

A UPLC-MS Based Exploration of the Xenobiotic
and Endogenous Metabolic Phenotypes of
Pre-Clinical Models of Hepatotoxicity

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Declaration of Originality

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Abstract

To reduce late stage attrition during drug development, and improve the diagnosis of drug induced liver injury (DILI), a greater mechanistic understanding of DILI and improved predictive biomarkers are required. In this thesis, the xenobiotic and endogenous metabolic phenotypes of model hepatotoxins are studied in the rat using an ultra-performance liquid chromatography- mass spectrometry (UPLC-MS) based metabonomics approach.

The idiosyncratic hepatotoxin Tienilic Acid (TA), was compared to its structural analogue, Tienilic Acid Isomer (TAI), which is an intrinsic hepatotoxin. TAI dosing resulted in elevated ALT activity and liver necrosis, whereas TA showed no signs of toxicity. The untargeted UPLC-MS approach revealed both previously reported and novel TA drug metabolites, including likely acyl-glucuronides and amino acid conjugates. In contrast, the TAI metabolites detected were predominantly glutathione (GSH) related; reflective of higher reactive metabolite formation. Untargeted UPLC-MS and targeted ion-pair-LC-MS revealed numerous endogenous metabolic alterations, including an elevation in hepatic and plasma ophthalmic acid, common to TA and TAI treated animals. In addition, a unique elevation in pyroglutamate was detected in response to TAI. Interestingly, both ophthalmic acid and pyroglutamic acid have previously been associated with hepatic GSH depletion and oxidative stress. Hepatic oxidative stress is a well-established mechanism in intrinsic toxicity, but has a less established role in idiosyncratic toxicity.

To enable further assessment of these compounds, and other glutathione related metabolites, as potential biomarkers of hepatic oxidative stress, a quantitative UPLC-MS/MS assay was developed. Interestingly, despite TA and TAI both depleting hepatic GSH and elevating ophthalmic acid, they were found to impact other circulating metabolites in different ways. To further explore the dynamics of these metabolites, the assay was applied to plasma from paracetamol (APAP) dosed rats; a model GSH depleting hepatotoxin. Quantitative data such as these may contribute to the further development and validation of mathematical models to predict hepatic glutathione status from multiple circulating plasma biomarkers. This thesis demonstrates the utility of a UPLC-MS based approach for hypothesis generation and biomarker development.

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“(3/4/5/6)” refers to the chapter the work is featured in. (*) data are referred to but not presented

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INTRODUCTION

MECHANISMS AND BIOMARKERS OF DRUG-INDUCED LIVER
INJURY

1.1 INTRODUCTION

It has been estimated to take an average of 13.5 years and \$1.78 billion to bring a new drug to market (Figure 1.1-1; Paul et al., 2010), consequently late stage attrition of drug candidates is extremely costly. An analysis of data collected from 835 drug developers between 2003 to 2011 found just 10.4% of the 7300 drugs entering the clinical phase of development made it to FDA approval (Hay et al., 2014). Aside from a lack of clinical efficacy, the biggest cause of attrition is adverse drug reactions (ADRs). Toxicity or safety concerns were attributed to 30% of clinical phase failures in 2000 (Figure 1.1-2, Kola and Landis, 2004), and cited in a third of suspended New Drug Application and Biologic License Application (NDA/BLA) filings to the Food and Drug Administration (FDA) between 2003- 2011 (Hay et al., 2014). ADRs are also a significant cause of post-release withdrawals and restrictions; of 548 drugs approved between 1975-1999, 2.9% were subsequently withdrawn due to previously undetected toxicity, and a further 10.2% were given black box warnings, restricting their usage (Lasser et al., 2002).

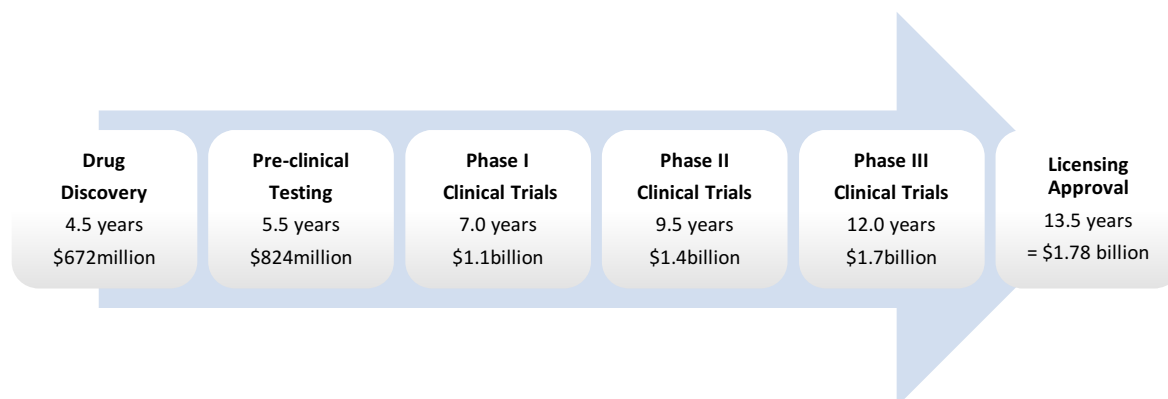


Figure 1.1-1 The cumulative time and cost of new drug development, data from Paul et al., 2010.

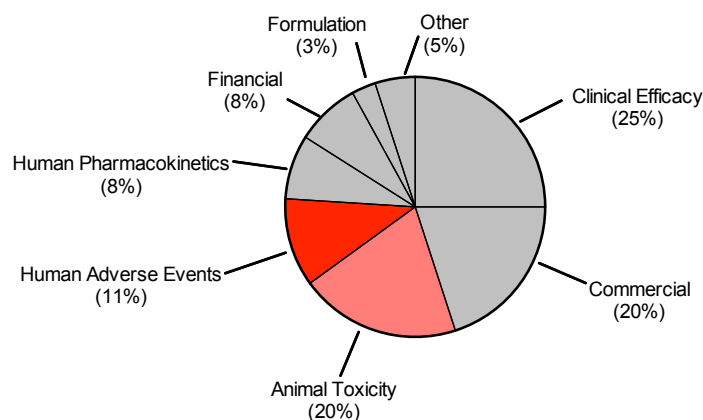


Figure 1.1-2 Causes of drug attrition in the development stage, reported for 2000, adapted from Kola and Landis, 2004..

The liver is often the target organ for ADRs (Lee, 2003), and it is estimated that over 1000 drugs have been associated with drug induced liver injury (DILI) (Kaplowitz, 2004b). DILI is not only implicated in a significant proportion of clinical phase failures, but is also the most common cause of withdrawal of approved drugs from the market (Lee, 2003). Clinically, DILI poses significant challenges and remains the biggest cause of acute liver failure in both the U.S and the U.K (Bernal and Wendon, 2013). As a consequence, an important research area, both for reducing late stage drug attrition and improving clinical outcomes, will be finding better biomarkers to predict DILI in both preclinical and clinical settings.

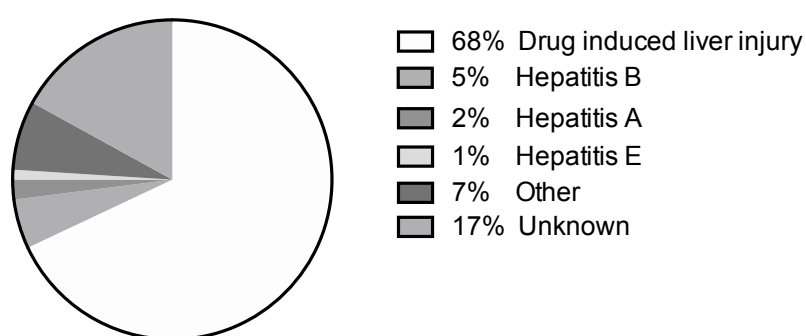


Figure 1.1-3 Causes of acute liver failure in the UK, data from Bernal and Wendon, 2013.

1.1.1 Aims

The overarching objective of this thesis is to contribute towards an improved understanding of DILI, and to aid in the search for improved predictive biomarkers.

The aim of this Chapter is to provide an overview of DILI. The later part will focus on the toxins that are the focus of this thesis: Tienilic Acid (TA), Tienilic Acid Isomer (TAI), and paracetamol (APAP).

1.2 DRUG INDUCED LIVER INJURY

DILIs are a type of ADR that impact liver function or structure. A wide spectrum of hepatic injury can be sustained as a result of DILI (Table 1.2-1), which can be seen as analogous to naturally occurring liver diseases. Differing clinical phenotypes are often characteristic of different toxins, such as hepatitis in paracetamol overdose, or methotrexate-induced fibrosis, although a single toxin can be associated with multiple types of hepatic injury. The severity of DILI can range from mild and asymptomatic to hepatic failure and death (Lee, 2003).

Table 1.2-1 Clinical features associated with DILI, adapted from Lee, 2003.

CLINICAL FEATURE	CHARACTERISTICS	EXAMPLES
HEPATITIS	Acute inflammation resulting in hepatocellular damage	Paracetamol
	Chronic- Hepatitis lasting over 6 months	Isoniazid Diclofenac
CHOLESTASIS	Dysfunction in bile flow	Oestrogen
CIRRHOSIS /FIBROSIS	Scarring resulting in liver dysfunction and distorts normal liver architecture	Methotrexate
HEPATIC STENOSIS-NAFLD	Microvesicular - due to mitochondrial dysfunction and inhibition of beta oxidation	Tamoxifen
	Macrovesicular - altered hepatic lipid trafficking	
STEATOHEPATITIS	Fat in liver cells, accompanied by inflammation and fibrosis	Tamoxifen
AUTO-IMMUNE-LIKE	Cytotoxic lymphocyte mediated hepatocyte damage	Lovastatin
IMMUNOALLERGIC	Enzyme-drug conjugates, antibody mediated response	Halothane

1.3 MECHANISMS OF DILI

ADRs, including DILI, can be classified as 'on-target' or 'off-target', depending on whether the toxicity arises from the intended pharmacology of the drug or not (Rudmann, 2013). On-target reactions usually stem from an exaggerated pharmacological action of the drug, and should therefore be predictable from the primary or secondary pharmacology. On-target reactions can usually be avoided or resolved by lowering the dose, and rarely result in serious injury (Guengerich and MacDonald, 2007). Conversely, off-target toxicity is unrelated to the target pharmacology of the drug, making them far harder to predict. Although less frequent, off-target ADRs can result in severe and life-threatening reactions (Guengerich and MacDonald, 2007).

Table 1.3-1 A comparison of the characteristics of on-target and off-target toxicity

On-target	Off-target
Related to the intended pharmacology of the drug	Unrelated to intended pharmacology of the drug
Fairly common	Quite rare
Predictable	Less predictable
Dose related	Not always dose related
Rarely cause severe injury	Can cause very severe reactions
	Includes: paracetamol, tienilic acid, tienilic acid isomer

ADRs can be further characterised as either intrinsic or idiosyncratic (IDRs). In intrinsic toxicity, the drug or its metabolites are directly toxic to cells or the target organ, resulting in a dose-dependent toxicity that is ultimately toxic to all individuals (Guengerich and MacDonald, 2007). This is a relatively predictable toxicity that has an acute onset. In contrast, idiosyncratic drug reactions only appear very rarely and only in susceptible individuals. These are often dose independent, and have a delayed onset, consequently these are highly unpredictable reactions (Guengerich and MacDonald, 2007). Our understanding of the mechanisms underlying IDRs is less well established, however, the discovery of antibodies directed against several idiosyncratic toxins implicates the adaptive immune system in some IDRs (Kaplowitz, 2005). The liver is often the site of both intrinsic and idiosyncratic off-target ADRs; most likely due to its function as the dominant site of drug metabolism. Over three-quarters of IDRs lead either to liver transplantation or death (Ostapowicz et al., 2002).

Table 1.3-2 A comparison of the characteristics of intrinsic and Idiosyncratic toxicity

Intrinsic	Idiosyncratic
Affects all individuals, at variable doses	Affects only susceptible individuals
Relatively common occurrence	Rare occurrence ($<1/10^4$)
Dose dependent	Not dose dependent
More predictable	Unpredictable
Often detected pre-clinically	Not detected until post-launch
Acute or sub-acute onset	Delayed onset
	Adaptive immune involvement
Includes: tienilic acid Isomer, paracetamol	Includes: tienilic acid

1.3.1 Drug metabolism and chemically reactive metabolites (CRMs)

Drug metabolism is broadly divided into three phases, phase 1 consisting of the 'functionalisation' of a drug, predominantly involving oxidation, reduction and hydrolysis reactions. Approximately 75% of drug transformations are thought to involve cytochrome

P450 (CYP450) enzymes, which are predominantly expressed in the smooth endoplasmic reticulum of hepatocytes (Wienkers and Heath, 2005). The subsequent phase 2 reactions, involve conjugation to endogenous compounds such as sulphate, glucuronide, glutathione and amino acids. These reactions are catalysed by enzymes including uridine 5'-diphospho-glucuronosyltransferase (UGT) and sulfotransferase (SULT) enzymes. Phase 3 drug metabolism involves the further metabolism and excretion of a phase 2 metabolite including the conversion of glutathione (GSH) conjugates to mercapturic acids.

Overall, drug metabolism acts to generate more hydrophilic metabolites enabling or enhancing excretion into urine and bile, and results in the 'detoxification' of a drug. However, the 'toxification' of susceptible drugs can also occur (Park et al., 2011a, Nelson, 1982). Toxification involves phase 1-3 reactions that result in the bioactivation of a drug to produce unstable, chemically reactive metabolites (CRMs). CRMs have been implicated in both intrinsic and idiosyncratic ADRs, and are particularly associated with hepatotoxicity (Park et al., 2011a). For example, 64% of drugs withdrawn in the U.S. due to hepatotoxicity and 71% of drugs given a "black box" warning for hepatotoxicity, have been found to generate CRMs (Guengerich and MacDonald, 2007). Although this is indicative of reactive metabolites playing a significant role in DILI, as "safe" drugs are less often the focus of research studies, it is not known what proportion also produce CRMs.

Expression of enzymes involved in drug metabolism differs between individuals, as a result of inter-individual differences including those as a result of polymorphisms in CYP450s, diet, and interactions with other drugs (Guengerich and Rendic, 2010, Kaplowitz, 2005).

Chemical structures, or functional groups within drugs that are commonly associated with different types of toxicity are known as 'structural alerts', and can aid understanding of toxicity, and the design of safer drugs (Park et al., 2011a, Nelson, 2001).

1.3.2 Intrinsic toxicity

CRM, which are typically electrophilic, can be neutralised by endogenous nucleophiles, such as glutathione (GSH; Ketterer et al., 1983). GSH is a tripeptide, consisting of glycine, glutamate and cysteine, that can either bind to CRMs to form a stable conjugate or reduce the CRM back to its original form, which will lead to the oxidation of GSH to oxidized glutathione (GSSG). Prior

to excretion, the glutamate and then glycine molecules are removed, leading to the production of cysteinyl conjugates which can be further metabolised to mercapturate/N-acetyl-cysteinyl conjugates (Ketterer et al., 1983). A high CRM burden results in the depletion of hepatic GSH stores, incomplete neutralization of CRM molecules, and binding of CRMs to other intracellular molecules such as proteins and DNA (Nelson, 1982). This can result in cellular dysfunction, apoptosis, necrosis, and eventually liver dysfunction and failure (Antoine et al., 2008).

Intrinsic CRM mediated reactions are dose dependent as the reactive metabolite formation will increase with dosage, and GSH will be increasingly depleted, allowing toxicity to progress. Much of our understanding of intrinsic toxicity, and the role of CRM and GSH has come from the study of paracetamol, which is further explored in Section 1.6 of this Chapter.

1.3.3 Idiosyncratic drug reactions

The mechanisms underlying IDRs are less well established (Kaplowitz, 2005). However, the discovery of antibodies directed against several idiosyncratic toxins (for example, halothane, and tienilic acid) has led to the development of one of the key hypotheses around IDRs, the hapten hypothesis. The hapten hypothesis implicates both adaptive immune reactions and CRM formation, summarised in Figure 1.3-1.

Although small molecules, such as drugs, are unable to induce an immune response alone (Landsteiner and Jacobs, 1935), CRM can act as 'haptens' and irreversibly bind with a protein, in a process, termed 'haptensisation' (Roth and Ganey, 2010). Haptensisation results in the formation of a neoantigen, which can then be recognised as foreign or 'non-self' by antibodies or T-cells. However, this neoantigen alone is not sufficient to activate an adaptive immune response, as a second signal must also be present to signal danger (Gallucci and Matzinger, 2001, Matzinger, 1994). These danger signals are thought to regulate the immune response and prevent autoimmunity. Danger signals can arise from external sources such as a pathogen, which are termed pathogen associated molecular patterns (PAMPs), or from endogenous molecules which are termed damage associated molecular patterns (DAMPs). Both PAMPs and DAMPs can activate immune signalling pathways (Matzinger, 2007). In relation to IDRs, it is unknown where the danger signal that enables the activation of the immune system originates.

Two key hypotheses have been proposed, suggesting either drug dependent (Uetrecht, 1999,

Utrecht, 2008) or drug independent danger signals may be responsible for IDRs (Roth et al., 2003, Roth et al., 1997, Roth and Ganey, 2010). Drug dependent danger signals would result from some level of cell stress or damage caused by the drug, and evidence of such signals has been suggested for several drugs, for example in response to Nevirapine (Zhang et al., 2013). Alternatively, drug independent danger signals have also been suggested to arise from co-infection, diseases associated with chronic inflammation, or excessive alcohol consumption. Evidence for this has been shown in rodents models demonstrating that a non-hepatotoxic dose of lipopolysaccharide (LPS) can prime rodents to respond to certain drugs with hepatotoxic endpoints, when the isolated compound is not hepatotoxic (Roth et al., 1997, Roth and Ganey, 2010).

Due to the inherent rarity of IDRs, toxicity is typically not detected in animal models or clinical trials in humans. As a result, it is only after licensing, when a sufficient post-marketing data are available, that they are often detected (Kaplowitz, 2005). Further mechanistic understanding of IDRs could help develop animal models to test for IDRs, or find biomarkers to monitor risk.

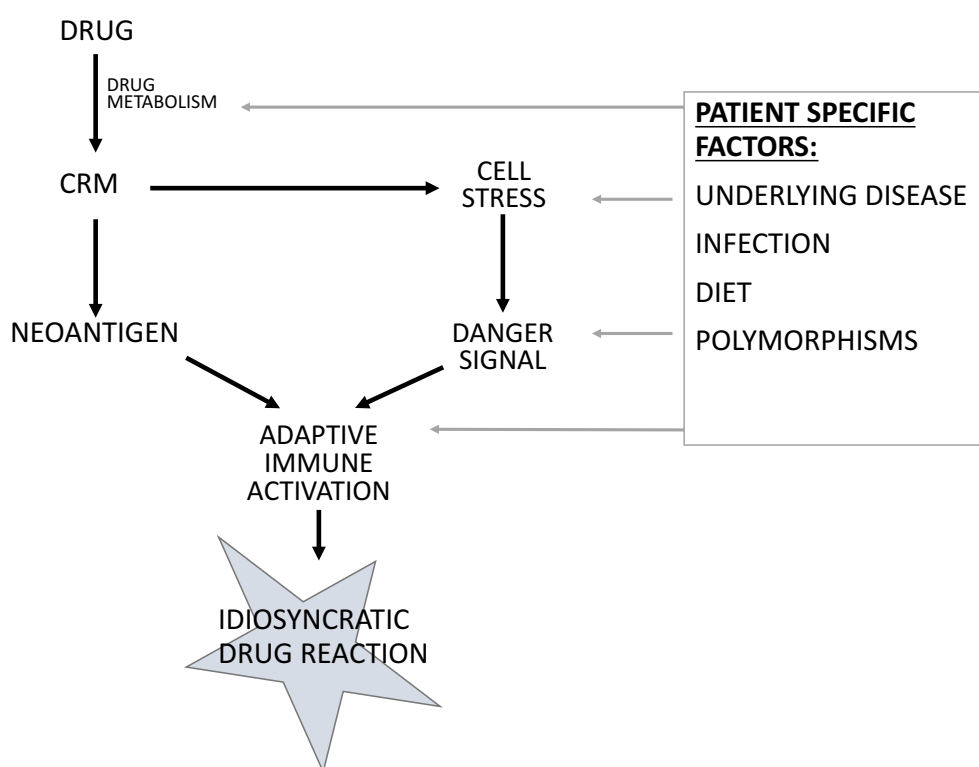


Figure 1.3-1 Summary of suggested pathways for immune activation in IDR

1.4 BIOMARKERS OF DILI

DILI is identified and clinically diagnosed based on changes in several blood biochemical parameters which are indicative of hepatocellular injury (alanine aminotransferase, ALT; aspartate aminotransferase; AST), choleostatic injury (alkaline phosphatase, AP; gamma glutamyl-transpeptidase, GGT) and general liver function (total and conjugated bilirubin, albumin concentration and pro-thrombin time) (FDA, 2016, Antoine et al., 2009). However, these markers are only evident once DILI has occurred, and have limited specificity for the type of injury sustained. Pre-clinical animal models rely on histopathological examination following necropsy to confirm and characterise DILI. Similarly, in the clinic, the only conclusive diagnostic test is an invasive liver biopsy to allow for histological examination. Currently there is no specific, non-invasive diagnosis, treatment or prevention available for DILI, except withdrawal of medication where it is suspected (Antoine, 2009).

New biomarkers of DILI are required that are more sensitive, are detectable earlier (are predictive), and that are specific to different types of toxicity. The FDA have recently published a letter of support for new DILI biomarkers, highlighting the lack of specificity and sensitivity of current approaches. Biomarkers currently considered by the FDA as most promising include cytokeratin 18 (CK-18), total and hyperacetylated high mobility group protein B1 (HMGB1), osteopontin, and macrophage colony-stimulating factor 1 receptor (CSF1R) (FDA, 2016).

1.4.1 New approaches to biomarker discovery: ‘-omics’

‘-Omics’ approaches such as genomics, transcriptomics, proteomics and metabolomics, involve the global study of genes, gene expression, proteins and metabolites, respectively (Hood and Galas, 2003). They can be useful in hypothesis generation and biomarker discovery as they require little/ no prior mechanistic knowledge, and could therefore be useful in finding novel off-target effects of a drug (Nicholson et al., 2002). For example, proteomic approaches can be used to find protein targets of CRMs (McGill et al., 2012b, Park et al., 2011b, Elrick et al., 2006), and transcriptomics approaches have been used to define signatures of expression changed indicative of toxicity (Zhang et al., 2013, Pacitto et al., 2007).

