is a dose-limiting factor. Reduction of toxicity without loss of pharmacological activity would greatly enhance the usefulness of gentamicin.

Overall this work has provided further evidence of the benefits of a metabonomic-based approach in toxicity studies. Metabolic profiling identified clear endogenous and exogenous metabolic signatures reflective of INH and gentamicin therapy. The application of pharmacometabonomics enabled determination of a baseline, pre-treatment metabolic signature that was predictive of the differential metabolic fate of INH. The integrated metabonomic approach for the investigation of gentamicin toxicity demonstrated the combined use of conventional solution state NMR spectroscopy and MAS NMR spectroscopy for the analysis of biofluids and intact tissues, and the application of pattern recognition techniques in probing the biochemical response to a xenobiotic, in this case, gentamicin.
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APPENDIX I – SUPPLEMENTAL DATA

Chapter 3 – Isoniazid

Table 7-1 Integral values for key INH-related urinary metabolites.

All integral values have been divided by $10^6$.

### Control, 0-7 Hours

<table>
<thead>
<tr>
<th>No.</th>
<th>INH</th>
<th>AcINH</th>
<th>INA</th>
<th>INH-GLC</th>
<th>INH-PA</th>
<th>INH-KA</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
<td>33</td>
<td>70</td>
<td>1.6</td>
<td>112</td>
<td>69</td>
<td>26</td>
</tr>
<tr>
<td>002</td>
<td>28</td>
<td>78</td>
<td>1.2</td>
<td>104</td>
<td>64</td>
<td>26</td>
</tr>
<tr>
<td>003</td>
<td>25</td>
<td>79</td>
<td>1.3</td>
<td>108</td>
<td>71</td>
<td>28</td>
</tr>
<tr>
<td>004</td>
<td>28</td>
<td>68</td>
<td>1.7</td>
<td>101</td>
<td>62</td>
<td>26</td>
</tr>
<tr>
<td>005</td>
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<td>61</td>
<td>1.6</td>
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<td>55</td>
<td>28</td>
</tr>
<tr>
<td>006</td>
<td>25</td>
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<td>0.5</td>
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<td>15</td>
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<tr>
<td>007</td>
<td>18</td>
<td>79</td>
<td>7.9</td>
<td>110</td>
<td>81</td>
<td>21</td>
</tr>
<tr>
<td>008</td>
<td>26</td>
<td>71</td>
<td>1.3</td>
<td>104</td>
<td>69</td>
<td>22</td>
</tr>
<tr>
<td>009</td>
<td>21</td>
<td>82</td>
<td>1.5</td>
<td>101</td>
<td>79</td>
<td>27</td>
</tr>
<tr>
<td>010</td>
<td>26</td>
<td>68</td>
<td>1.5</td>
<td>100</td>
<td>65</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>26 ± 4.1</td>
<td>75 ± 8.6</td>
<td>1.3 ± 0.4</td>
<td>105 ± 4.4</td>
<td>71 ± 11</td>
<td>25 ± 4.1</td>
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</tbody>
</table>

### Low Dose, 0-7 Hours

<table>
<thead>
<tr>
<th>No.</th>
<th>INH</th>
<th>AcINH</th>
<th>INA</th>
<th>INH-GLC</th>
<th>INH-PA</th>
<th>INH-KA</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>304</td>
<td>440</td>
<td>248</td>
<td>125</td>
<td>225</td>
<td>194</td>
</tr>
<tr>
<td>102</td>
<td>921</td>
<td>443</td>
<td>347</td>
<td>151</td>
<td>353</td>
<td>198</td>
</tr>
<tr>
<td>103</td>
<td>201</td>
<td>342</td>
<td>178</td>
<td>118</td>
<td>182</td>
<td>233</td>
</tr>
<tr>
<td>104</td>
<td>151</td>
<td>301</td>
<td>166</td>
<td>115</td>
<td>126</td>
<td>166</td>
</tr>
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<td>105</td>
<td>1150</td>
<td>409</td>
<td>375</td>
<td>146</td>
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<td>106</td>
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<td>137</td>
<td>337</td>
<td>175</td>
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<td>128</td>
<td>255</td>
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<td>108</td>
<td>1270</td>
<td>441</td>
<td>377</td>
<td>149</td>
<td>584</td>
<td>311</td>
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<tr>
<td>109</td>
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<td>485</td>
<td>229</td>
<td>100</td>
<td>183</td>
<td>245</td>
</tr>
<tr>
<td>110</td>
<td>959</td>
<td>298</td>
<td>262</td>
<td>163</td>
<td>369</td>
<td>265</td>
</tr>
<tr>
<td>Total</td>
<td>292 ± 165</td>
<td>390 ± 78</td>
<td>206 ± 34</td>
<td>117 ± 11</td>
<td>194 ± 49</td>
<td>205 ± 3</td>
</tr>
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</table>