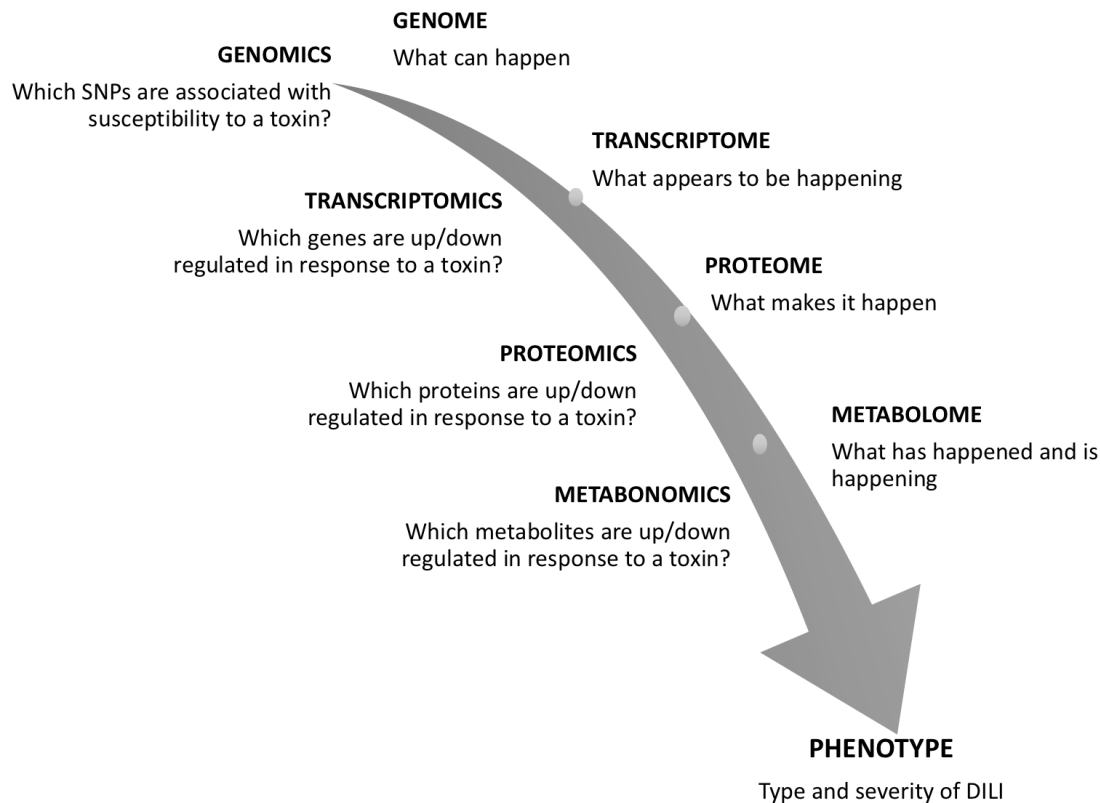


Figure 1.4-1 Overview of how 'omics' approaches relate to one another and can be used to study DILI, adapted from Dettmer et al., 2007.

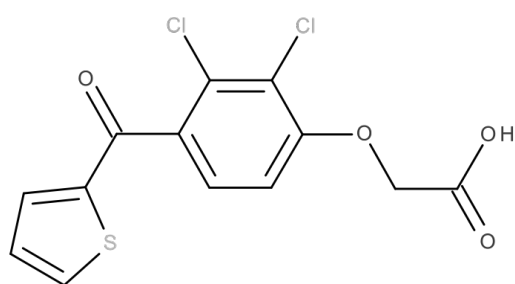
Metabonomics is the more recent and emerging 'omics' field, which has been defined as '*the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification*' (Nicholson et al., 1999). The metabolome can be defined as the complete small molecule complement of a biological fluid or tissue, and there are over 4229 compounds reported in the human serum metabolome (Psychogios et al., 2011), while 2651 compounds have been identified in urine (Bouatra et al., 2013).

Metabonomics is thought to have particular potential in the field of biomarker discovery as it can be readily applied to accessible biofluids, such as urine and plasma. Additionally, as it reflects both genetic background and environmental factors such as an individual's gut microbiome and diet, it can be seen as a closer representation of phenotype than the other 'omics' approaches (Nicholson et al., 1999). It is hoped that this approach could offer far more informative biomarkers than the current gold standard techniques, such as monitoring ALT (Coen, 2010).

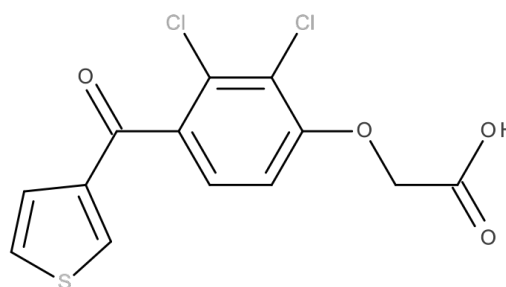
1.5 TIENILIC ACID AND TIENILIC ACID ISOMER

Tienilic acid (TA) is a uricosuric and potassium sparing diuretic designed to treat hypertension (Lau et al., 1977). When TA was discovered in 1967 it was seen as an important advancement in diuretic therapy due to its unique uricosuric properties, which would reduce the risk of gout and kidney failure; complications associated with other diuretics in use at the time. TA was marketed in the 1970s in Europe and U.S., however, just a few months after its release it was banned by the U.S. FDA due to several reported ADRs and a number of deaths. Since that time, interest into the mechanism of this idiosyncratic toxin has continued, but remains elusive.

In contrast, Tienilic acid Isomer (TAI) is a structural analogue of TA that has reported intrinsic hepatotoxic properties in rodents, and has never been used clinically (Mansuy, 1997).



Tienilic Acid- idiosyncratic toxin



Tienilic Acid Isomer (TAI)- intrinsic toxin

Table 1.5-1 Chemical structures of Tienilic acid and the Tienilic acid isomer

1.5.1 Summary of the clinical features associated with TA toxicity

In 1984 Zimmerman et al. published what remains the most comprehensive report on the clinical impact of TA toxicity in an analysis based on the ADRs reported to the manufacturer (Zimmerman et al., 1984a). Of the 526 incidences of hepatic injury reported, 340 cases were deemed to be either 'likely' or 'possibly' caused by TA based on an analysis of the patients' circumstances, and became the focus of further analysis. The remainder were excluded from further analysis due to 'scanty data' or an alternative cause of hepatotoxicity being identified. The reported clinical features are summarised in Table 1.5-2.

Table 1.5-2 Clinical features of TA induced adverse drug reaction in 340 patients. Adapted from Zimmerman 1984.

CLINICAL FEATURES ASSOCIATED WITH ADVERSE REACTIONS TO TA (340 PATIENTS)

JAUNDICE (N=42, 48%)
NAUSEA/VOMITING (N=33, 38%)
ABDOMINAL DISCOMFORT (17, 19.5%)
ANOREXIA (22, 25%)
FATIGUE (16, 18%)
MALAISE (14, 16%)
ALT > 300 UNITS (80% OF 322 TESTED)
ALT > 1000 UNITS (38% OF 322 TESTED)
DEATH (N=25, 7% (10% OF JAUNDICE PATIENTS))

In 80% of cases the dose was 250mg/day, whilst the remaining 20% were taking 500mg/day. As is typical of IDR, there were no observed correlations between dose or duration of therapy and severity of hepatotoxicity. The authors noted the delayed onset of overt injury, which ranged from 14-240 days, with 90% of cases appearing after 30 days. There were 16 cases in which treatment was stopped and then re-started- and in 15 of these cases of re-challenge there was a rapid relapse and return of symptoms.

Those most at risk were females over 60 who, despite making up just 16% of those taking TA, accounted for 31% of the cases of hepatotoxicity. There were no observed correlations between concomitant use of other medications, infections or history of alcohol abuse. In addition to the clinical presentations above, available autopsy data showed massive/sub-massive necrosis in the majority of cases, and cirrhosis with chronic inflammation reported in the remainder of cases.

1.5.2 TA/TAI metabolism and chemically reactive metabolite formation

More recent *in vitro* work in microsomes and cell-based models (Rademacher, 2011a) has taken advantage of the advances in tandem mass spectrometry (MS) and improvements in both liquid chromatography (LC) and MS sensitivity/resolution to further our previous understanding of both TA and TAI metabolism (Neau et al., 1990, Dansette et al., 1990, Dansette et al., 1991, Belghazi et al., 2001, Lim et al., 2008, Masubuchi et al., 2007). A summary of the key metabolic pathways of TA and TAI established using *in vitro* techniques is depicted in Figure 3.

Whilst it was originally hypothesised that the thiophene ring of TA and TAI both produced a reactive S-oxide to cause toxicity (Dansette et al., 1990, Lopez Garcia et al., 1993), the

possibility that an arene oxide was responsible for TA toxicity was also suggested (Koenigs et al., 1999). This has recently been supported by theoretical, *in silico* and experimental data, using isotope incorporations, which found no evidence that TA can form an S-oxide (Rademacher et al., 2012, Rademacher, 2011a). This is suggested to be due to the different binding orientations of TA and TAI in the CYP2C9 active site which had previously been modelled using nuclear magnetic resonance (NMR) spectroscopy (Poli-Scaife et al., 1997).

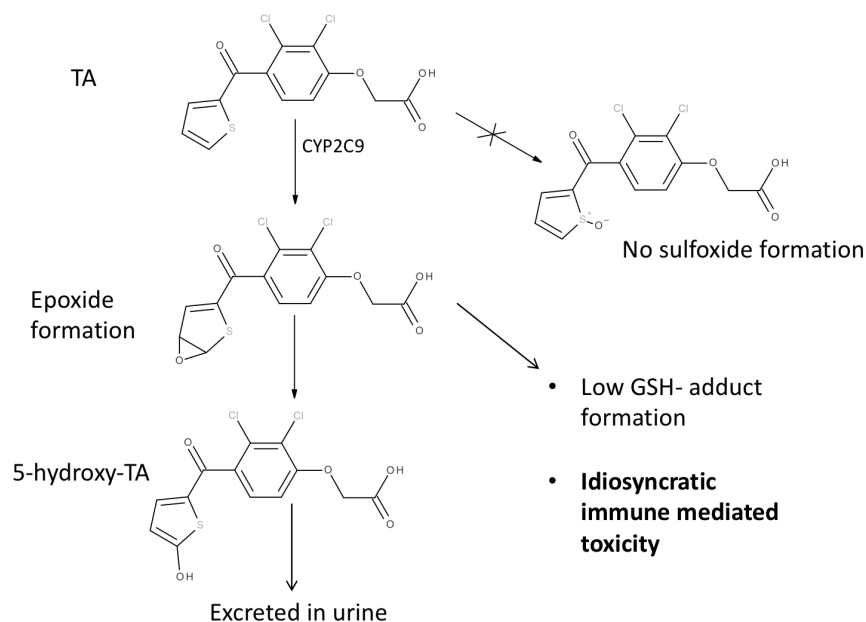


Figure 1.5-1 Summary figure of TA metabolism, adapted from Rademacher, 2011.

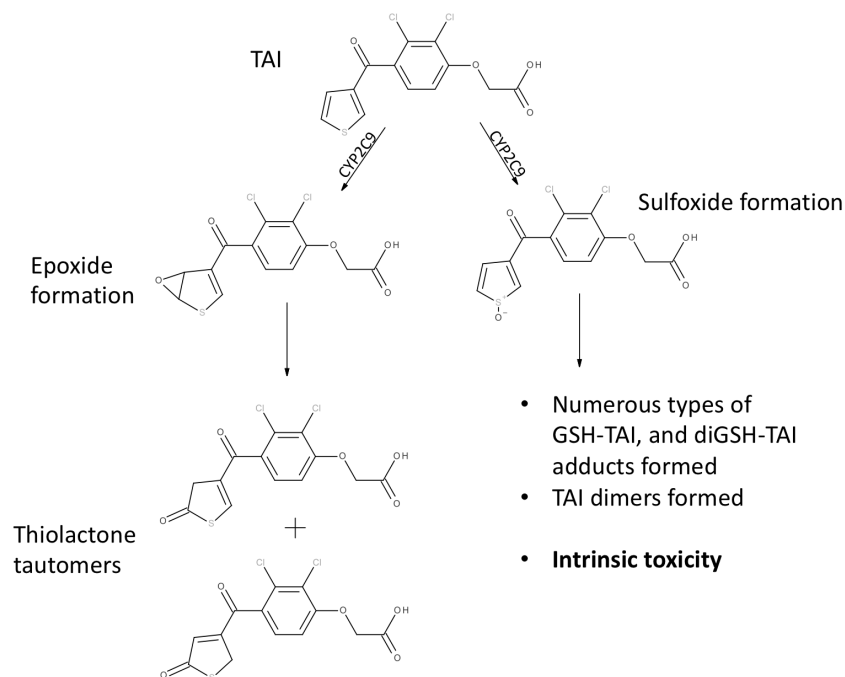


Figure 1.5-2 Summary figure of TAI metabolism, adapted from Rademacher, 2011.

1.5.3 Immune mechanism of TA toxicity

Although not all clinical features of TA toxicity were thought to be indicative of an immune mediated pathway, several features were suggestive of immune cell involvement, including: prompt recurrence of hepatic injury following re-challenge, fever and chills in 6/15 confirmed cases and prominent eosinophils in some histological data (Zimmerman et al., 1984a).

Data have been presented to support involvement of the immune system by the discovery of an autoantibody associated with TA induced hepatitis (Beaune et al., 1987, Homberg et al., 1984). This was a fortuitous discovery by two labs in France that had been collecting serum samples between 1973 and 1979 from patients with cirrhosis. The groups examined rat sections by techniques including immunofluorescence staining to look for organ specific and non-specific antibodies related to cirrhosis. Of the 70 000 serum samples tested, 373 from 131 patients were found to have anti-Liver-Kidney Microsome (LKM) antibodies. From 1977, a few months after the release of TA in France, they noticed a different immunofluorescent pattern emerging in some of the LKM positive samples; suggesting a different target of autoimmunity had emerged at this time. They defined new selection criteria for this auto-antibody and called it anti-LKM2. All LKM positive sera samples from the cirrhosis patients were re-tested and 45 were found to be positive for LKM-2. Of these, 43 medical records were available and all of those patients were found to be taking TA. The authors then tested samples from patients taking TA but without presentation of hepatotoxicity and reported that these patients were anti-LKM2 negative; suggesting a strong association with the autoantibody and TA toxicity. However, this antibody was later found to be absent in 35% of patients tested with TA induced hepatotoxicity (Neuberger and Williams, 1989). Overall, the discovery of the TA-hepatotoxicity related auto-antibody strongly suggests an immune mechanism in some, but not all, cases of TA toxicity.

Further investigations revealed that anti-LKM2 were specific for rat CYP2C11 (Pons et al., 1991), and human CYP2C9 (Lopez Garcia et al., 1993), and that the conformational epitope was located near the active site (Lecoeur et al., 1996). In addition, TA was found to be a suicide substrate /mechanism based inhibitor of CYP2C9 (Lopez Garcia et al., 1993, Jean et al., 1996). In addition, both the TA-CYP2C11 and unmodified CYP2C11 can be expressed on plasma membranes and be recognized by LKM-2 positive sera (Robin et al., 1996). Overall, these data suggest that during its metabolism by CYP2C9, a chemically reactive TA metabolite is created

and forms a hapten-complex with CYP2C9 which can be expressed on the cell surface. It is therefore possible that idiosyncratic TA hepatotoxicity could have occurred via antibody-dependent cell cytotoxicity.

1.5.4 Intrinsic/ direct toxicity of TA and TAI

In vivo studies have reported conflicting results about whether TA has any intrinsic cytotoxicity. Histological signs of liver damage following TA administration were reported in Sprague-Dawley rats, however, this was only at high doses 120-480 mg/kg of TA; far greater than the approximate dose of 3.5 mg/kg prescribed in humans (Oker-Blom et al., 1980b). Additionally, TA increased hyperbilirubinemia in Eisai hyperbilirubinemia rats (EHBR), with the authors suggesting that TA may precipitate jaundice in humans by potentiating bilirubin biosynthesis and its sinusoidal transport, which occurs as a consequence of stress in the liver (Nishiya et al., 2006). Most recently, although ALT was not increased in Sprague-Dawley rats with doses up to 1000 mg/kg, some toxicity was observed when GSH was depleted prior to treatment; suggesting a link between the mechanism of liver damage and oxidative stress pathways (Nishiya et al., 2008a, Nishiya et al., 2008b).

There have also been contrasting reports on *in vitro* toxicity of TA. For example, in 1982, Zimmerman *et al.* showed that in perfused rat liver, signs of toxicity were evident (Zimmerman et al., 1982). However, primary rat liver cells incubated with TA have shown no signs of cytotoxicity (Acosta et al., 1982) and a more recent study in primary cultures of male rat liver cells identified TA-protein adducts but, again, in the absence of cytotoxicity (Lopez-Garcia et al., 2005). In addition, a comparison of TA and TAI cytotoxicity in several cell-lines including HepG2, HepG2-2C9, THLE-2C9 as well as cryopreserved rat and human hepatocytes found only very mild TA toxicity was reported, which was slightly increased when GSH was depleted. In contrast TAI was far more cytotoxic when compared to TA, and was significantly more toxic when GSH was depleted (Rademacher, 2011a).

1.5.5 New approaches to study TA toxicity

Two studies have focused on identifying the protein targets of the low-level, non-CYP2C9/11, binding of TA that had previously been observed (Lopez-Garcia et al., 2005). The first used western blotting of rat liver after *in vivo* TA administration (Methogo et al., 2007) whilst the other incubated carbon-14 labelled TA with human hepatocytes then performed 2D gel

electrophoresis and LC- MS/MS (Koen et al., 2012). Both studies identified numerous targets, however, only one protein was found in both studies: fumarylacetoacetase.

In addition, studies have utilized a transcriptomic approach to study the impact of TA on the liver and found pathways associated with regeneration, immune response and oxidative stress were upregulated (Pacitto et al., 2007). The authors went on to suggest this may be indicative of TA producing a drug induced danger signal (Pacitto et al., 2007). Similar results have since been replicated in TA treated rodents, again showing oxidative/electrophilic stress related genes being upregulated (Nishiya et al., 2008b). Markers of electrophilic stress have also been reported in response to TA an *in vitro* human hepatocyte study (Takakusa et al., 2008).

NMR based metabonomic analyses of urine, serum and liver samples from TA and TAI treated rodents was recently completed (Coen et al., 2012). The current project utilises the same samples collected during this study (Figure 1.5-3).

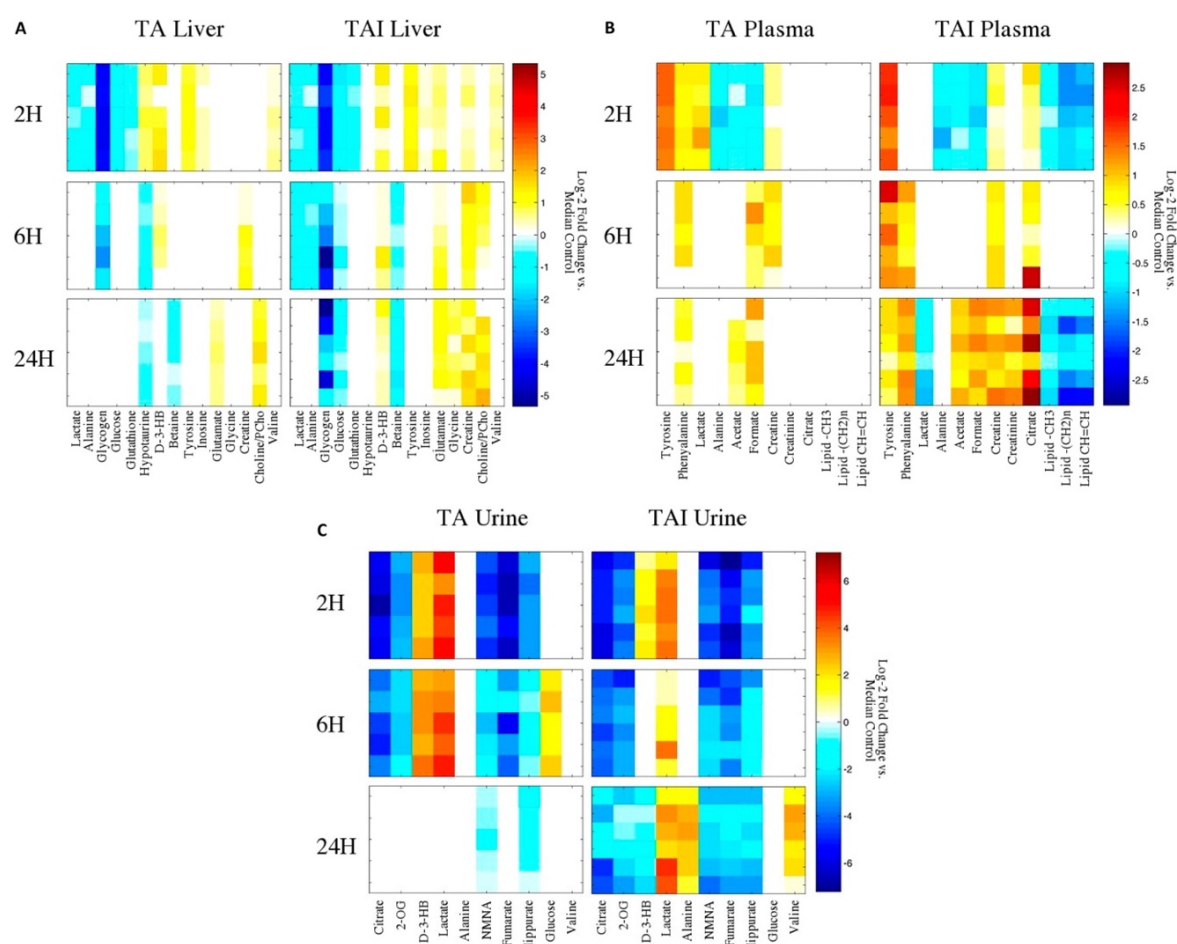


Figure 1.5-3 NMR based metabonomic analyses of urine, serum and liver samples from TA and TAI treated rodents. Reproduced with permission from Coen M, et al., 2012, Comparative NMR-Based Metabonomic Investigation of the Metabolic Phenotype Associated with Tienilic Acid and Tienilic Acid Isomer, CHEMICAL RESEARCH IN TOXICOLOGY, Vol: 25, Pages: 2412-2422, ISSN: 0893-228X. Copyright 2012 American Chemical Society.

1.6 PARACETAMOL

Paracetamol, or N-acetyl-p-aminophenol (APAP), is a widely used antipyretic and analgesic drug that was introduced in the USA and UK in the mid-twentieth century. APAP is generally considered to be safe at therapeutic doses but it can cause predominantly hepatocellular damage at excessive doses; first reported approximately 10 years after its release (Davidson and Eastham, 1966). It is the leading cause of acute liver failure in the developed world, including both intentional and unintentional overdose (Bernal and Wendon, 2013). The hepatotoxic properties of APAP have been extensively studied (Lee, 2003), leading to the U.S. FDA recommendation to avoid prescribing medication with a high APAP content due to the risk of liver injury (Mitka, 2014).

1.6.1 APAP metabolism

The major drug metabolites of APAP are sulphate and glucuronide conjugates, however a reactive metabolite can also be formed, N-acetyl-p-benzoquinone (NAPQI), that is considered to be responsible for toxicity (McGill and Jaeschke, 2013), Dahlin et al., 1984, Jollow et al., 1973). Different members of the CYP450 superfamily are thought to be involved in NAPQI generation, although CYP2E1 is thought to be the most important in humans (Thummel et al., 1993, Hu et al., 1993). Other products of CYP450 catalysed reactions include 3-hydroxyparacetamol and 3-methoxyparacetamol (Bessemers and Vermeulen, 2001).

There is significant intra- or inter-species, and gender variation in APAP metabolism (Savides et al., 1984, Chen et al., 2003, Lee et al., 2009). For example, the rat has much higher glucuronidation capacity and makes less CYP-generated NAPQI compared with mouse (McGill et al., 2012b). The respective proportion of each metabolite can also be dependent on other factors such as liver health and metabolic status, for example, stenosis has been associated with higher SULT enzyme activity (Hardwick et al., 2013).

GSH can bind to NAPQI to form an APAP-glutathione conjugate or reduce NAPQI back to APAP, leading to the oxidation of GSH to oxidized glutathione (GSSG) (Rashed et al., 1990). APAP-glutathione conjugates undergo further metabolism in the intestine and kidney prior to excretion (Newton et al., 1986), including the removal of the glutamate, then glycine molecules, leading to the production of APAP-cysteinyl (APAP-Cys). This molecule can be further metabolized by N-acetyltransferase enzymes to form the mercapturate; APAP-N-acetyl-

cysteinyl (APAP-NAC) (Tate, 1980). An overdose of APAP can result in the irreversible depletion of hepatic GSH stores, leading to incomplete neutralization of NAPQI molecules (Mitchell et al., 1973). Figure 1.6-1 provides an overview of the major hepatic routes of APAP metabolites.

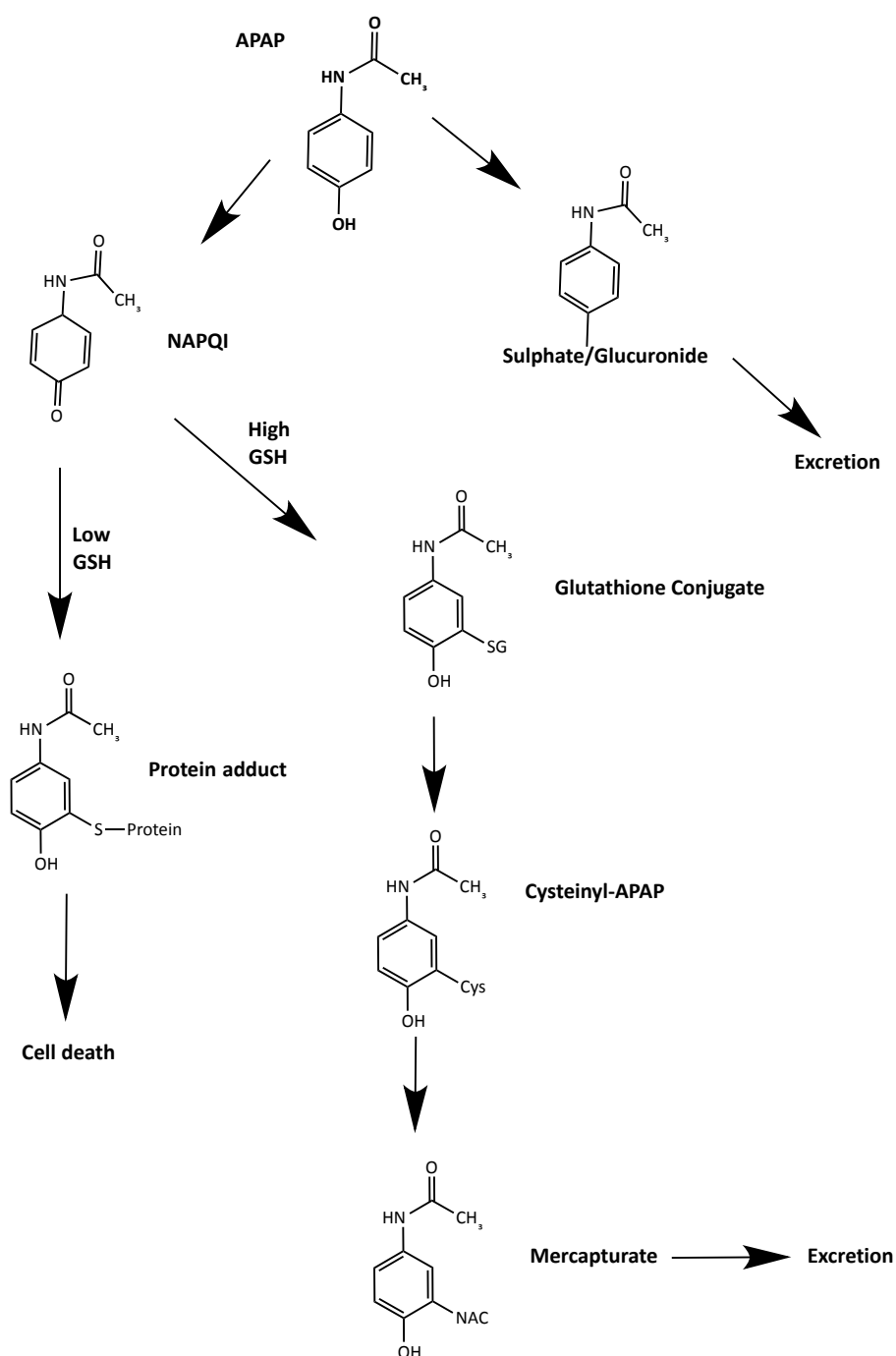


Figure 1.6-1 The major routes of APAP metabolism

1.6.2 Mechanism of toxicity

NAPQI can covalently bind to mitochondrial proteins (Landin et al., 1996, Halmes et al., 1996), leading to altered mitochondrial morphology, oxidative stress, ATP depletion and reduced respiration (Katyare and Satav, 1989, Placke et al., 1987). Mitochondrial damage has been directly correlated to the mortality of acute liver failure in APAP overdose patients (McGill et al., 2012a). The disruption in mitochondrial membrane permeability also leads to the release of nucleases from the mitochondrial inter-membrane space resulting in nuclear DNA fragmentation (Bajt et al., 2006). The predominant mechanism of cell death in APAP-induced toxicity is considered to be necrosis (McGill and Jaeschke, 2013).

A metabonomic approach has been applied to study the endogenous impact in pre-clinical models of APAP toxicity (Ghauri et al., 1993, Coen et al., 2004, Coen et al., 2003, Soga et al., 2006, Sun et al., 2008, Sun et al., 2009), and in human studies (Bhattacharyya et al., 2014). In the mouse, metabolic alterations have been linked to a disturbance of energy metabolism, including increased rates of glycolysis and impaired β -oxidation (Coen et al., 2004, Coen et al., 2003), as well as elevated levels of long-chain acyl-carnitines (Chen et al., 2008), which has since been found in children {Bhattacharyya et al., 2014}. Others have reported a significant perturbation of metabolites involved in glutathione biosynthesis in the mouse (Soga et al. 2006, Kyriakides et al, 2016) and rat (Ghauri et al. 1993, Sun et al., 2008). Further work understanding these metabolites will be required to determine their use as potential biomarkers of APAP induced DILI.

METHODOLOGY

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY BASED
METABONOMICS

2.1 INTRODUCTION

Metabonomic studies involve the global measurement (profiling) of small molecules present in a tissue or biological fluid. A metabonomic profile from a global analysis can comprise of both endogenous (e.g. amino acids, organic acids, nucleotides, and lipids) and xenobiotic metabolites. Metabonomic profiling is typically achieved using nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry (MS) coupled to liquid (LC) or gas (GC) chromatography (Nicholson et al., 2002). Due to the diversity of chemical classes in the metabolome it can be advantageous to use multiple platforms in parallel, and NMR and LC-MS approaches can be seen as complementary to each other (Dettmer et al., 2007). NMR provides greater structural information important for metabolite identification, whereas MS has superior sensitivity, resolution and a greater breadth of metabolite classes that can be profiled (Want et al., 2005). A UPLC-MS based approach was selected for this work to complement previous NMR analyses of samples from an *in vivo* hepatotoxicity study of tienilic acid and tienilic acid isomer (Coen et al., 2012).

A schematic overview of the UPLC-MS based metabonomics methodology applied in this thesis is presented in Figure 2.1-1. This figure also highlights the role of collaborators in this work, and the intended scope of this thesis.

2.1.1 Aims

The objective of this thesis is to apply a UPLC-MS based metabonomic approach to study DILI. The aim of this Chapter is to provide a brief overview of the methodologies applied in this thesis, including the LC, MS, data processing, and statistics.

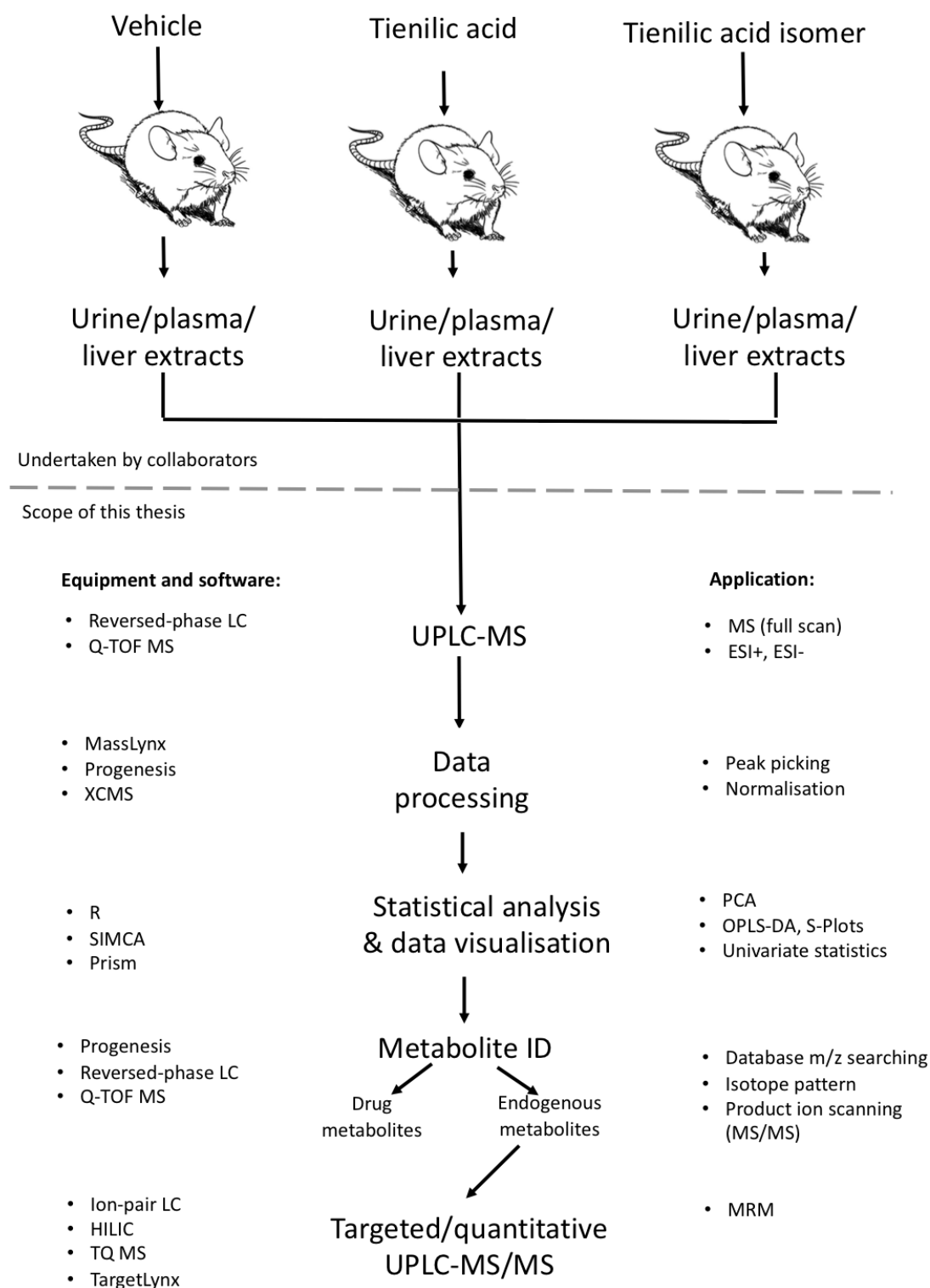


Figure 2.1-1 A schematic overview of the LC-MS based metabonomic approach taken to study TA and TAI in this thesis.

2.2 LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) and gas chromatography (GC) platforms are commonly used in tandem with mass spectrometry (MS) to separate the sample prior to MS analyses (Dettmer et al., 2007). This reduces ion suppression, which is the reduced ionisation efficiency due to the presence of another compound, and enables more accurate metabolite detection and quantification (Want et al., 2007). LC consists of a stationary phase; a column packed with derivatized silica, and two mobile phases; organic and aqueous solvents. One of the more recent developments in LC is ultra-performance liquid chromatography (UPLC; Waters Corp., MA, USA), where the column is packed with smaller particle sizes (1.7 μm compared to 3-5 μm). UPLC is performed under higher pressures (10,000 – 15 000 psi) enabling increased chromatographic resolution and shorter run times (Wilson et al., 2005).

As the sample is injected onto the column, a gradient is created by progressively altering the relative composition of the mobile phases, and enables the selective elution of compounds based on their differing chemical properties. LC separation will therefore depend on the different chemistries of analytes, and how they interact with the stationary and mobile phases. Parameters such as the column type (stationary phase composition, length, width), solvents, additives, column temperature, flow rates, gradient and run time can all be optimised to separate compounds of interest.

2.2.1 RP chromatography for medium polar and non-polar metabolite analyses

Reversed-phase chromatography (RP) is the most commonly applied chromatographic approach in metabonomics (Dettmer et al., 2007). RP chromatography utilises a column packed with a hydrophobic stationary phase; typically C-18 derivatised silica particles, and the solvent gradient applied runs from relatively high aqueous to high organic (MeOH/ACN) content. Metabolites generally elute in order of decreasing polarity (Dettmer et al., 2007). For the untargeted analyses presented in Chapters 3 and 4, previously reported reversed-phase methods were applied. For both applications a High Strength Silica (HSS) T-3 column was utilised (Figure 2.2-1).

2.2.2 Ion-pairing and HILIC for polar metabolite analyses

Although RP chromatography can be used to separate a broad range of medium polar and non-polar analytes, RP columns do not retain very polar metabolites well. To retain polar

metabolites, derivatisation, ion-pairing and hydrophilic interaction liquid chromatography (HILIC) can be applied. Derivatisation can enable polar compounds to be better retained using a RP approach, however, it requires time consuming sample preparation and is limited to a select chemical class e.g. amines depending on the derivatisation agent. Ion-pair chromatography also allows the use of RP; this is achieved through adding an ion-pairing reagent to the mobile phase. The hydrophobic surfactant can interact with the stationary phase and the polar metabolites, which enables polar metabolites to be retained on the column. To profile a broad range of polar metabolites in Chapter 4 and 6, a previously developed IPC approach (Michopoulos et al., 2014) was applied, using a HSS T3 column.

A limitation of ion-pairing is that ion-pairing reagents contaminate the MS with the surfactant, requiring a dedicated MS to run the method (Michopoulos et al., 2014). An alternative approach to study polar metabolites is hydrophilic interaction LC (HILIC), this approach uses different columns and run from higher organic to high aqueous gradients (Dettmer et al., 2007). HILIC stationary phases are often silica or derivatised silica, and require a high water content in the mobile phase to allow the formation of an aqueous layer on the stationary phase. Polar metabolites are thought to partition into the water layer, before interacting with the stationary phase. The retention of compounds in HILIC is through multiple mechanisms, including hydrophilic interaction, ion-exchange and reversed-phase retention, which can be useful to improve selectivity (Naidong, 2003, Greco and Letzel, 2013). However, multimodal retention can result in abnormal peak shape, especially if a compound is retained by multiple retention mechanisms (Naidong, 2003). In Chapter 5, a HILIC approach was applied in the development of a targeted assay for polar metabolites using a BEH amide column Figure 2.2-1

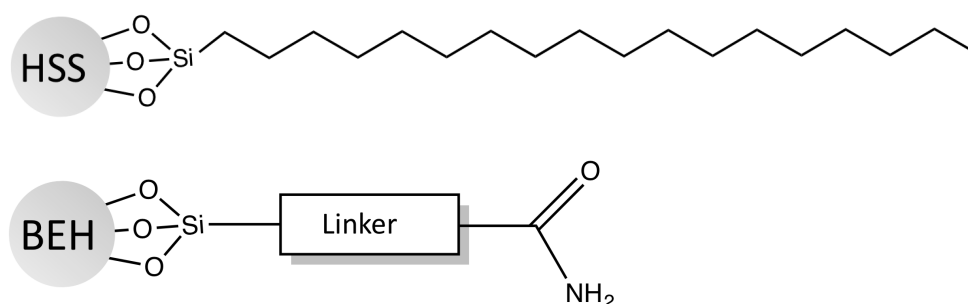


Figure 2.2-1 An illustrative representation of a HSS T3 particle and BEH Amide particle (Waters Corp.)

2.3 MASS SPECTROMETRY

Mass spectrometry (MS) is a highly sensitive analytical technique used to determine the mass-to-charge ratio (m/z) of molecules in a substance (Maher et al., 2015). Compounds are first ionized to create gas phase ions, separated by their m/z , and then detected to create a mass spectrum. The mass spectrum consists of the ion m/z versus the relative intensity/abundance. MS can be used in a qualitative or quantitative way to both identify and accurately quantify compounds (Maher et al., 2015).

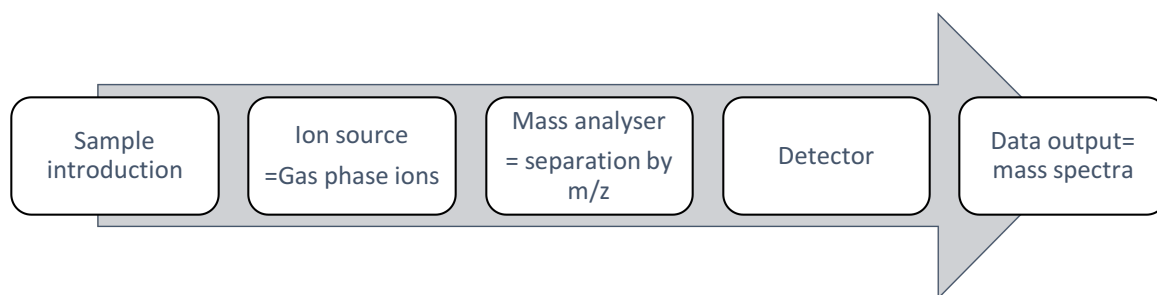


Figure 2.3-1 Key steps in mass spectrometry, (adapted from Maher et al., 2015).

2.3.1 Electrospray ionisation

Electrospray ionization is commonly used in metabolomic studies of biological fluids and tissue extracts as it is a “soft” ionization technique. This means that a large proportion of the ions are formed without breaking chemical bonds, so the majority of ions analysed are the parent molecule not fragments, which can help in the identification of molecules. It is considered advantageous over earlier ionisation methods such as the “hard” ionization technique electron impact ionization, or chemical ionization that required gaseous phase molecules, so could only ionize volatile compounds (Feng et al., 2008).

ESI is composed of a needle with a high voltage (1-3kV) applied to it, and an opposing metal plate/counter electrode of the opposite charge to create an electric field gradient (Kearle and Verkerk, 2009). As the analyte is pumped through the needle, the electric field gradient charges the liquid as it is expelled from the needle tip. This creates charged liquid droplets, as the solvent evaporates, the charged analytes repel each other, and the charged analytes are drawn into the MS Figure 2.3-2.

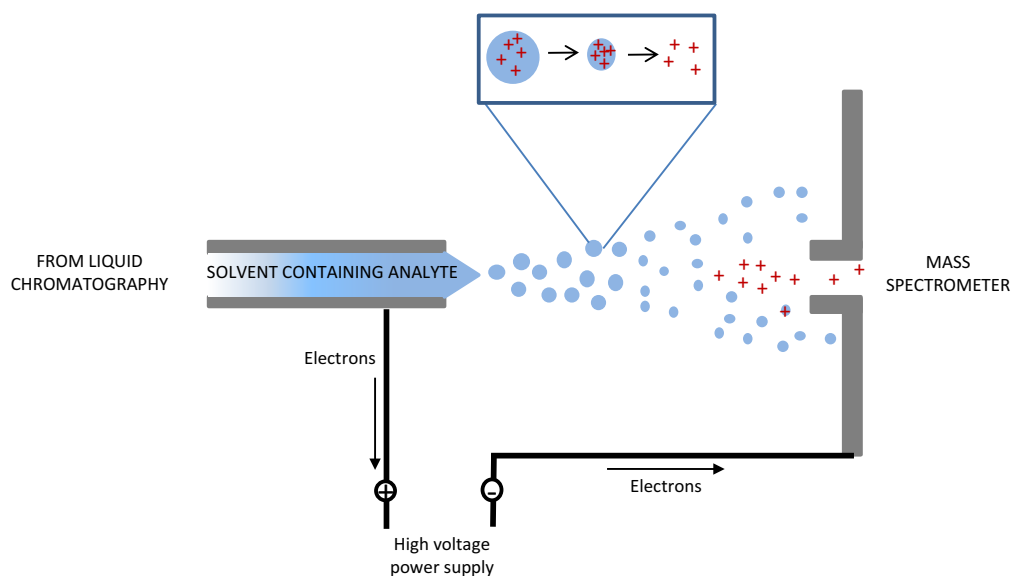


Figure 2.3-2 A diagram depicting stages of electrospray ionisation (ESI), (adapted from Kobarle and Verkerk, 2009).