### High Dose (CNS Non-Responders), 0-7 Hours

<table>
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<th>INA</th>
<th>INH-GLC</th>
<th>INH-PA</th>
<th>INH-KA</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>539</td>
<td>392</td>
<td>283</td>
<td>150</td>
<td>298</td>
<td>232</td>
</tr>
<tr>
<td>202</td>
<td>866</td>
<td>461</td>
<td>364</td>
<td>127</td>
<td>309</td>
<td>250</td>
</tr>
<tr>
<td>203</td>
<td>600</td>
<td>415</td>
<td>296</td>
<td>117</td>
<td>289</td>
<td>199</td>
</tr>
<tr>
<td>208</td>
<td>762</td>
<td>560</td>
<td>332</td>
<td>188</td>
<td>404</td>
<td>221</td>
</tr>
<tr>
<td>210</td>
<td>524</td>
<td>436</td>
<td>361</td>
<td>113</td>
<td>171</td>
<td>126</td>
</tr>
<tr>
<td>Total</td>
<td>658 ± 150</td>
<td>452 ± 65</td>
<td>327 ± 37</td>
<td>139 ± 31</td>
<td>294 ± 83</td>
<td>206 ± 48</td>
</tr>
</tbody>
</table>

### High Dose (CNS Responders), 0-7 Hours

<table>
<thead>
<tr>
<th>No.</th>
<th>INH</th>
<th>AcINH</th>
<th>INA</th>
<th>INH-GLC</th>
<th>INH-PA</th>
<th>INH-KA</th>
</tr>
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<tbody>
<tr>
<td>204</td>
<td>744</td>
<td>353</td>
<td>552</td>
<td>209</td>
<td>1010</td>
<td>294</td>
</tr>
<tr>
<td>205</td>
<td>498</td>
<td>211</td>
<td>316</td>
<td>152</td>
<td>776</td>
<td>214</td>
</tr>
<tr>
<td>207</td>
<td>957</td>
<td>210</td>
<td>301</td>
<td>871</td>
<td>679</td>
<td>308</td>
</tr>
<tr>
<td>Total</td>
<td>733 ± 230</td>
<td>258 ± 82</td>
<td>390 ± 141</td>
<td>1490 ± 608</td>
<td>821 ± 170</td>
<td>272 ± 51</td>
</tr>
</tbody>
</table>
### High Dose (CNS Non-Responder*), 0-7 Hours

<table>
<thead>
<tr>
<th>No.</th>
<th>INH</th>
<th>AcINH</th>
<th>INA</th>
<th>INH-GLC</th>
<th>INH-PA</th>
<th>INH-KA</th>
</tr>
</thead>
<tbody>
<tr>
<td>206</td>
<td>671</td>
<td>379</td>
<td>478</td>
<td>1080</td>
<td>815</td>
<td>292</td>
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</table>

### Control, 7-24 Hours

<table>
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<tr>
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<th>INA</th>
<th>INH-GLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>001</td>
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<td>0.7</td>
<td>89</td>
</tr>
<tr>
<td>002</td>
<td>26</td>
<td>62</td>
<td>1.6</td>
<td>81</td>
</tr>
<tr>
<td>003</td>
<td>22</td>
<td>68</td>
<td>1.3</td>
<td>77</td>
</tr>
<tr>
<td>004</td>
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<td>70</td>
</tr>
<tr>
<td>005</td>
<td>23</td>
<td>64</td>
<td>1.7</td>
<td>86</td>
</tr>
<tr>
<td>006</td>
<td>23</td>
<td>66</td>
<td>1.1</td>
<td>82</td>
</tr>
<tr>
<td>007</td>
<td>19</td>
<td>73</td>
<td>1.7</td>
<td>76</td>
</tr>
<tr>
<td>008</td>
<td>24</td>
<td>65</td>
<td>3.7</td>
<td>76</td>
</tr>
<tr>
<td>009</td>
<td>20</td>
<td>65</td>
<td>1.8</td>
<td>75</td>
</tr>
<tr>
<td>010</td>
<td>12</td>
<td>70</td>
<td>0.9</td>
<td>71</td>
</tr>
<tr>
<td>Total</td>
<td>24 ± 3.5</td>
<td>66 ± 3.3</td>
<td>1.9 ± 0.5</td>
<td>78 ± 6.2</td>
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</table>