2.3.2 Quadrupole and Time-of-Flight Mass Analysers

Mass analysers commonly used in metabonomics include time-of-flight (TOF) and quadrupole (Q) analysers. TOF MS instruments calculate the ion transit time through a drift region to separate ions of different masses based on the principle that ions of the same kinetic energy but different masses take different times to traverse a fixed distance (Maher et al., 2015). In contrast, a Q-MS consists of four parallel electrodes, used to create an electric field where only ions of the selected m/z can pass through to the detector without hitting the quadrupoles (Maher et al., 2015). QMS can have greater sensitivity (detecting analytes at very low concentrations), whereas the TOF has greater resolution and mass accuracy (differentiate between analytes that are close in mass).

Different combinations of mass analysers are often used together, such as a triple quadrupole (TQ) or the Q-TOF. In a Q-TOF a quadrupole is first used to scan either a specific mass or range of masses, a collision cell can be used to fragment ions before a TOF mass analyser separates ions by m/z (Maher et al., 2015). Q-TOF MS is often used for untargeted metabonomic analyses due to their high resolution and mass accuracy (Want et al., 2007). In TQ-MS, the middle quadrupole is used as a collision cell, and first and final quadrupoles can select for known precursor and product ion m/z . TQM is commonly used for accurate quantification of a known metabolite, for example in xenobiotic pharmacokinetic studies or the quantification of a known biomarker, as they provide greater sensitivity and reproducibility compared to Q-TOF MS (Want et al., 2005).

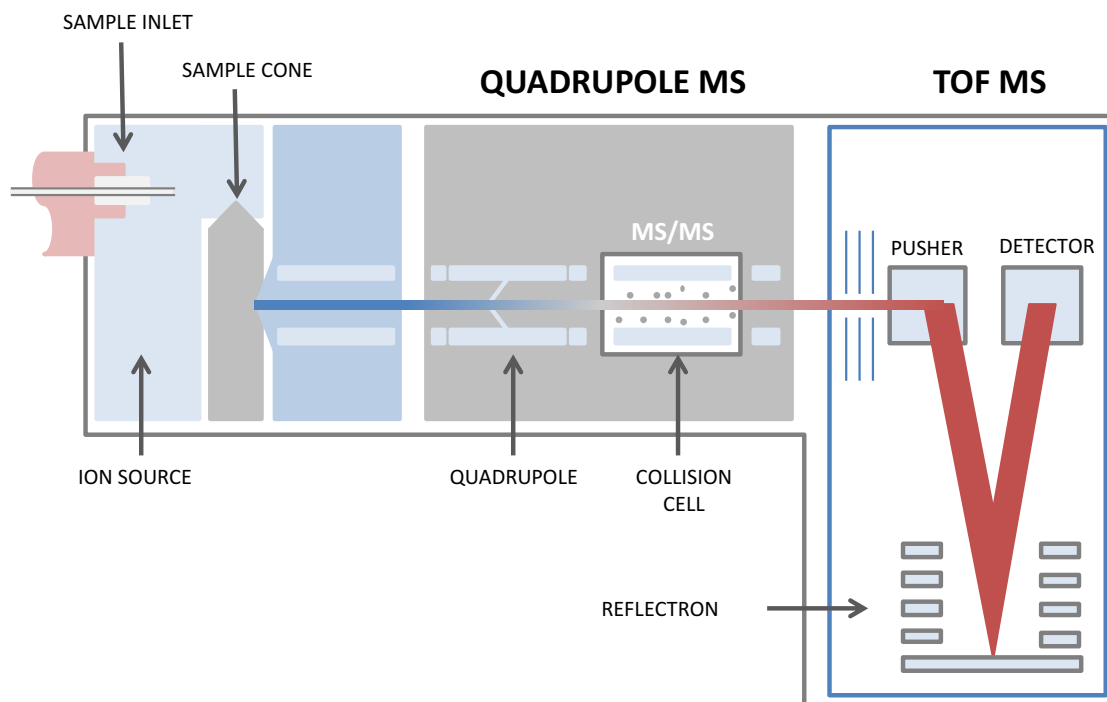


Figure 2.3-3 A illustrative representation of a Q-TOF MS, adapted from Waters promotional material

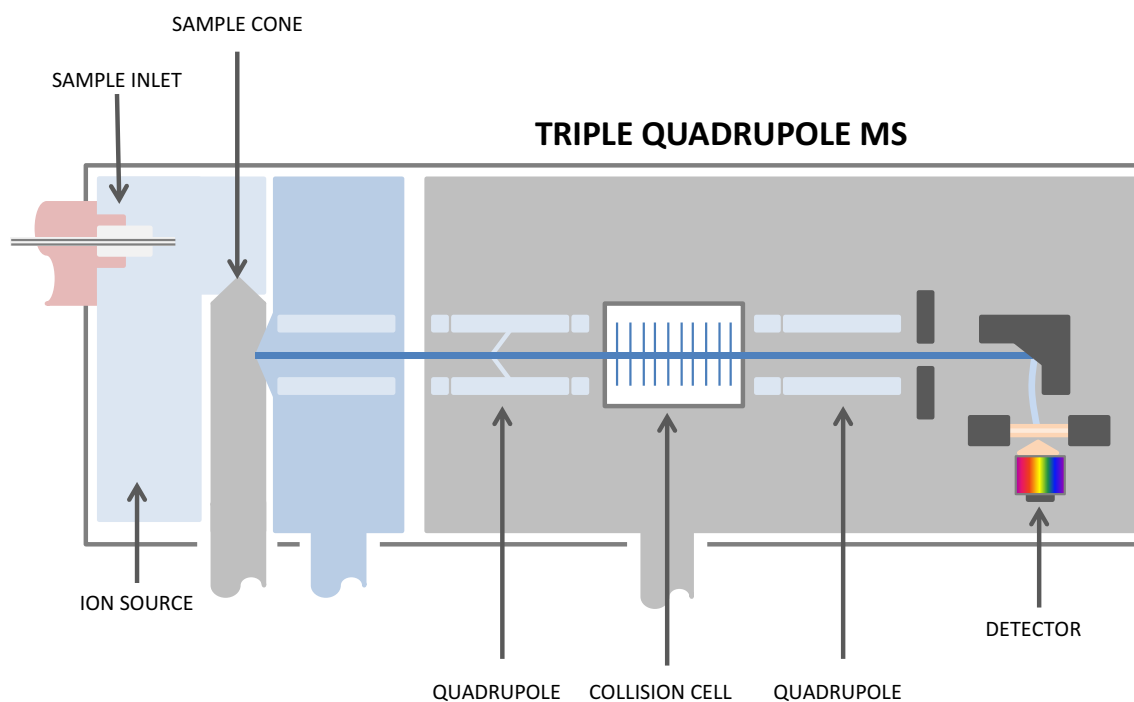


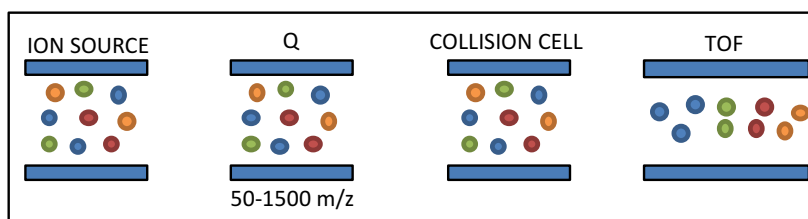
Figure 2.3-4 Figure 4 A illustrative representation of a TQ-MS, adapted from Waters promotional material

For untargeted analyses in Chapters 3 and 4, a Q-TOF was used in full scan mode. The quadrupole was set to scan masses 50-1500m/z, and the ions were then separated by the TOF-MS, enabling accurate m/z detection of unknown metabolites.

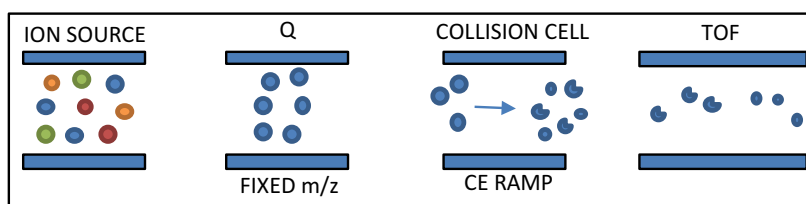
A Q-TOF was subsequently used for product ion scanning experiments (MS/MS). The first quadrupole was set to select only the m/z of the compound of interest. The compound was fragmentation in the collision cell, before the fragments were analysed in the TOF-MS. In Chapter 3 this approach was used to determine likely drug metabolite identifications.

A TQ-MS was used for multiple reaction monitoring (MRM) experiments, for both targeted and quantitative analyses. The first quadrupole was used to select the known precursor ions, before they were fragmented in the middle quadrupole (collision cell). The final quadrupole was also fixed for known product ions. Where quantification was obtained, a standard curve and internal standards of known concentrations were run to enable unknown concentrations to be extrapolated.

A. Full scan- untargeted profiling



B. Product ion scanning- metabolite identification



C. Multiple reaction monitoring- targeted analyses & quantification

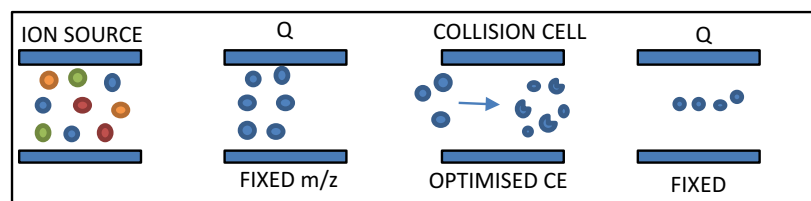


Figure 2.3-5 Schematic depicting different mass spectrometry experiments. Q-Quadrupole, TOF- time of flight, CE- collision energy. Adapted from Maher et al., 2015.

2.4 DATA PRE-PROCESSING OF UNTARGETED LC-MS DATA

To extract information from the raw data generated from LC-MS analyses, pre-processing is required. Key stages include alignment, peak picking, deconvolution, and normalization (Want and Masson, 2011), that can be performed in slightly different orders depending of the software used. LC-MS pre-processing can be undertaken using freeware such as XCMS (Smith et al., 2006), or commercial products such as Progenesis (Waters), different software offer different advantages and disadvantages. For example, XCMS provides greater user control for optimization for parameters, whereas, Progenesis provides a more streamlined approach for endogenous metabolite identification and visualization.

Alignment. Retention time alignment is required to correct for retention time shifts that can occur during an analytical run, this is important for comparing compounds between samples (Want and Masson, 2011), as a compound is defined by m/z and retention time so poorly aligned spectra can lead to compounds incorrectly being identified as separate compounds. This can be performed prior to peak detection (e.g. in Progenesis), or after peak detection (e.g. in XCMS). An example of a chromatographic alignment correction performed in Progenesis, can be seen in Figure 2.4-1.

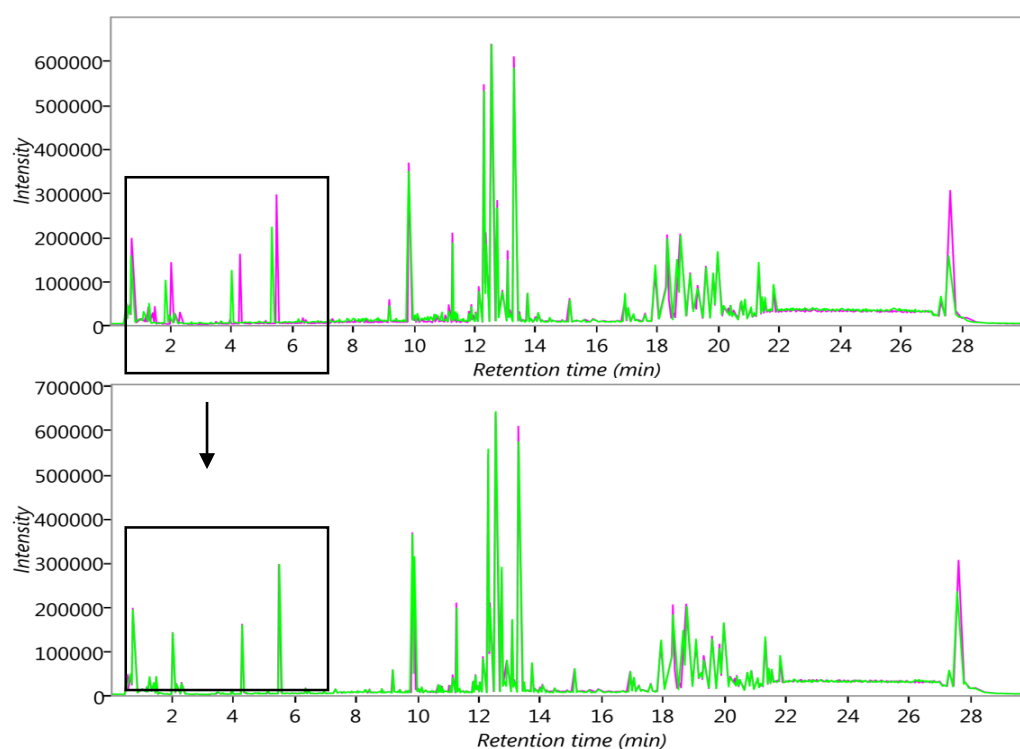


Figure 2.4-1 Total ion chromatogram of a plasma QC sample analysed in ESI-positive mode: (A) is a poorly aligned spectra, and (B) is a sample after alignment

Peak detection and integration. Peak detection or “picking” refers to the determination of what compounds or “features” are in the raw data (Want and Masson, 2011). During this process each ion detected with a unique m/z and retention time will be defined as a feature/compound. For example, Figure 2.4-2 shows an ion map for the peaks picked from a plasma sample in Progenesis. Peak matching involves the alignment and matching of peaks across samples, this ensures features are correctly defined in each sample by accounting for small m/z and retention time differences. Accurate peak matching is crucial in enabling comparisons of compound abundance between samples. The features found during peak detection are then integrated to determine the relative abundance of each feature in each sample (Want and Masson, 2011).

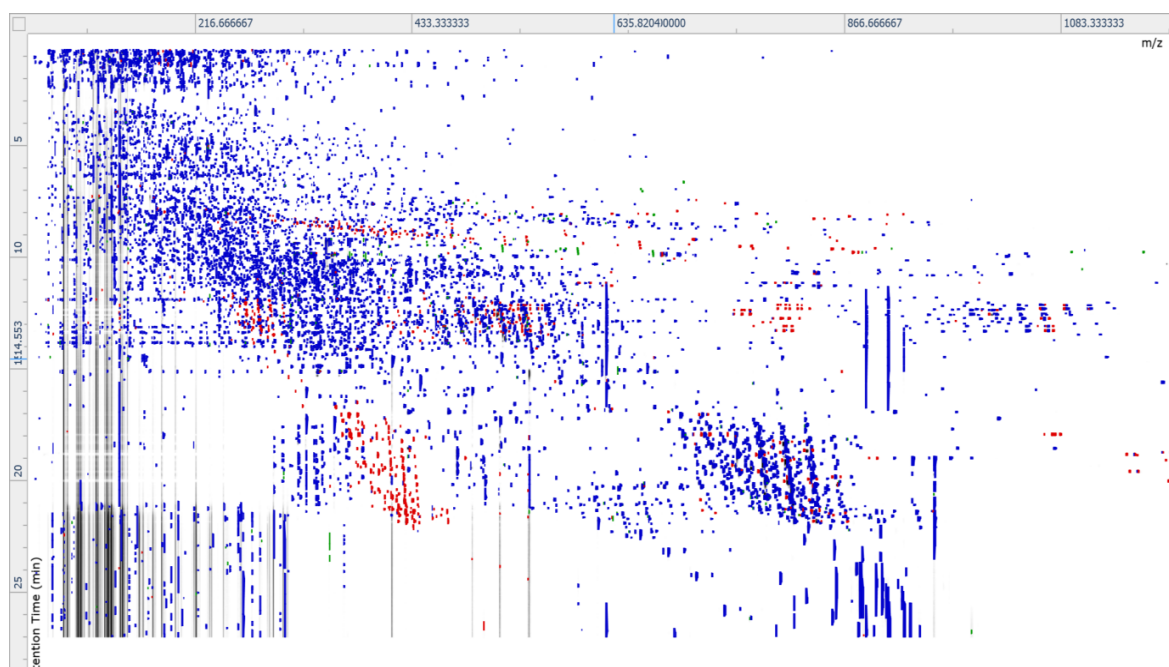


Figure 2.4-2. An ion map ions peak picked in a plasma QC sample analysed in ESI –positive mode. Blue indicates singly charged ions, red indicates doubly charged ions.

Deconvolution. Multiple features can be detected for a single analyte, due to their isotopes and any dimers, adducts or fragments formed during ionization. To enable easier data interpretation, ideally these would be identified as a single compound for further analysis. This cannot be achieved through assuming co-eluting peaks are related, as all analytes not separated by LC will also co-elute (Want and Masson, 2011). Therefore, deconvolution approaches also look for specific m/z differences known to relate to common adducts/losses. An example is demonstrated in Figure 2.4-3, where the neutral mass has been calculated from the proposed fragment ($M+H-H_2O$) and adducts ($M+H$, $M+Na$), and have been overlaid. In this

example, the neutral mass and corresponding chromatographic peaks both show a close match, indicating a high likelihood that they have been correctly assigned. Deconvolution is particularly helpful for metabolite identification, as knowing the neutral mass of an unidentified compound greatly reduces the number of possible identifications when using m/z for database searching.

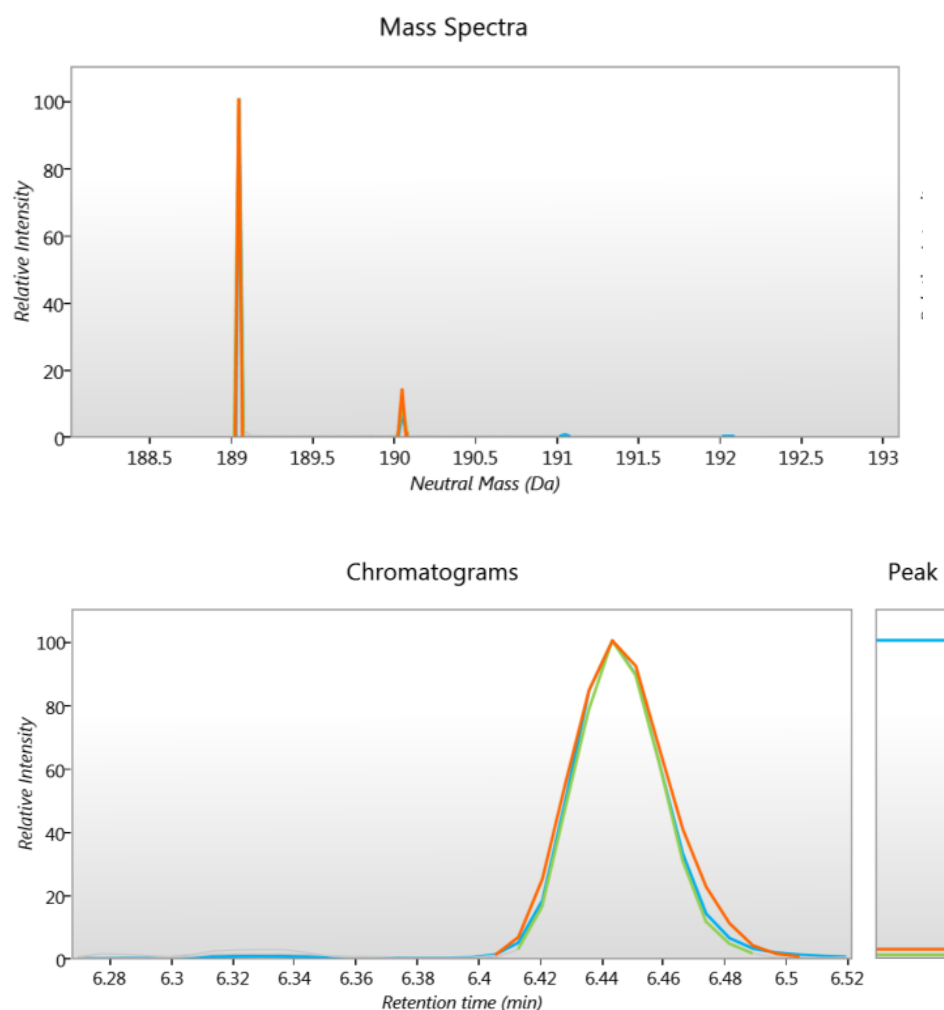


Figure 2.4-3 Deconvolution of adducts to determine neutral mass of a compound, overlay of m/z of co-eluting ions. blue is the $M+H$, orange is $M+H-H_2O$, green is $M+Na$ adduct.

Normalisation. A transformation across variables (feature abundance) within one sample—designed to account for global differences in detected ion intensity in a sample run (Want and Masson, 2011). This can be due to analytical changes such as source contamination or differences in global sample concentration such as urine. Various statistical approaches exist that can be applied to determine the different scaling factors for each sample, for example total area normalization and probabilistic quotient normalization. Total area divides 1 by the

total intensity of features, this approach assumes total intensity will be equal, which may not be the case with drug metabolites in urine. On the other hand, probabilistic quotient normalization determines the normalization factor by calculating the median fold change of feature intensity to a reference spectrum (Dieterle et al., 2006). The assumption with median fold change is that 50% of the data does not change.

2.5 CHEMOMETRICS

The vast data sets produced from metabonomic studies can be made interpretable using advanced chemometric techniques (Lindon and Nicholson, 2008). These include 'unsupervised' approaches, such as principal components analysis (PCA), which require no input of sample class, and 'supervised' approaches, such as orthogonal partial least squares discriminant analysis (OPLS-DA), which requires sample class to be defined. Both of these approaches improve visualisation of data by reducing its dimensionality, and can reveal metabolic patterns that could not otherwise be readily determined (Lindon and Nicholson, 2008).

Discriminatory metabolites between phenotypes of interest, such as toxicity, will contribute to separation of classes, which can be visualised on a 'scores plot'. The 'loadings plot' from a PCA or O-PLS- DA analysis indicates the relative contribution to variation from the specific features. S-plots can be used to show the modelled covariation/magnitude against the modelled correlation/ reliability. The features with the highest magnitude and reliability are seen at the extremities, and can be selected for further examination and identification.

To avoid over-fitting, it is important to validate models by assessing their goodness of fit (R^2) and goodness of prediction (Q^2). Model validity can be assessed through the use of permutation tests, or analysis of variance of the cross-validated residuals (C.V ANOVA) calculations(Eriksson et al., 2008).

RESULTS

A COMPARATIVE STUDY OF THE METABOLISM OF TIENILIC ACID
AND TIENILIC ACID ISOMER IN THE RAT

3.1 INTRODUCTION

3.1.1 Rationale and aims

The thiophene moiety is a five membered ring containing a sulphur atom (Figure 3.1-1). Several thiophene containing drugs have been associated with chemically reactive metabolite (CRM) formation and toxicity (Gramec et al., 2014). As a result, the thiophene moiety is considered a 'structural alert'; indicating an increased risk of toxicity. Despite this, there are several clinically important drugs that contain a thiophene, such as the antidepressant duloxetine, that do not undergo bioactivation and are considered safe (Chan et al., 2011). An improved understanding of the metabolism of different thiophene containing compounds could therefore be central to identifying which specific chemical factors lead to thiophene toxicity; contributing to safer drug design.