### Low Dose, 7-24 Hours

<table>
<thead>
<tr>
<th>No.</th>
<th>INH</th>
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<th>INA</th>
<th>INH-GLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>68</td>
<td>318</td>
<td>119</td>
<td>85</td>
</tr>
<tr>
<td>102</td>
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<td>103</td>
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</tr>
<tr>
<td>105</td>
<td>62</td>
<td>227</td>
<td>104</td>
<td>82</td>
</tr>
<tr>
<td>106</td>
<td>27</td>
<td>193</td>
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<td>107</td>
<td>75</td>
<td>248</td>
<td>100</td>
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</tr>
<tr>
<td>108</td>
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<tr>
<td>109</td>
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<td>262</td>
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</tr>
<tr>
<td>110</td>
<td>55</td>
<td>209</td>
<td>77</td>
<td>88</td>
</tr>
<tr>
<td>Total</td>
<td>55 ± 14</td>
<td>259 ± 5</td>
<td>100 ± 18</td>
<td>84 ± 8.3</td>
</tr>
</tbody>
</table>

### High Dose (CNS Non-Responders), 7-24 Hours

<table>
<thead>
<tr>
<th>No.</th>
<th>INH</th>
<th>AcINH</th>
<th>INA</th>
<th>INH-GLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>201</td>
<td>403</td>
<td>217</td>
<td>141</td>
</tr>
<tr>
<td>202</td>
<td>171</td>
<td>395</td>
<td>200</td>
<td>128</td>
</tr>
<tr>
<td>203</td>
<td>216</td>
<td>478</td>
<td>228</td>
<td>135</td>
</tr>
<tr>
<td>208</td>
<td>168</td>
<td>477</td>
<td>245</td>
<td>92</td>
</tr>
<tr>
<td>210</td>
<td>122</td>
<td>378</td>
<td>167</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>176 ± 36</td>
<td>418 ± 59</td>
<td>211 ± 29</td>
<td>114 ± 3</td>
</tr>
</tbody>
</table>

### High Dose (CNS Non-Responder*), 7-24 Hours

<table>
<thead>
<tr>
<th>No.</th>
<th>INH</th>
<th>AcINH</th>
<th>INA</th>
<th>INH-GLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>206</td>
<td>52</td>
<td>275</td>
<td>121</td>
<td>93</td>
</tr>
</tbody>
</table>

Integrals were measured for resonances at δ 2.150 (AcINH), 2.194 (INH-PA), 4.263 (INH-GLC), 7.705 (INH), 7.908 (INH-KA) and 8.619 (INA).

Key: AcINH – acetylisoniazid; INA – isonicotinic acid; INH – isoniazid; INH-GLC – glucosyl isonicotinylhydrazide; INH-KA – 2-oxoglutarate isonicotinylhydrazone; INH-PA – pyruvate isonicotinylhydrazone.
(* This individual exhibited no overt clinical signs of adverse CNS effects but shared some of the metabolic features of the CNS responders)
Chapter 4 – Gentamicin

To account for the low animal numbers used in this study, integrals were calculated for the metabolites responsible for separation between the groups. Individual values for each animal are listed; group values are expressed as mean ± standard deviation. All integral values have been divided by $10^6$.