Tienilic Acid (TA) is a thiophene containing drug that was released in the 1970s to treat hypertension (Lau et al., 1977). However, it was withdrawn after just a few months on the market, due to previously undetected idiosyncratic hepatotoxicity (Zimmerman et al., 1984b). This was suspected to be immune mediated following the discovery of an antibody directed against TA bound to CYP2C9, the enzyme involved in its metabolism (Beaune et al., 1987, Homberg et al., 1984, Dansette et al., 1991). In contrast, a structural analogue of TA, Tienilic Acid Isomer (TAI), despite having similar pharmacological actions as TA, is an intrinsic hepatotoxin (Bonierbale et al., 1999, Dansette et al., 1991, Mansuy, 1997). It is interesting that although TA and TAI are structurally very similar (Figure 3.1-1), differing only at the site of thiophene conjugation, they elicit very different toxicological responses. This has given rise to studies comparing their metabolism, with the aim of finding links to their respective mechanisms of toxicity.

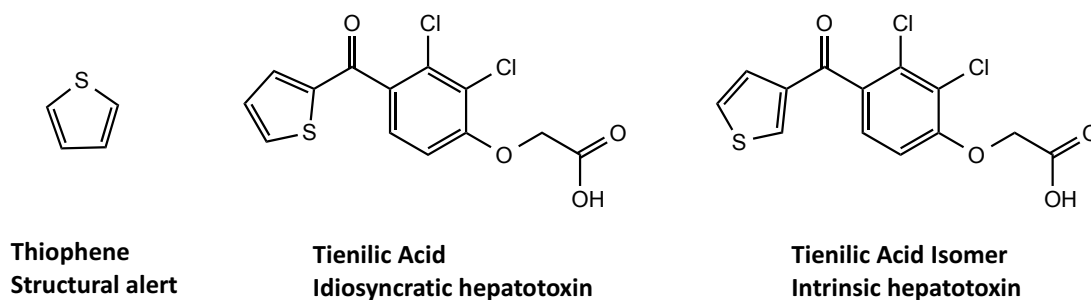


Figure 3.1-1 The chemical structures of a thiophene moiety, and the thiophene containing hepatotoxins Tienilic Acid and Tienilic Acid Isomer

Previous studies have assessed TA and TAI metabolism using *in vitro* microsomal incubations (Dansette et al., 1990, Lopez-Garcia et al., 1994, Mansuy et al., 1984, Koenigs et al., 1999, Lopez Garcia et al., 1993, Dansette et al., 1991, Belghazi et al., 2001, Rademacher et al., 2012, Rademacher, 2011b), and have found evidence of glutathione (GSH) conjugation to the thiophene ring in response to both drugs (Belghazi et al., 2001, Rademacher, 2011b), however, it was found to be a more minor route in TA than TAI. As with TA the major drug metabolite is known to be an hydroxylated metabolite (Mansuy et al., 1984). There is also some limited *in vivo* evidence of CRM formation in both drugs, with separate studies reporting a TA-GSH conjugate detected in rat bile (Nishiya et al., 2006), and TAI associated mercapturate and cysteine conjugates in rat urine (Valadon et al., 1996). However, since the 1980s there has not been a comprehensive characterisation of TA drug metabolites *in vivo*, and there are only limited reports of the *in vivo* metabolites of TAI (Valadon et al., 1996).

The aim of the work presented in this chapter was to characterise the *in vivo* metabolites of TA and TAI, taking advantage of advances in LC-MS technology, such as the improvements in resolution, sensitivity, and tandem MS (MS/MS). For this study, the rat was chosen as it has previously been shown as comparable and translatable to human TA metabolism (Mansuy et al., 1984). Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) was applied to generate global metabolic profiles (including xenobiotic and endogenous compounds) from liver extracts, plasma and urine. UPLC-MS/MS was then used to further characterise the drug metabolites. A parallel exploration of the impact of TA and TAI on endogenous metabolism is presented in Chapter 4.

3.1.2 Hypothesis

The hypothesis central to the work presented in this Chapter was that, consistent with *in vitro* studies, the metabolism of TA and TAI would differ, and that TAI would show greater evidence of CRM formation than TA.

3.2 MATERIALS AND METHODS

3.2.1 Contributions of others

The animal study, histopathology and ALT activity analyses were undertaken by collaborators at Michigan State University, U.S (Professor Roth). The TA/TAI synthesis and quantification in plasma were performed by collaborators at the University of Washington, U.S (Professor Nelson). All analytical sample preparation, UPLC-MS, UPLC-MS/MS analyses and the related data processing were performed by the author at Imperial College London.

3.2.2 Animal handling and sample collection

3.2.2.1 Animals

Male Sprague–Dawley rats [CrI:CD(SD)IGS BR] weighing 250–300g were obtained from Charles River Laboratories (Portage, MI). The animals were housed under controlled conditions with a temperature range of 294–300 K and relative humidity between 30–70%. A 12 h light/dark cycle was maintained throughout the study. Water and food (Standard Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) were provided *ad libitum*. There was a one-week acclimatisation period prior to the study commencing.

3.2.2.2 TA/TAI preparation

TA and TAI (99% purity) were synthesised as previously reported (Rademacher et al., 2012), and were found to have a purity of 99%. TA and TAI (75mg/mL) were prepared in Trizma base with HPLC-grade water to a molar ratio of 1:1.1, with a pH 7.4. TA/TAI preparations were vortexed, sonicated, and vortexed again to ensure their full suspension in solution.

3.2.2.3 TA/TAI administration

Animals were fasted overnight for 15h (6.00pm-9.00am) prior to intravenous administration (1.5 mL/min) of 250 mg/kg of TA (n=5 per time-point, total of n=15), 250 mg/kg TAI (n=5 for 2h and 6h time-points n=6 for 24h time-point, total n=16), or vehicle (Trizma base in HPLC-grade water; n=5 per time-point). Food was provided *ad libitum* for the remainder of the study, and animals were housed in individual metabolism cages.

3.2.2.4 Sample collection

At 2h, 6h or 24h post-treatment animals were euthanised with isoflurane. Blood was collected from the vena cava into vacutainer tubes containing heparin, and then centrifuged to separate

the plasma. Immediately after euthanasia necropsies were performed and sections of the left lateral lobe of the liver were removed and snap frozen in liquid nitrogen until UPLC-MS analysis. For liver histopathology, three representative slices 3-4mm thick were taken, and fixed in 10% buffered formalin.

Urine was collected only for the animals euthanized at the 24h time-point. The collection periods were between 0–2h, 2–6h, and 6–24h post-dose. The urine was collected via cage funnel and immediately cooled with dry ice, and at the end of the time period samples were frozen at –80°C. The total urine volume per animal at each time point was recorded, to compare the diuretic action of TA/TAI. Due to insufficient urine not all urine samples were available for analysis in this study (vehicle: 2h group n=0, 6h n=4 and 24h n=5; TA n=5 per time-point; TAI n=6 for 2h and 24h time-points, and n=5 for the 6h time-point).

3.2.2.5 Overview of animal study design and sample collection

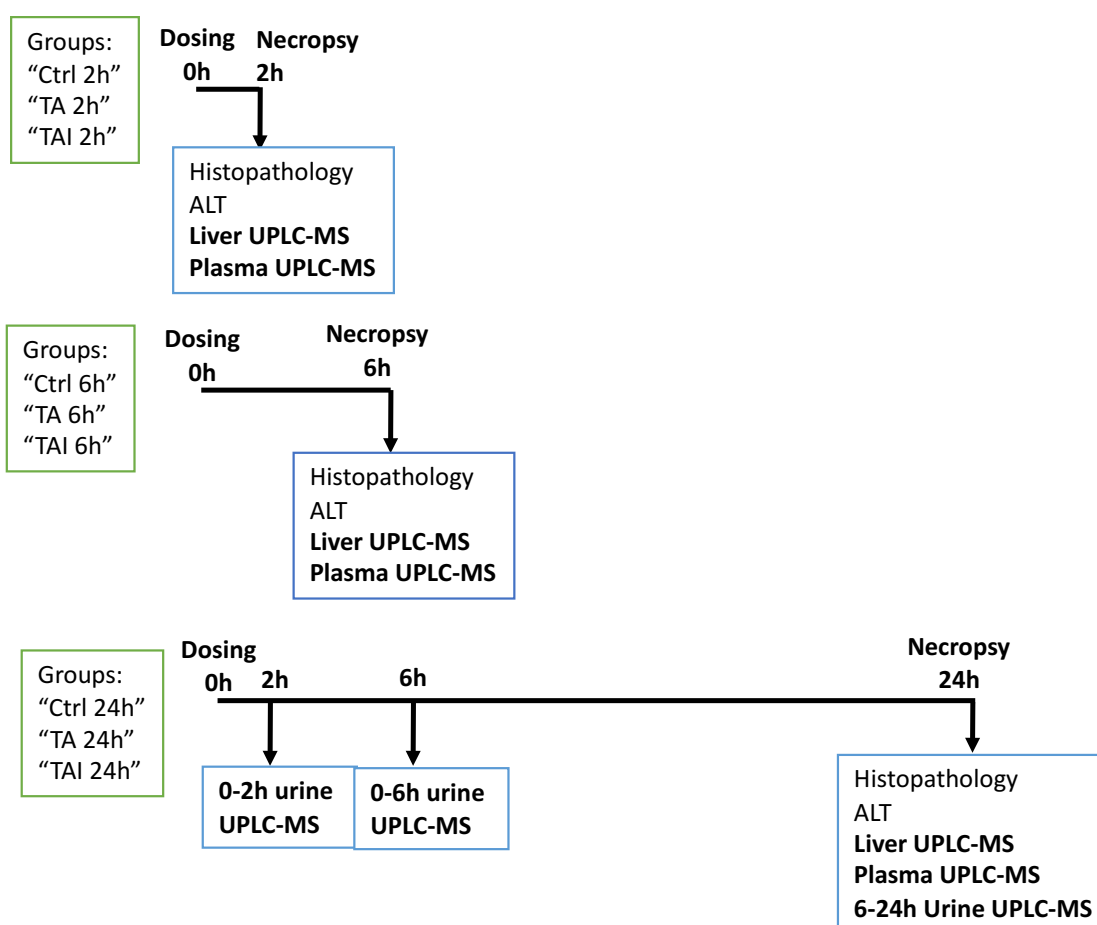


Figure 3.2-1 A figure depicting the study design, sample collection points and analyses performed. Ctrl: control/vehicle treated animals, TA: Tienilic Acid, TAI: Tienilic Acid Isomer, ALT: alanine aminotransferase activity, UPLC-MS: ultra-performance liquid chromatography mass spectrometry.

3.2.3 Liver histopathology and plasma ALT activity

The formalin-fixed, paraffin-embedded liver slices were cut into 6µm sections and stained with hematoxylin and eosin. The liver sections were examined under light microscope by a pathologist masked to time and drug treatment information, except which were from vehicle treated animals. Scores (0-3) were given for lipid like vacuolation, cell death, presence of glycogen, and inflammation. A “0” score represented “no abnormalities detected”, and scores of 1, 2 and 3 represented “mild”, “moderate” and “marked” abnormalities detected, respectively. The plasma alanine transferase (ALT) activity was determined using an Infinity-ALT kit (Thermo Corporation, Waltham, MA, USA). Statistical significance was calculated in Prism 6.0 (GraphPad, La Jolla, California, USA) using two-tailed Mann Whitney test.

3.2.4 Quantification of TA/TAI in plasma

Full details have been published elsewhere (Coen et al., 2012). Briefly, the quantification of TA and TAI in plasma samples was performed using an LC-MS/MS method, operated in positive ion mode. Transitions of 331 → 247 were used for TA and TAI, and 325 → 247 for the internal standard (4- benzoyl-2,3-dichlorophenoxy) acetic acid.

3.2.5 Untargeted UPLC-MS

The untargeted UPLC-MS analyses were performed with the aim of obtaining both xenobiotic and endogenous metabolite information, therefore, the sample preparation and UPLC-MS conditions were based on previously reported metabonomic approaches, as detailed below.

3.2.5.1 Reagents

Optima LC-MS grade 0.1% formic acid in water was obtained from Fisher Scientific, Loughborough, UK. LC-MS Chromasolv grade methanol, LC-MS Chromasolv grade acetonitrile, and formic acid (approximately 98% for MS) were obtained from Sigma Aldrich, Gillingham, UK.

3.2.5.2 Quality control and sample preparation

Aqueous Liver extracts were prepared following a protocol developed by Want et al. (2013) for LC-MS based tissue metabonomics. Briefly, in 2mL polypropylene tubes (Precellys) containing approximately 100µL of zirconium beads, 1.5ml of ice cold methanol/water (1:1) was added to 50.0mg (±1.8mg) liver. The tissue was homogenized using a Precellys bead beater, at 6 500Hz speed for 40s, cooled on ice, and then subjected to another cycle of 40s at 6 500Hz. The samples were then centrifuged at 4°C for 20min at 10 000g. Supernatants were dried down in

a Savant Vacuum Concentrator (180 minutes at 45°C, V-AQ mode). The dry residue was then re-dissolved with 120µL of methanol/water (1:1), centrifuged for 5 min at 13 000g, before being placed in MS vials.

Plasma samples were extracted based on a previously reported protocol, shown to provide effective, straightforward and reproducible results for metabonomic profiling of plasma (Want et al., 2006). The plasma samples were thawed and then vortexed. 150µL of ice cold methanol were added to 50µL of plasma and vortexed briefly. Samples were kept at -20°C for 40 minutes before centrifugation at 13 000g for 10 min. The supernatants were transferred to eppendorfs and dried down in a Savant Vacuum Concentrator (45°C, AQ mode). The dry residues were re-suspended in 100µL of water and briefly sonicated before being transferred to MS vials.

The urine samples were prepared following a sample preparation procedure reported by Want et al. (2010). Urine samples were thawed at room temperature before being vortexed. 70µL of urine was added to 210µL of water, vortexed again, and centrifuged at 13 000g for 10 min. The supernatants were then transferred into a 96-well plate prior to UPLC-MS analysis.

Instrument/sample stability was monitored using an approach previously reported for tissue extracts (Want et al., 2013), and urine (Want et al., 2010). Composite Quality Control (QC) samples were made by from combining aliquots of all prepared samples in the sample set to be analysed. For liver extracts and plasma, 20µL of each prepared sample were used for the QC, in urine analyses 30µL of each prepared sample was combined. These were injected ten times prior to the analytical run to condition the column, and were also used to monitor instrument and sample stability, by injecting the QC after every five samples for aqueous liver extracts and plasma, and after every 10 samples for urine.

3.2.5.3 Chromatography

The chromatography used for liver and plasma analyses were based on previously developed methods (Want et al., 2013), as were the urine samples (Want et al., 2010). Chromatography was performed on an Acquity UPLC system (Waters, Elstree, U.K.), with a temperature controlled auto-sampler set to 4°C to minimise sample degradation.

For aqueous liver and plasma sample analysis a Waters Acquity UPLC HSS T3 Acquity column (2.1 x 100mm, 1.8 µm; Waters Corporation, Milford, USA) was used with an injection volume of 8µL. A temperature of 50°C and a flow rate of 0.4 mL/min were maintained throughout. A

26 min gradient was employed with mobile phase A of 0.1% formic acid (FA) in water and mobile phase B 0.1% FA in methanol. The gradient started at 0.1% B and was held for 2min. The composition was increased linearly to 25% B over 4 min, increasing to 80% B by 8min, to 90% B by 12min, and to 99.9% B by 21min. It was held at 99.9%B for 2 min before being brought back to 0.1% B by 24min, when it was held for 2min prior to the next injection.

For urine sample analysis a Waters Acquity UPLC HSS T3 Acquity column (2.1 x 100mm, 1.8 μ m; Waters Corporation, Milford, USA) column was utilised with a temperature of 40°C and a flow rate of 0.5 mL/min. A 12 min gradient was employed with mobile phase A of 0.1% formic acid (FA) in water and mobile phase B 0.1% FA in ACN. The composition was increased linearly from 0 - 15% B over 1 min, then to 50% B over 2 min, 95% B over 3 min, retained at 95% B until 9.9 mins then returned to 100% A over 0.2 min, and re-equilibrated over the next 1.9 min prior to injection of the next sample.

3.2.5.4 Mass Spectrometry

An LCT Premier time-of-flight mass spectrometer (Waters, Manchester, U.K.) was used to analyse all samples in both positive and negative electrospray ionisation (ESI) modes, based on the MS methods previously reported (Want et al., 2013, Want et al., 2010). The source temperature was 120°C and desolvation temperature was set to 350°C. The cone gas flow was 25 L/h with a desolvation gas flow of 900 L/h. The capillary voltage was set to 3200 V and the cone voltage to 35 V. The V optics mode was selected for optimal sensitivity. Data were acquired over the m/z range of 50-1000 with scan time of 0.2s and an interscan delay of 0.01s. Data were collected in centroid mode.

The instrument was calibrated before analysis using a sodium formate solution (0.5 mM). Leucine enkephalin (MW = 555.62) at 200 pg/ μ L in acetonitrile/water 50:50 was used as a lock mass and injected every 3.5min. The run order was randomized to minimize the impact of any instrument changes over the course of the run.

3.2.5.5 Data processing of untargeted UPLC-MS data

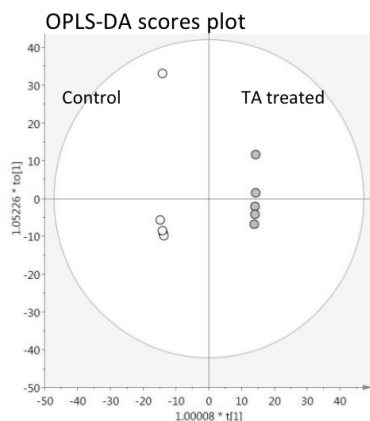
Automated peak integration was performed in XCMS (Smith et al., 2006), after the raw UPLC/MS data files were converted to netCDF format by using the DataBridge tool found in MassLynx V4.1 software (Waters). The CentWave algorithm was used for peak picking (Tautenhahn et al., 2008). A peak width window of 2-20s was used, and the m/z width for the

grouping step was changed to 0.05Da. The bandwidth parameter was optimised from the default 30s by performing retention time correction iterations. The output table consisting of retention time, m/z, and intensity (peak area) of detected features for each sample was obtained and viewed using Excel (Microsoft). Prior to multivariate statistical analysis data were normalised using probabilistic quotient normalisation (Veselkov et al., 2011, Dieterle et al., 2006), to adjust for differences in sample concentration.

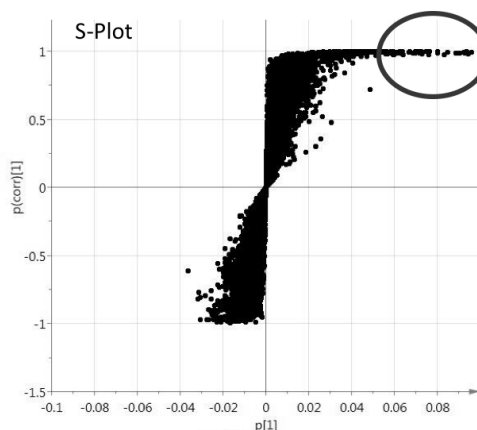
3.2.5.6 Isolation of xenobiotic from endogenous features

In SIMCA, data were log transformed and mean-centred to reduce the likelihood of the models being dominated by the largest features detected, which is an approach previously shown to be effective on tissue samples for metabonomic analyses (Masson et al., 2011). This was preferred as the intensity of the features detected are not necessarily correlated to the likelihood of them being a drug metabolite (i.e. not endogenous), and could help to reveal more minor or less well ionized drug metabolites. Multivariate statistical modelling was then used in conjunction with manual isotope recognition, to distinguish xenobiotic from endogenous features, as summarised in Figure 3.2-2. Both unsupervised and supervised multivariate statistical modelling was performed in SIMCA-P+ Version 14.1 (Umetrics). First, principal component analysis (PCA) models were computed and the scores plots were observed for an overview of the UPLC-MS data, and to identify any outliers. A supervised approach, using pairwise OPLS-DA models was then taken to increase the ease of identifying discriminating metabolites between treatments, and OPLS-DA S-plots were used to reveal features that correlated most to each class. The mass spectra of features most strongly correlated to class, as revealed in S-plots, were then manually assessed to determine the likelihood of them being a drug metabolite. Drug metabolite features were distinguished from endogenous metabolites based on the stable isotope pattern generated from the dichlorinated structure of TA and TAI.

1. Multivariate statistical analysis

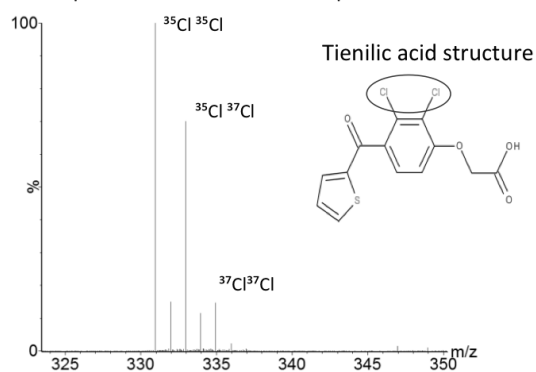


2. Select the most differentiating features between groups



3. Assess isotope ratio to differentiate likely xenobiotic from endogenous features

MS spectra characteristic isotope ratio of di-chlorination



4. Characterise drug metabolites using MS/MS

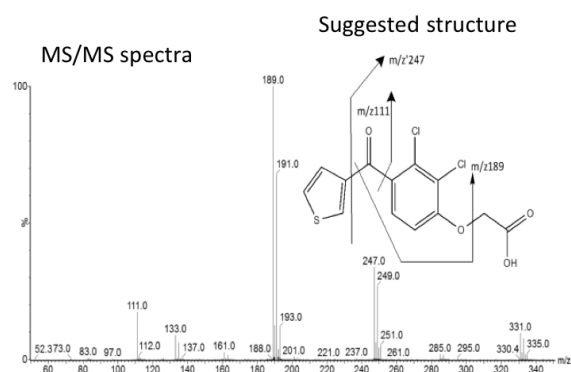


Figure 3.2-2 An overview of methodological approach taken to separate endogenous from TA/TAI related metabolites.