Table 7-2 Integral values for key urinary metabolites.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control, AM</th>
<th>Control, PM</th>
<th>Gentamicin, AM</th>
<th>Gentamicin, PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OG</td>
<td>130 ± 3.8</td>
<td>120 ± 4.6</td>
<td>120 ± 8.8</td>
<td>950 ± 110</td>
</tr>
<tr>
<td>3-IS</td>
<td>120 ± 2.8</td>
<td>120 ± 3.9</td>
<td>120 ± 7.9</td>
<td>90 ± 16</td>
</tr>
<tr>
<td>Alanine</td>
<td>140 ± 2.6</td>
<td>120 ± 4.1</td>
<td>130 ± 19</td>
<td>180 ± 16</td>
</tr>
<tr>
<td>Citrate</td>
<td>140 ± 2.6</td>
<td>140 ± 2.6</td>
<td>170 ± 30</td>
<td>1600 ± 130</td>
</tr>
<tr>
<td>Hippurate</td>
<td>66 ± 1.1</td>
<td>110 ± 3.5</td>
<td>190 ± 3.3</td>
<td>1600 ± 20</td>
</tr>
<tr>
<td>Lactate</td>
<td>190 ± 2.8</td>
<td>140 ± 2.8</td>
<td>150 ± 15</td>
<td>190 ± 2.8</td>
</tr>
<tr>
<td>PAG</td>
<td>310 ± 3.3</td>
<td>310 ± 3.3</td>
<td>350 ± 30</td>
<td>320 ± 30</td>
</tr>
<tr>
<td>Succinate</td>
<td>270 ± 3.9</td>
<td>270 ± 3.9</td>
<td>340 ± 45</td>
<td>300 ± 35</td>
</tr>
<tr>
<td>Taurine</td>
<td>290 ± 4.5</td>
<td>290 ± 4.5</td>
<td>340 ± 45</td>
<td>570 ± 55</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control, PM</th>
<th>Control, AM</th>
<th>Gentamicin, AM</th>
<th>Gentamicin, PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OG</td>
<td>140 ± 2.6</td>
<td>120 ± 4.6</td>
<td>120 ± 8.8</td>
<td>950 ± 110</td>
</tr>
<tr>
<td>3-IS</td>
<td>120 ± 2.8</td>
<td>120 ± 3.9</td>
<td>120 ± 7.9</td>
<td>90 ± 16</td>
</tr>
<tr>
<td>Alanine</td>
<td>140 ± 2.6</td>
<td>120 ± 4.1</td>
<td>130 ± 19</td>
<td>180 ± 16</td>
</tr>
<tr>
<td>Citrate</td>
<td>140 ± 2.6</td>
<td>140 ± 2.6</td>
<td>170 ± 30</td>
<td>1600 ± 130</td>
</tr>
<tr>
<td>Hippurate</td>
<td>66 ± 1.1</td>
<td>110 ± 3.5</td>
<td>190 ± 3.3</td>
<td>1600 ± 20</td>
</tr>
<tr>
<td>Lactate</td>
<td>190 ± 2.8</td>
<td>140 ± 2.8</td>
<td>150 ± 15</td>
<td>190 ± 2.8</td>
</tr>
<tr>
<td>PAG</td>
<td>310 ± 3.3</td>
<td>310 ± 3.3</td>
<td>350 ± 30</td>
<td>320 ± 30</td>
</tr>
<tr>
<td>Succinate</td>
<td>270 ± 3.9</td>
<td>270 ± 3.9</td>
<td>340 ± 45</td>
<td>300 ± 35</td>
</tr>
<tr>
<td>Taurine</td>
<td>290 ± 4.5</td>
<td>290 ± 4.5</td>
<td>340 ± 45</td>
<td>570 ± 55</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>11</th>
<th>12</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-OG</td>
<td>1300 ± 260</td>
<td>1300 ± 260</td>
<td>1300 ± 260</td>
</tr>
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<td>3-IS</td>
<td>67 ± 9.3</td>
<td>67 ± 9.3</td>
<td>67 ± 9.3</td>
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<td>120 ± 2.8</td>
<td>120 ± 2.8</td>
</tr>
<tr>
<td>Citrate</td>
<td>360 ± 33</td>
<td>360 ± 33</td>
<td>360 ± 33</td>
</tr>
<tr>
<td>Hippurate</td>
<td>160 ± 3.9</td>
<td>160 ± 3.9</td>
<td>160 ± 3.9</td>
</tr>
<tr>
<td>Lactate</td>
<td>140 ± 17</td>
<td>140 ± 17</td>
<td>140 ± 17</td>
</tr>
<tr>
<td>PAG</td>
<td>440 ± 130</td>
<td>440 ± 130</td>
<td>440 ± 130</td>
</tr>
<tr>
<td>Succinate</td>
<td>290 ± 45</td>
<td>290 ± 45</td>
<td>290 ± 45</td>
</tr>
<tr>
<td>Taurine</td>
<td>1200 ± 8.8</td>
<td>1200 ± 8.8</td>
<td>1200 ± 8.8</td>
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<table>
<thead>
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<th>15</th>
<th>16</th>
<th>Total</th>
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<td>1000 ± 8.8</td>
<td>1000 ± 8.8</td>
<td>1000 ± 8.8</td>
<td>1000 ± 8.8</td>
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<td>74 ± 3.2</td>
<td>74 ± 3.2</td>
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<tr>
<td>Alanine</td>
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<td>110 ± 4.6</td>
<td>110 ± 4.6</td>
<td>110 ± 4.6</td>
<td></td>
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<tr>
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<td>2200 ± 180</td>
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</table>

Subjects 1, 6, and 12 were excluded as outliers (see text for full details).