3.2.6 Characterisation of drug metabolites using UPLC-MS/MS

Drug metabolite features were further characterized using a UPLC-MS/MS approach. The same chromatography and mass spectrometry conditions were applied as previously stated, however, selected ions determined to be likely drug metabolites were targeted for fragmentation using a ramp of 10-40V. The ions selected for fragmentation are detailed in figure legends.

To assess the distribution of metabolites across treatment and time groups, dot-plots were generated from the XCMS output spreadsheet, using Prism 6.0 (GraphPad, La Jolla, California, USA).

3.3 RESULTS

The quantification of plasma TA and TAI, urine volume, ALT activity, and histopathology scores have previously been published (Coen et al., 2012). With permission, it remains detailed here to provide an overview, to anchor the drug metabolite data and help interpret the results presented.

3.3.1 TA and TAI plasma concentration

The plasma concentrations of TA and TAI were quantified (Figure 3.3-1 A) to compare the pharmacokinetics of TA/TAI. At 2h post dose the mean plasma concentration of TA was detected as 20.1 μM , which fell to 1.0 μM by 6h and was below the limit of detection (3nM) by 24h. Similarly, the mean concentration of TAI in plasma was detected as 23.4 μM at 2h, 0.6 μM at 6h and was also below the limit of detection (3nM) by 24h.

3.3.2 Urine volumes

To compare the diuretic action of TA and TAI, animals were individually caged and urine was collected and measured (Figure 3.3-1 B). There was a statistically significant, by Mann Whitney test, increase in the volume of urine collected at 2h from both TA and TAI treated animals compared to control animals, and no significant difference between TA and TAI. There were no significant differences at the later time-points.

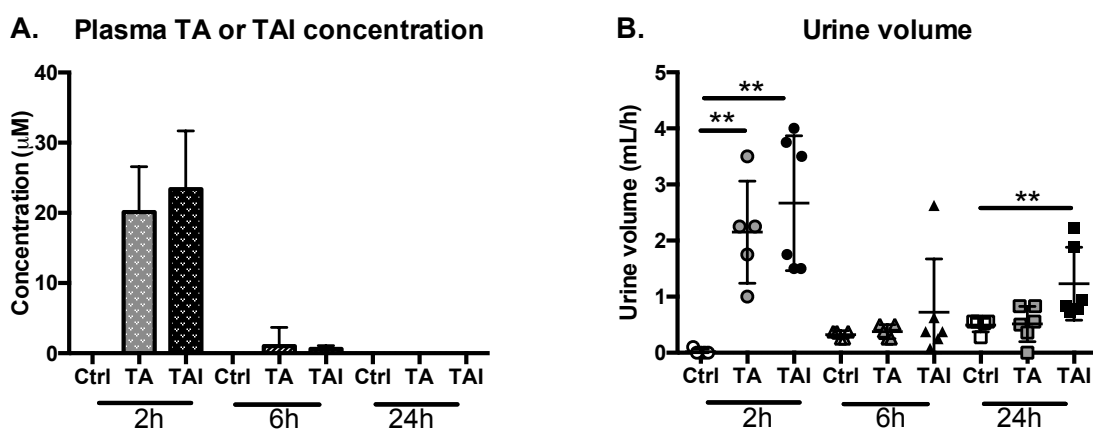


Figure 3.3-1. (A) The plasma TA and TAI concentration and (B) the collected urine volume, from rats treated with Tienilic Acid (TA), Tienilic Acid Isomer (TAI) or Vehicle/Control (Ctrl), collected at 0-2h, 2-6h and 6-24h post-dose. In (A) bars indicate mean, and lines show standard deviation. In (B) each, symbol represents an animal, lines indicate mean with standard deviation. Significance was calculated using Mann Whitney test $**p<0.01$. Adapted with permission from Coen M, et al., 2012, Comparative NMR-Based Metabonomic Investigation of the Metabolic Phenotype Associated with Tienilic Acid and Tienilic Acid Isomer, *CHEMICAL RESEARCH IN TOXICOLOGY*, Vol: 25, Pages: 2412-2422, ISSN: 0893-228X. Copyright 2012 American Chemical Society.

3.3.3 ALT Activity

The quantification of the traditional marker of liver damage, alanine transferase (ALT) activity, revealed a very slight, statistically significant increase in ALT at in TA treated animals compared to vehicle at 24h (Figure 3.3-2). In the TAI treated animals, there was large, but variable increase in ALT activity at 6h, and a statistically significant increase compared to both vehicle treated and TA treated animals by 24h.

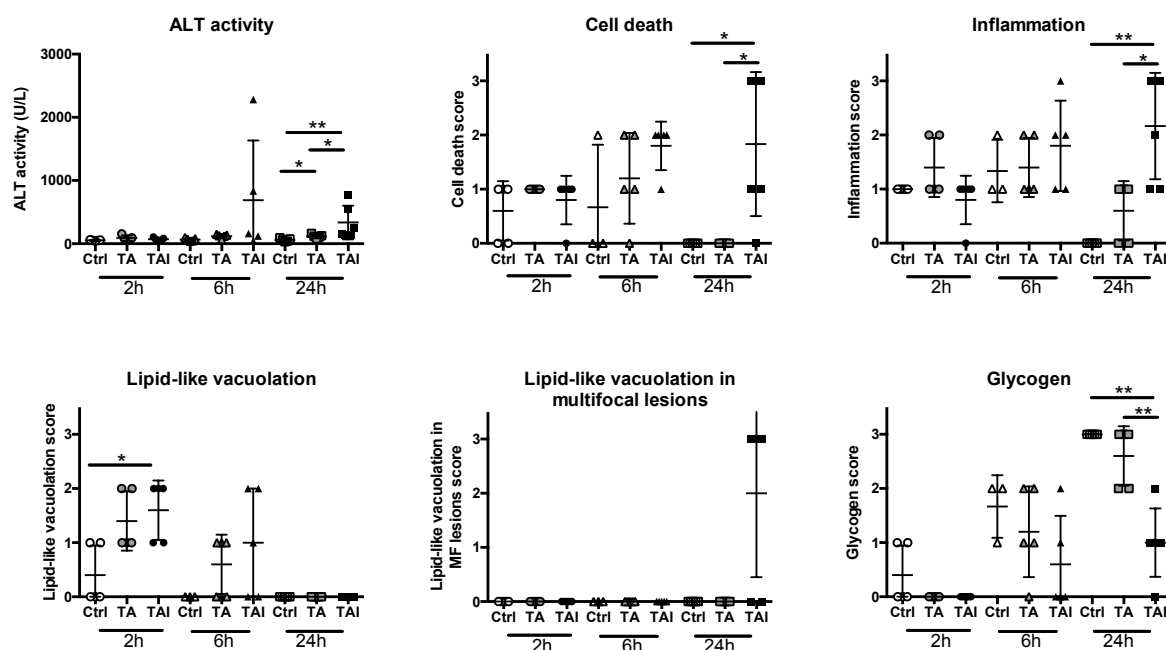


Figure 3.3-2 Alanine transferase activity (ALT) activity and liver histopathology scores. The scoring criteria are: 0= absent, 1= mild, 2= moderate and 3= marked. Each dot represents an animal, and lines indicate mean with standard deviation. Significance was calculated using Mann Whitney test *, $p < 0.01$. Adapted with permission from Coen M, et al., 2012.

3.3.4 Liver Histology

Histopathology examination of liver sections revealed mild cell death (hepatocyte necrosis), mild and moderate inflammation, and lipid-like vacuolation in some control, TA and TAI treated animals at 2 and 6h. However, at 24h there was no cell death, inflammation, or lipid like vacuolation in control or TA treated animals, whereas in TAI treated animals there was significant cell death and inflammation. The early time-point lesions were distinct in appearance to those observed in TAI treated animals at 24h, and were considered unrelated to drug treatment. Liver glycogen increased over time in control rats from absent/mild at 2h to marked at 24h. Liver glycogen in TA treated animals was absent at 2h, and recovered to moderate/marked by 24h. In contrast, hepatic glycogen in TAI treated animals was statistically lower than both TA and vehicle treated animals at 24h.

3.3.5 Drug metabolites

3.3.5.1 TA metabolites in aqueous liver extracts

OPLS-DA models computed from UPLC-MS profiles generated from aqueous liver extracts of control and TA treated animals were generated for each time point. The 2h OPLS-DA model between TA and control samples, is shown in Figure 3.3-3, and is predictive and robust (1 predictive and 2 orthogonal components, cumulative $R^2X=0.533$, predictive component $R^2X=0.253$, $Q^2=0.882$, $Q^2Y=1$, analysis of variance in the cross-validated residuals of the Y variable, C.V. ANOVA, $P=0.05$). The majority of the dominating features driving separation in the S-plot were found to be drug metabolites (and their isotopes), Figure 3.3-3 highlights some of these in the S-plot. The extracted ion chromatogram provides an overview of the metabolites found, and their relative retention times and intensities.

TA eluted at 9.80 min, having a protonated m/z of 330.961. The variation detected across time-points is shown in Figure 3.3-4, showing TA peaked at 2h, a decreasing amount was detected at 6h and it was absent by 24h. As TA and TAI have the same mass and retention time, they were grouped as one feature by XCMS and were therefore not separately integrated. Figure 3.3-4 therefore indicates TA or TAI abundance depending on the sample analysed. Subsequent UPLC-MS/MS analysis confirmed the major fragment ions of TA at m/z 246.966 (4-keto-2,3-dichlorophenoxyacetic acid), 188.968 (4-keto-2,3-dichlorophenol) and 111.011 (2-ketothiophene) (Figure 3.3-5). The previously reported major TA metabolite in rat and humans, 5-hydroxy-tienilic acid (5-OH-TA), was detected at 9.20 min with a protonated m/z of 346.959. It was detected in all 2h and 6h TA samples (Figure 3.3-4), and like TA it peaked at 2h, was reduced by 6h and was not detected at 24h. MS/MS fragmentation (Figure 3.3-6), showed fragments of 246.972 (4-keto-2,3-dichlorophenoxyacetic acid), 188.969 (4-keto-2,3-dichlorophenol) and 127.0 m/z (oxidised 2-ketothiophene).

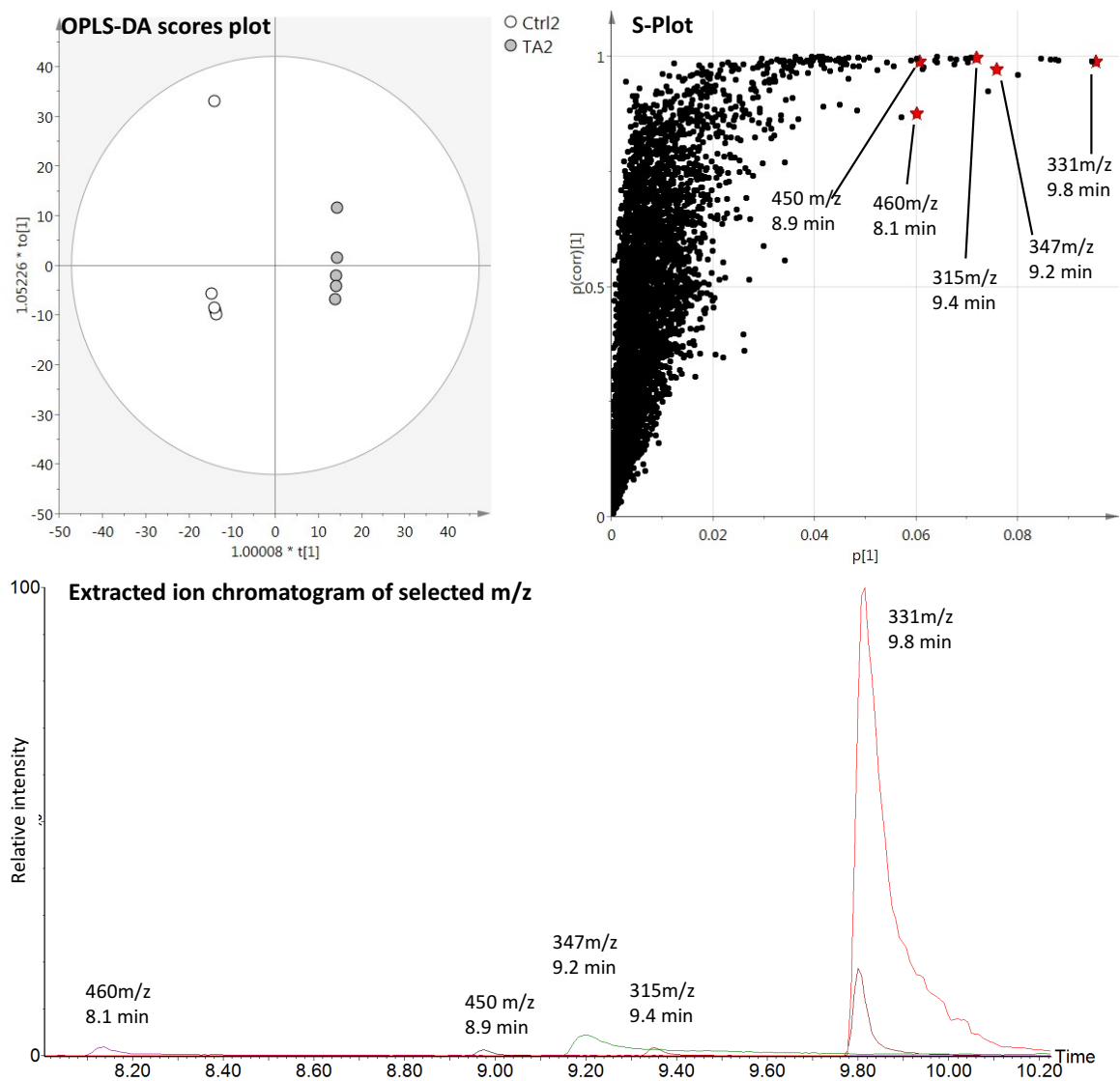


Figure 3.3-3 Tienilic Acid (TA) metabolites detected in aqueous liver extracts at 2h. (A) OPLS-DA scores plot (B) S-plot. (C) A representative extracted ion chromatogram of masses of selected drug metabolite

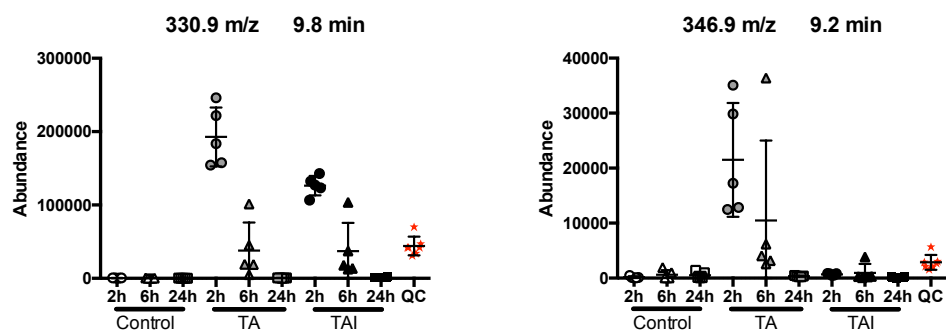


Figure 3.3-4 The abundance of selected features across treatment and time groups. Feature shown with m/z of 330.9, eluting at 9.8 min was identified as Tienilic acid or Tienilic acid Isomer. Feature shown with mass 346.9 m/z and eluting at 9.2 min was identified as 5-hydroxy-TA (5-OH-TA). Each symbol represents an animal, the line indicates the mean and error bars signify standard deviation.

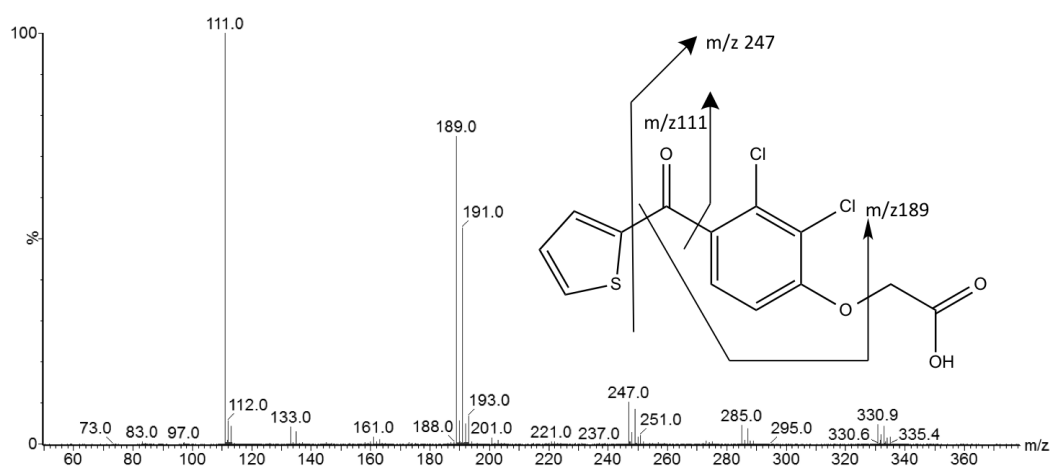


Figure 3.3-5 An ESI+ MS/MS spectra of ion 331 m/z at 9.8 min, identified as TA, as seen in liver, plasma and urine.

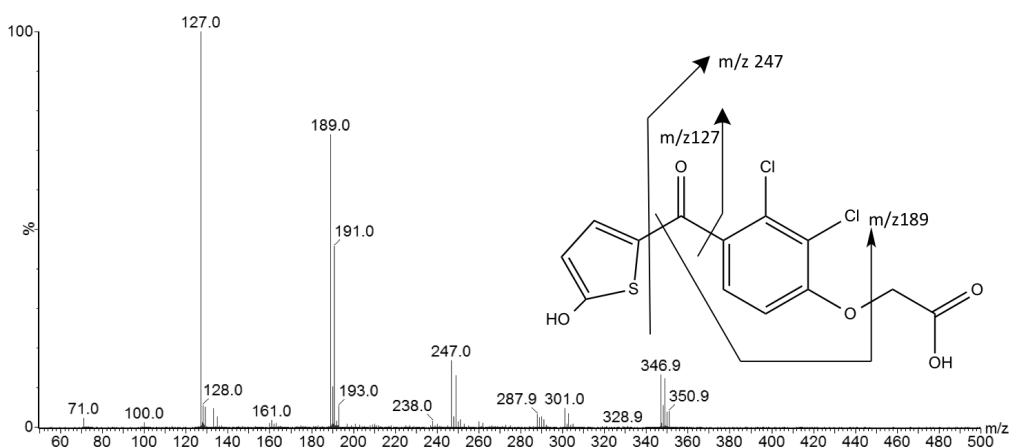


Figure 3.3-6 An ESI+ MS/MS spectra of ion 347 m/z at 9.2 min, identified as 5-OH-TA, as seen in liver, plasma and urine.

A feature with a retention time of 9.35 min, with a mass of 314.986 m/z was also prominent in the S-plot. MS/MS showed fragments at 269.0, 247.0, 189.0 and 95.0. This compound was present at a similar concentration in all 2h animals, a more variable concentration was detected at 6h, and was absent by 24h. This ion corresponded with another ion eluting in negative ionisation mode with a mass of 506.98m/z, which had MS/MS indicative of a glucuronic acid conjugate. The MS/MS, shown in Figure 3.3-8, of this ion showed fragments at 193.027 (deprotonated glucuronic acid), 175.009 (deprotonated anhydroglucuronic acid), 113.019 (after loss of CO₂, H₂O) and 85.056 (after further loss of CO). However, the expected mass of TA-glucuronic acid in negative mode would be 505m/z. This suggests this is a reduced TA glucuronide, where the keto group has been reduced to an alcohol, the suggested structure is depicted in Figure 3.3-8.

In addition, a metabolite was found at 8.2min with a mass of 460.003m/z, which would be the addition of 129.042 m/z to TA. This could therefore correspond to a glutamate conjugate (+C₅H₇O₃N). This metabolite had high inter-animal variation, and was nearing the limit of detection in two of the 2h group. MS/MS fragmentation is shown in Figure 3.3-9; the major fragments were seen at m/z 414.005, 246.974 and 188.970.

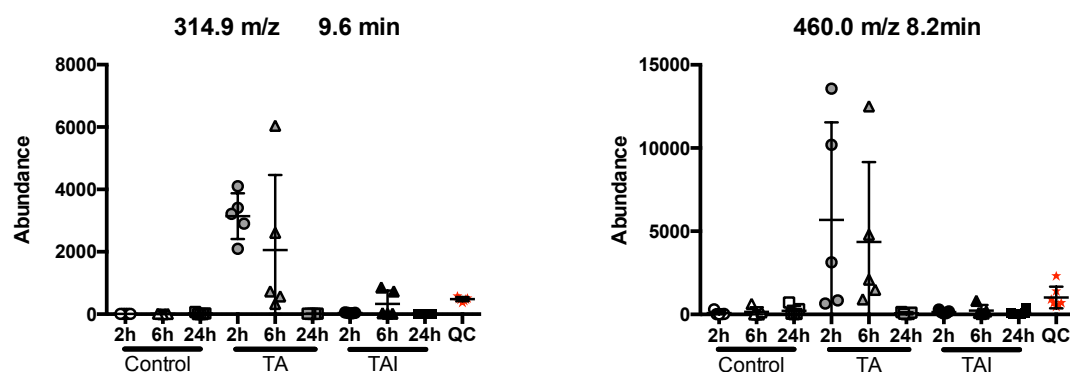


Figure 3.3-7 The abundance of selected features across treatment and time groups. Feature shown with mass 314.9m/z, tR 9.6 min was identified as a fragment of a reduced TA, glucuronide conjugate. Feature shown with mass 460.9 m/z and tR 8.2 min was provisionally identified as a glutamate conjugate. Each symbol represents an animal, the line indicates the mean and error bars signify standard deviation.

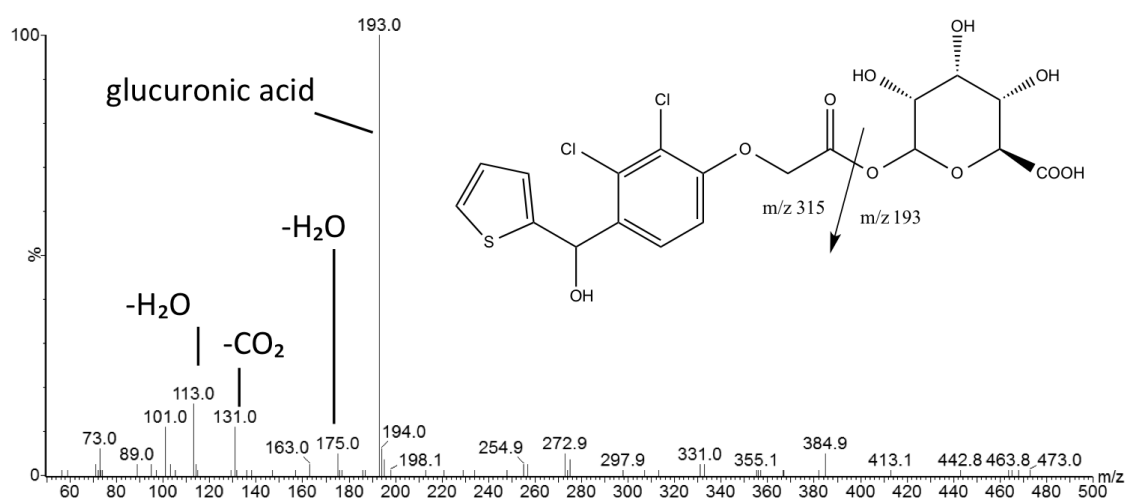


Figure 3.3-8 MS/MS spectra of an ion found in ESI- with a m/s of 507. Fragments are consistent with a glucuronide conjugated to a reduced TA. In ESI+ a fragment with m/z 315 was detected (indicated on structure).

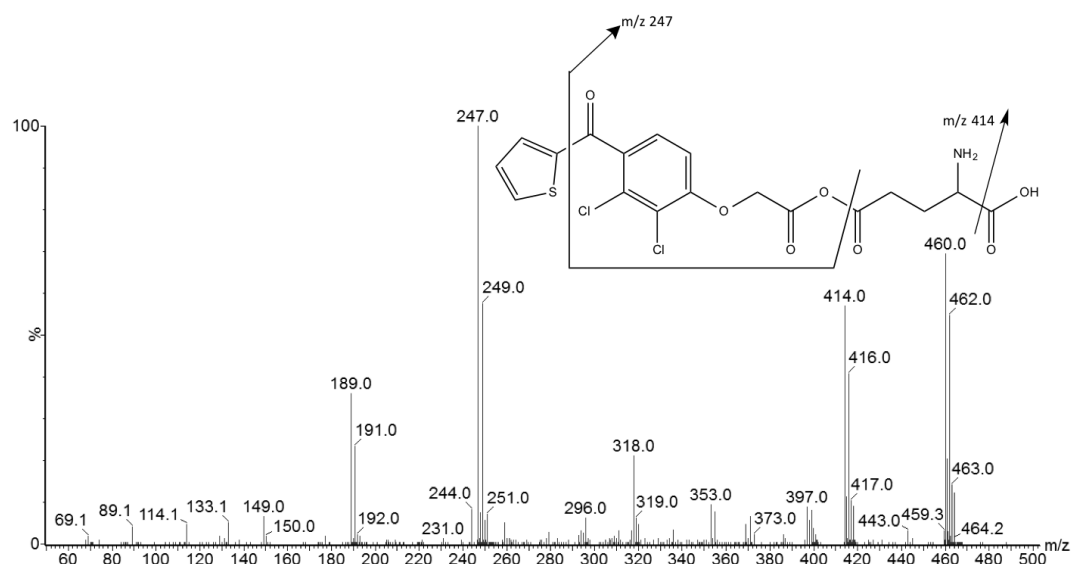


Figure 3.3-9 MS/MS spectra of TA drug metabolites with m/z 460 in positive ESI. The chemical structure indicates one potential glutamate-TA structure.

Another TA metabolite with a mass of 449.962 m/z was prominent in the 2h S-plot, its distribution is shown in Figure 3.3-10. This is an addition of 119.001 m/z to TA; suggestive of a cysteine conjugate. However, this was unfortunately not explored with MS/MS in TA samples. Interestingly, this metabolite was one of the few that was detected in both TA and TAI samples, the fragmentation of the corresponding TAI metabolite is found in Table 3.3-2.

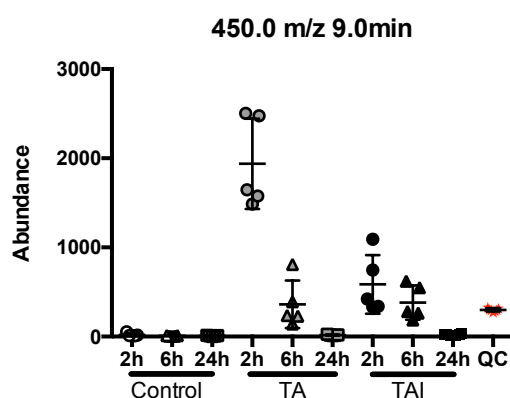


Figure 3.3-10 The abundance of selected features across treatment and time groups. Feature shown with mass 450.0 m/z , t_R 9.0 min was preliminarily identified as a TA-cysteine, and TAI-cysteine conjugates.

There were no TA-GSH adducts revealed in the S-plots or in manual searches for masses of previously reported TA-GSH adducts found in bile of TA-treated rats (636 m/z ; Nishiya et al., 2008) or *in vitro* (636, 654, 652, 672 m/z ; Belghazi et al., 2001, Lim et al., 2008, Rademacher et al., 2011).

3.3.5.2 TAI metabolites in aqueous liver extracts

The 2h OPLS-DA model between TAI and control samples (Figure 3.3-11), was predictive and valid (1 predictive and 2 orthogonal components, cumulative $R^2X=0.573$, predictive component $R^2X=0.293$, $Q^2=0.901$, $Q^2Y=1$, C.V. ANOVA $p=0.04$). As with TA v Ctrl, a large number of the prominent features in the S-plot were drug metabolites (and their isotopes), in Figure 3.3-11 some of these have been highlighted in the S-plot. The extracted ion chromatogram of TAI metabolites from a representative sample is shown in Figure 3.3-11, and reveals very different metabolites to those found in TA.

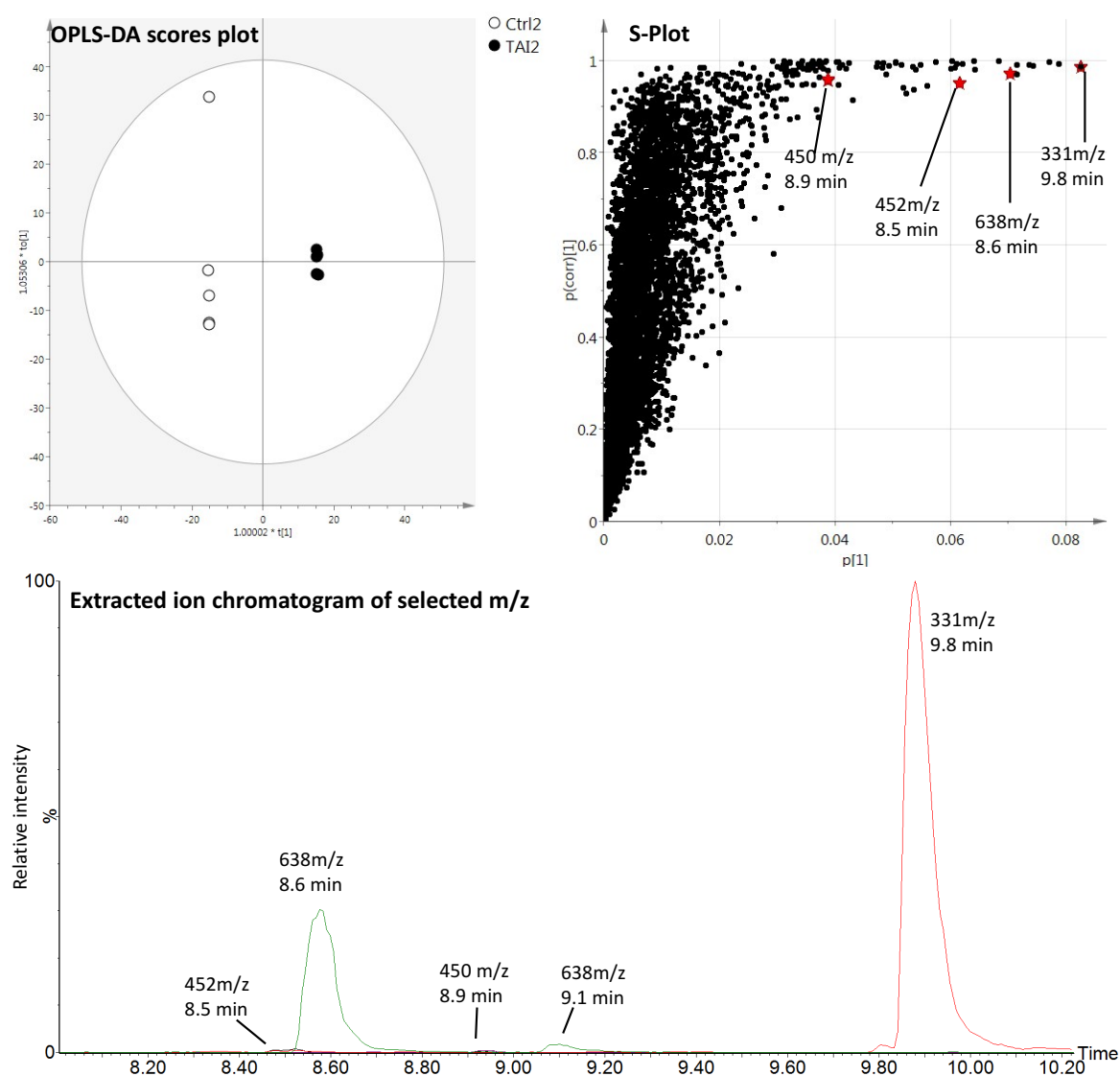


Figure 3.3-11. OPLS-DA, S-plots and extracted ion chromatograms of aqueous liver extracts from TA and TAI treated animals.

Like TA, TAI eluted at 9.80 min with a protonated m/z of 330.964, TAI was detected at a lower intensity to TA (Figure 3.3-4), but like TA was highest at 2h, most variable at 6h and not detected at 24h post dose. MS/MS confirmed fragments were the same as TA, including 246.966, 188.970, 132.982 and 111.013 m/z (Figure 3.3-12).

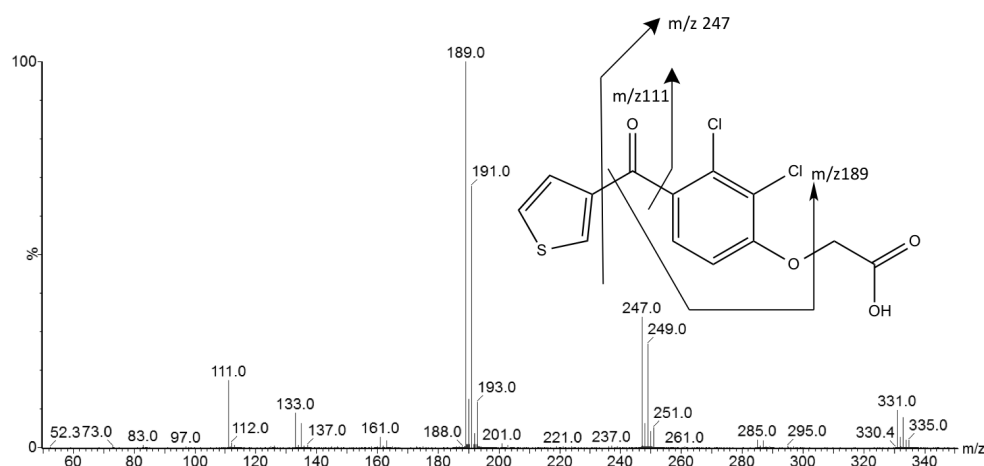


Figure 3.3-12 MS/MS spectra of TAI, and the chemical structure with suggested fragmentation.

In contrast to TA, the major metabolite observed with TAI was an ion with mass 638.030 m/z eluting at 8.60 min. This corresponds to a dihydro-TAI-GSH conjugate with fragments including 509.001 (after loss of pyroglutamic acid), 330.974 (re-ionised TAI), 308.101 (protonated GSH), 179.066 (loss of pyroglutamic acid from GSH), and 162.040 (Figure 3.3-14). Another metabolite with mass 638 m/z was also observed, that eluted earlier, indicating another GSH conjugate, likely to be conjugated at another position on the thiophene ring. This metabolite was found in all TAI liver samples at 2h, at lower concentrations at 6h and was almost absent in 24h samples (Figure 3.3-13).

Another dominant feature in the S-plot was an ion with mass 451.980 m/z at 8.50 min. This corresponds to a dihydro-cysteine-TAI conjugate with fragments shown in Figure 3.3-15, including 330.973 (protonated TAI), 246.970 (4-keto-2,3-dichlorophenoxyacetic acid), 188.971 (4-keto-2,3-dichlorophenol), 122.049 (protonated cysteine). Although present in all 2h samples it was more variable than the glutathione conjugate (Figure 3.3-13).

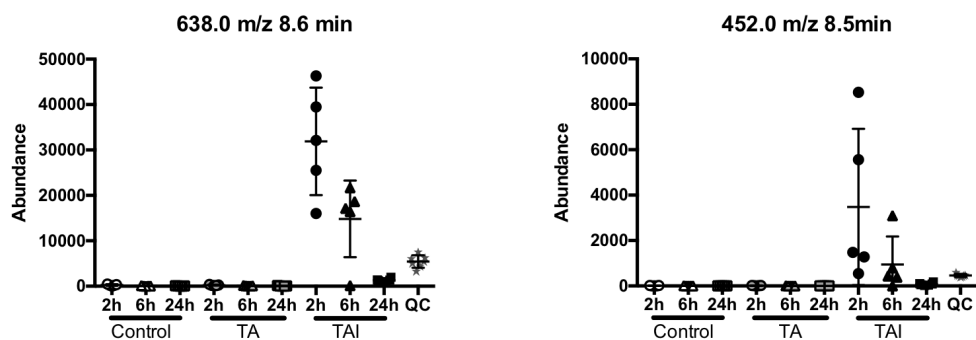


Figure 3.3-13 The abundance of selected features across treatment and time groups. Feature shown eluting at 8.6min with m/z 638 was identified as a dihydro-TAI-GSH conjugate. Feature shown eluting at 8.5min and m/z of 452.0 m/z was identified as a dihydro-TAI-cysteine conjugate. Each symbol represents an animal, the line indicates the mean and error bars signify standard deviation.

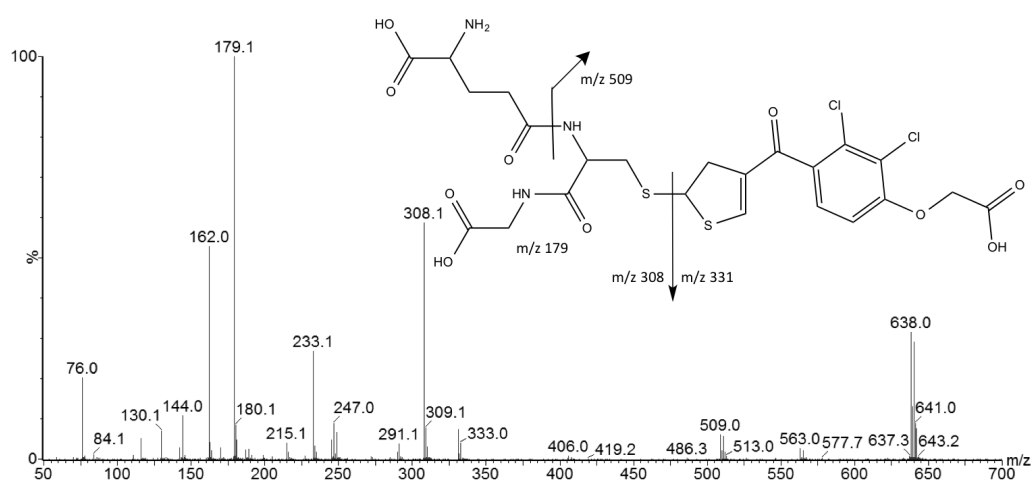


Figure 3.3-14 MS/MS spectra selecting for 638 m/z . Identified as dihydro-TAI GSH conjugate, and the potential chemical structure with suggested fragmentation.

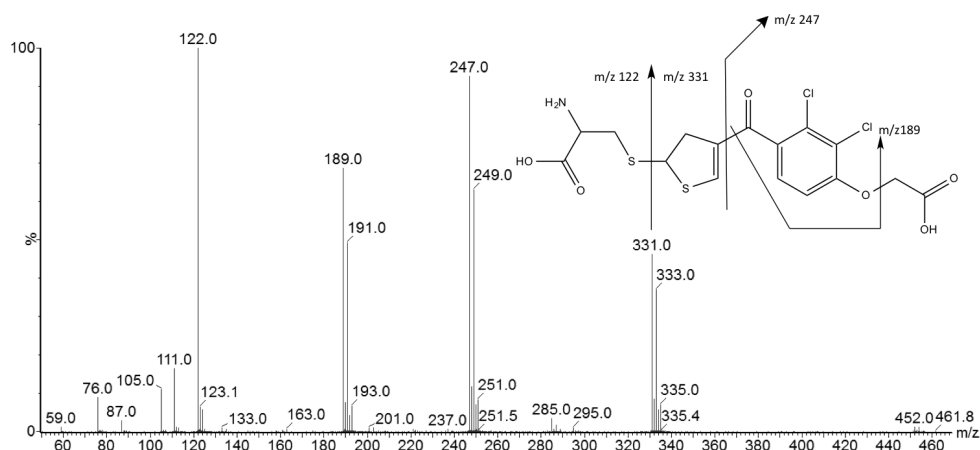


Figure 3.3-15 Mass spectra from 8.5 min of an MS/MS experiment selecting for 452 m/z . Identified as dihydro-TAI cysteine conjugate, and the potential chemical structure with suggested fragmentation.

The metabolite with a mass of 450m/z that was observed in TA samples were also detected in TAI treated animals with a mass of 450.015m/z. The acquired MS/MS showed fragments of 360.933, 314.914, 246.972, 188.967 and 120.095. A likely structure has not been determined, but the mass difference is suggestive of another cysteine conjugate.

3.3.5.3 TA metabolites in plasma

The 2h OPLS-DA model between TA and control samples (Figure 3.3-11), was predictive and valid (1 predictive and 1 orthogonal component, cumulative R^2X of 0.49, with predictive component R^2X of 0.406, Q^2 of 0.962 and an R^2Y of 1, C.V. ANOVA $p = 0.001$). The S-plot highlights metabolites found, indicating the same metabolites seen in liver extracts; 5-OH-TA (m/z 346.9), the potential fragment of a reduced-TA glucuronide (m/z 315) and the potential cysteine conjugate (m/z 450.0). The potential glutamate conjugate, with a mass of 460m/z, was not observed.

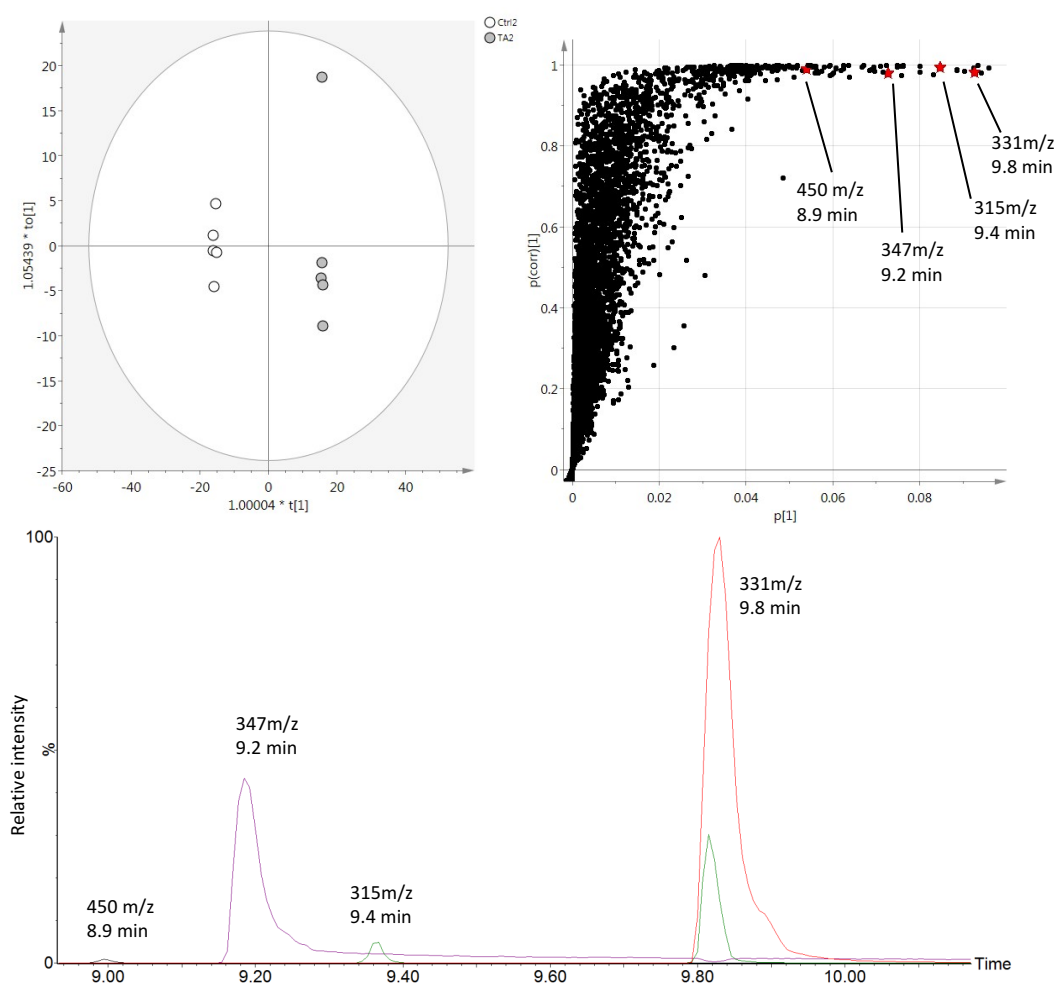


Figure 3.3-16 Tienilic Acid (TA) metabolites detected in plasma at 2h. (A) OPLS-DA scores plot (B) S-plot. (C) A representative extracted ion chromatogram of masses of selected drug metabolite

The abundance of each metabolite across treatment groups is shown in Figure 3.3-17. Feature 330.0 m/z, the coeluting TA and TAI, shows a slightly higher intensity for TA compare to TAI. This is in contrast to the quantification of TA/TAI in plasma previously performed on these samples (Coen et al., 2012), and may be due to slightly different ionisation of TA and TAI not accounted for here with internal standard. Alternatively, it could originate from the degradation of conjugates in plasma, e.g. TA-glucuronide conversion to glucuronic acid and TA.