Key: 2-OG – 2-oxoglutarate; 3-IS – 3-indoxylsulfate; DMG – dimethylglycine; PAG – phenylacetylglycine.
Table 7-3 Integral values for key metabolites in kidney MAS-NMR models.

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<td>110 ± 7.6</td>
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</tbody>
</table>

Subjects 1, 6, and 12 were excluded as outliers (see text for full details).

Key: MI – myo-inositol.
Chapter 5 – Statins

To account for the low animal numbers used in this study, integrals were calculated for the metabolites responsible for separation between the groups. Individual values for each animal are listed; group values are expressed as mean ± standard deviation. Integral values have been divided by $10^6$.

**Table 7-4 Integral values for key urinary metabolites.**

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<td>95</td>
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</tr>
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</tr>
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<td>79</td>
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</tr>
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<td>-----</td>
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<td>63</td>
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</table>

Subject 2 was excluded as an outlier (see text for full details).

Key: 2-OG – 2-oxoglutarate; 3-IS – 3-indoxylsulfate; DMA – dimethylamine; DMG – dimethylglycine; PAG – phenylacetylglycine.
Table 7-5 Integral values for key metabolites in kidney MAS-NMR models.

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<tr>
<th>Metabolite</th>
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<th>Gentamicin</th>
<th>Atorvastatin</th>
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<td>5 6 7 8  Total</td>
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<td>66 39 42</td>
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<td>24 ± 3.2</td>
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<tr>
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<td>72 92 84</td>
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<td>9.1 30 24</td>
<td>8.9 ± 0.8</td>
</tr>
<tr>
<td>Valine</td>
<td>39 29 28</td>
<td>32 ± 5.9</td>
<td>34 30 24</td>
<td>30 ± 3.9</td>
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### Gentamicin + Atorvastatin

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<th>Total</th>
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<td>84</td>
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<td>Glycine</td>
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<td>74</td>
<td>71</td>
<td>92</td>
<td>75 ± 12</td>
</tr>
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### Gentamicin + Atorvastatin + Mevalonate

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Subject 2 was excluded as an outlier (see text for full details).

Key: MI – myo-inositol.
APPENDIX II — PUBLICATION

Pharmacometabonomic characterization of xenobiotic and endogenous metabolic phenotypes that account for inter-individual variation in Isoniazid-induced toxicological response.

Katharine Cunningham,† Sandrine P. Claus,‡ John C. Lindon,† Elaine Holmes,† Jeremy R. Everett,§ Jeremy K. Nicholson,† and Muireann Coen,†

Journal of Proteome Research, 2012

†Biomolecular Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, Sir Alexander Fleming Building, South Kensington, London SW7 2AZ, U.K.
‡Department of Food and Nutritional Sciences, The University of Reading, Whiteknights, PO Box 226, Reading RG6 6AP, U.K.
§Pharmaceutical, Chemical and Environmental Sciences, School of Science, University of Greenwich at Medway, Central Avenue, Chatham Maritime, Kent ME4 4TB, U.K.

ABSTRACT: An NMR-based pharmacometabonomic approach was applied to investigate inter-animal variation in response to isoniazid (INH; 200 and 400 mg/kg) in male Sprague–Dawley rats, alongside complementary clinical chemistry and histopathological analysis. Marked inter-animal variability in central nervous system (CNS) toxicity was identified following administration of a high dose of INH, which enabled characterization of CNS responders and CNS non-responders. High-resolution post-dose urinary $^1$H NMR spectra were modelled both by their xenobiotic and endogenous metabolic information sets, enabling simultaneous identification of the differential metabolic fate of INH and its associated endogenous metabolic consequences in CNS responders and CNS
non-responders. A characteristic xenobiotic metabolic profile was observed for CNS responders, which revealed higher urinary levels of pyruvate isonicotinylhydrazone and β-glucosyl isonicotinylhydrazide and lower levels of acetylisoniazid compared to CNS non-responders. This suggested that the capacity for acetylation of INH was lower in CNS responders, leading to increased metabolism via conjugation with pyruvate and glucose. In addition, the endogenous metabolic profile of CNS responders revealed higher urinary levels of lactate and glucose, in comparison to CNS non-responders. Pharmacometabonomic analysis of the pre-dose $^1$H NMR urinary spectra identified a metabolic signature that correlated with the development of INH-induced adverse CNS effects and may represent a means of predicting adverse events and acetylation capacity when challenged with high dose INH. Given the widespread use of INH for the treatment of tuberculosis, this pharmacometabonomic screening approach may have translational potential for patient stratification to minimize adverse events.