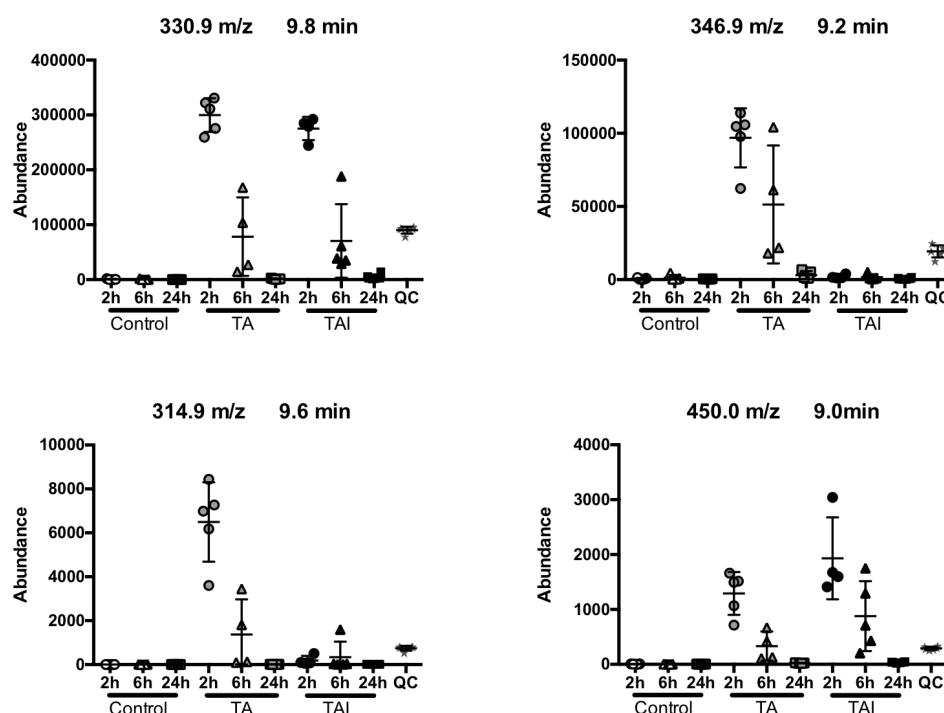


Figure 3.3-17 The abundance of selected features across treatment and time groups. Feature shown with mass 330.9m/z, tR 9.6 min was identified as a Tienilic Acid or the co-eluting Tienilic Acid Isomer. Feature shown with mass 346.9 m/z and tR 9.2 min was identified as 5-OH-TA. Feature shown with mass 314.9m/z, tR 9.6 min was identified as a fragment of a reduced TA, glucuronide conjugate. Feature shown with mass 450.0.9m/z, tR 9.0 min was preliminarily identified as a TA-cysteine, and TAI-cysteine conjugates. Each symbol represents an animal, the line indicates the mean and error bars signify standard deviation.

An additional metabolite, not seen in liver, had a mass of 435.934 m/z and was eluting at 9.1min. This is potentially a taurine conjugate- 329m/z (TA) with addition of 107 (+C₂H₃O₂NS). There was a fragment present with a mass of 124.008m/z- which would correspond to a deprotonated taurine. This corresponds to an ion of 315m/z in positive mode, which could correlate to TA after loss of taurine with loss of oxygen at the site of conjugation. It was seen in all TA samples collected at 2h, and an analogous TAI conjugate eluting at the same time was also detected.

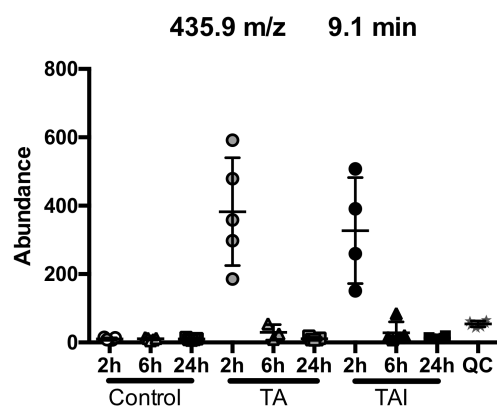


Figure 3.3-18 The abundance of feature with mass 435.9m/z in ESI-. It has been preliminarily identified as TA-taurine, and TAI-taurine conjugates. Each symbol represents an animal, the line indicates the mean and error bars signify standard deviation.

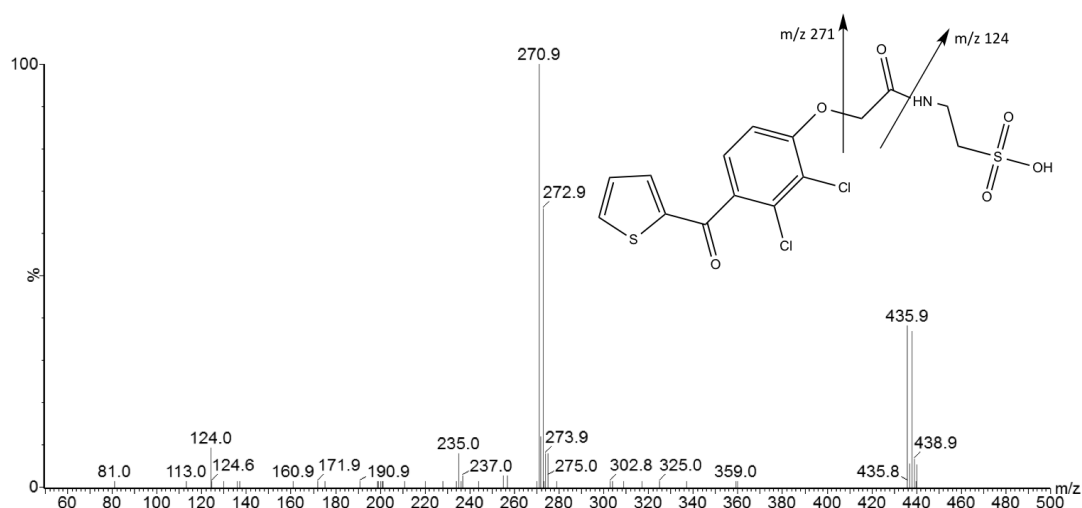


Figure 3.3-19 MS/MS of an ion found in ESI- analysis of plasma with m/z of 435.9. Mass and fragments are indicative of a taurine conjugate, suggested structure is shown.

3.3.5.4 TAI metabolites in plasma

The 2h OPLS-DA model between TAI and Control samples (Figure 3.3-20), was predictive and valid (1 predictive and 1 orthogonal component, cumulative $R^2X=0.598$, predictive component $R^2X=0.479$, $Q^2=0.975$, $Q^2Y=1$, C.V. ANOVA $p=0.002$). In plasma, at 2h the TAI metabolites were dominated by high levels of TAI, but the metabolites detected include masses of 450m/z and 452m/z as seen in liver. There were also two separately eluting ions with a mass of 570.988m/z, and one with 602.969 m/z.

These are likely to be products of GSH conjugates as they shared a characteristic fragment of re-ionised cysteine at mass 122m/z, a fragment at 418.014, which has previously been seen in

GSH related metabolites in cell cultures (Rademacher, 2011b). Other fragments included the 247 and 189m/z from the TAI. A TAI metabolite with mass 571m/z has previously been seen in cell incubations with TAI (Rademacher, 2011b), and was identified as a di-cysteinyglycine-dihydro-TAI conjugate.

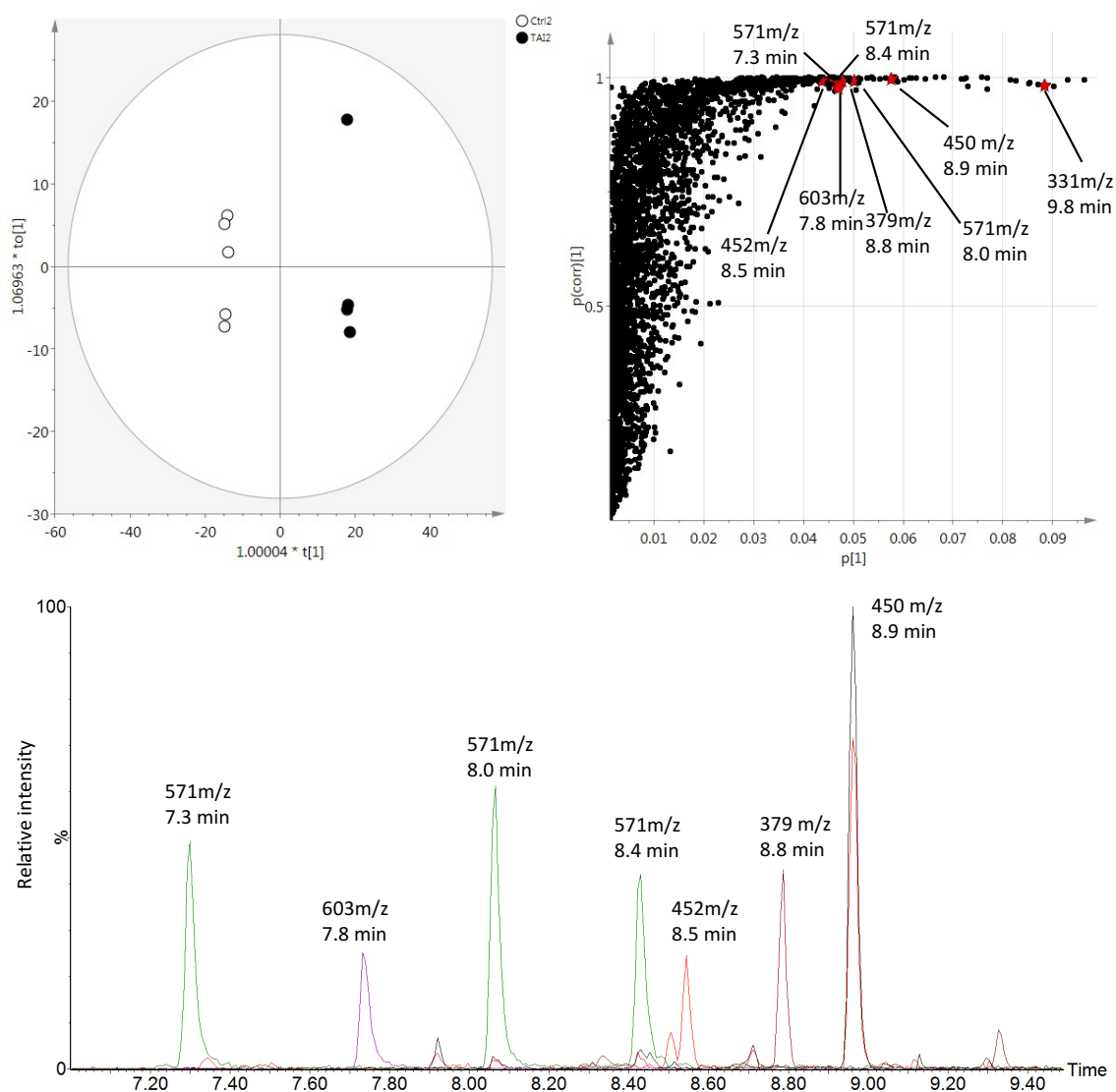


Figure 3.3-20. OPLS-DA, S-plots and extracted ion chromatograms of plasma from TAI treated animals 2h post dose.

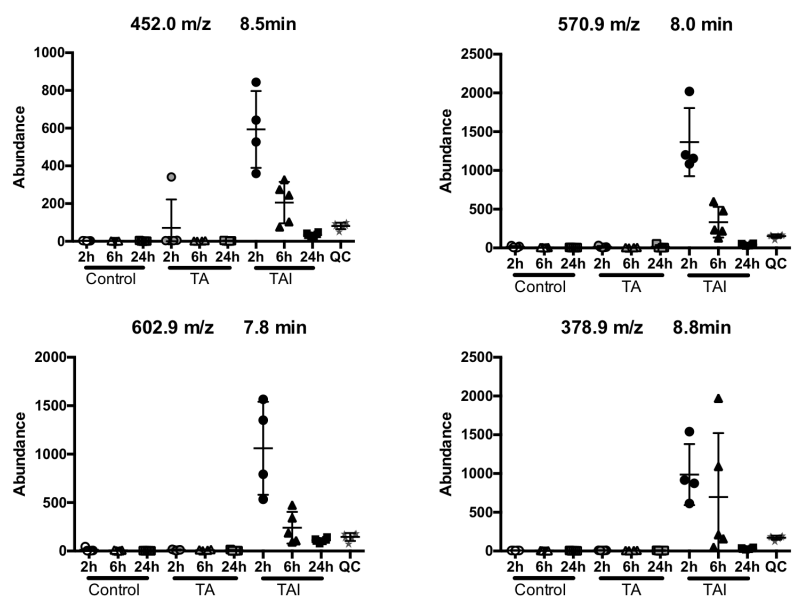


Figure 3.3-21 The abundance of selected features across treatment and time groups. Feature shown with mass 452.0m/z, tR 8.5 min was identified as dihydro-cysteinyl-TAI. Features shown with mass 570.9 m/z tR 8.0 min, and 602.9m/z tR 7.8 min were identified as hydrolysed di-GSH-TAI conjugates. Feature shown with mass 378.9m/z, tR 8.8 min was identified as an opened ring TAI metabolite. Each symbol represents an animal, the line indicates the mean and error bars signify standard deviation.

In addition, a metabolite with mass of 378.986 m/z was also prominent in the S-plot. The major fragments observed by MS/MS were at 362.0 and 287.0, after loss of C₂H₄O₂S (Figure 3.3-22). This may correspond to an open-ringed carboxylic acid, which was also previously detected in cell incubations (Rademacher 2011), and in the metabolism of other thiophene containing xenobiotics (e.g. Suprofen).

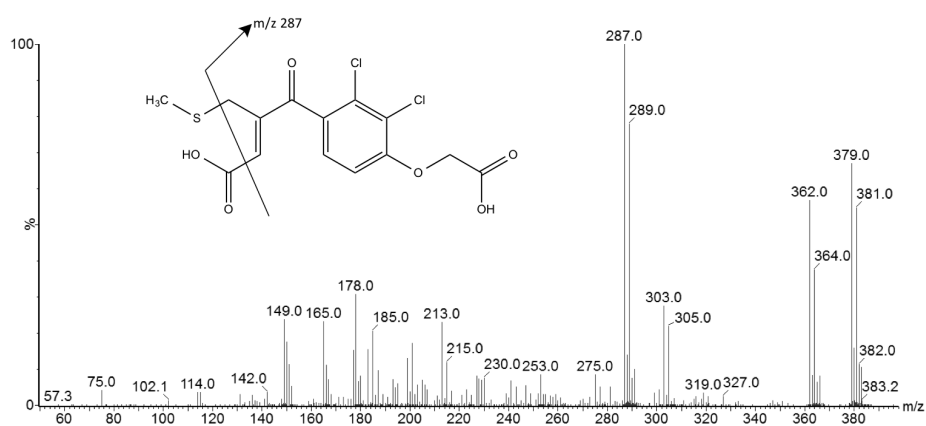


Figure 3.3-22 MS/MS of an ion found in plasma of TAI treated rats with m/z of 378.9. A potential chemical structure and fragmentation is shown.

3.3.5.5 TA metabolites in urine

The OPLS-DA scores plot between TA and Control urine samples collected between 2-6h is shown in Figure 3.3-23, (1 predictive and 1 orthogonal component, cumulative $R^2X=0.625$, predictive component $R^2X= 0.534$, $Q^2= 0.984$, $Q^2Y=1$, C.V. ANOVA $p=0.0008$). The S-plot was dominated by features corresponding to drug metabolites, which have been highlighted in the S-plot and shown in a representative extracted ion chromatogram.

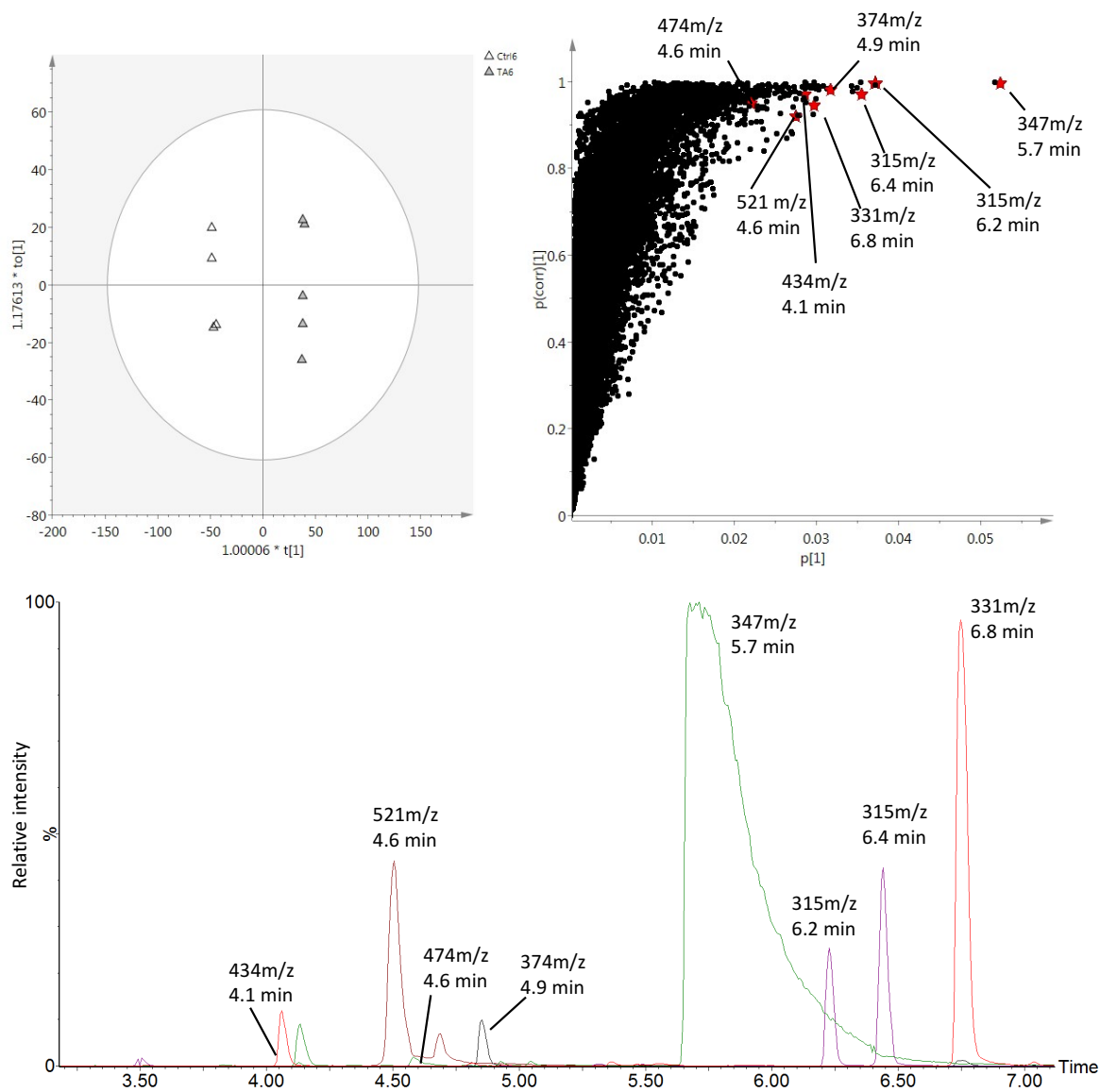


Figure 3.3-23 OPLS-DA scores plot, S-plot and extracted ion chromatograms of urine from TA treated animals, collected between 2-6h post dose.

The LC gradient used for urine analysis was 11min, compared to a 30min run used for the liver and plasma samples, so the retention times for the same metabolites are earlier. Under this gradient, TA eluted at 6.8min, the relative abundance across the time-points is shown in Figure 3.3-25. The most prominent features in the S-plot correspond to the 5-hydroxy-TA metabolite, which eluted at 5.8min, and a feature with a mass of 315m/z at 6.5 min. These ions had the same fragmentation patterns as the metabolite of the same mass observed in plasma and aqueous liver extracts. The abundance of these is shown in Figure 3.3-25.

In addition, other metabolites included another 315m/z eluting at 6.4min, using complementary negative mode data, an ion with m/z in negative of 331.0, and a dimer at 663m/z suggesting this is a fragment of an ion with a neutral mass of 332m/z, reduced TA, this was also seen in TAI metabolites, with a co-eluting metabolite seen in TAI samples.

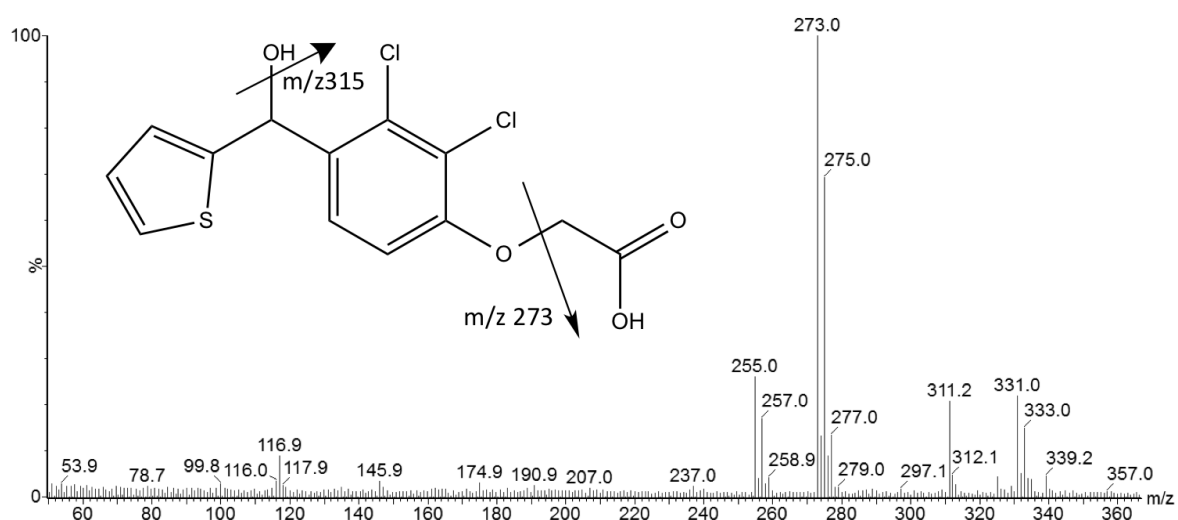


Figure 3.3-24 MS/MS of an ion for in ESI- with m/z 331 indicative of a reduced TA, structure shown.

In negative mode, an ion with a mass of 507m/z, eluting at 5.5min, was present and may correspond to the suspected glucuronide metabolite seen in the aqueous liver samples, although this has not been confirmed by MS/MS. A metabolite with mass 520.9m/z, which was present in all samples, corresponds to the mass of a glucuronide, of a methylated TA metabolite, this was seen in all samples at 0-2h and peaking at 2-6h (Figure 3.3-25).

There were also a number of more minor, early eluting/polar metabolites as shown in the extracted ion chromatogram. The major metabolites include a mass 474.0, 373.9 and 434.0 m/z. Fragments are listed in Table 3.3-1, the identity of these metabolites are unknown.

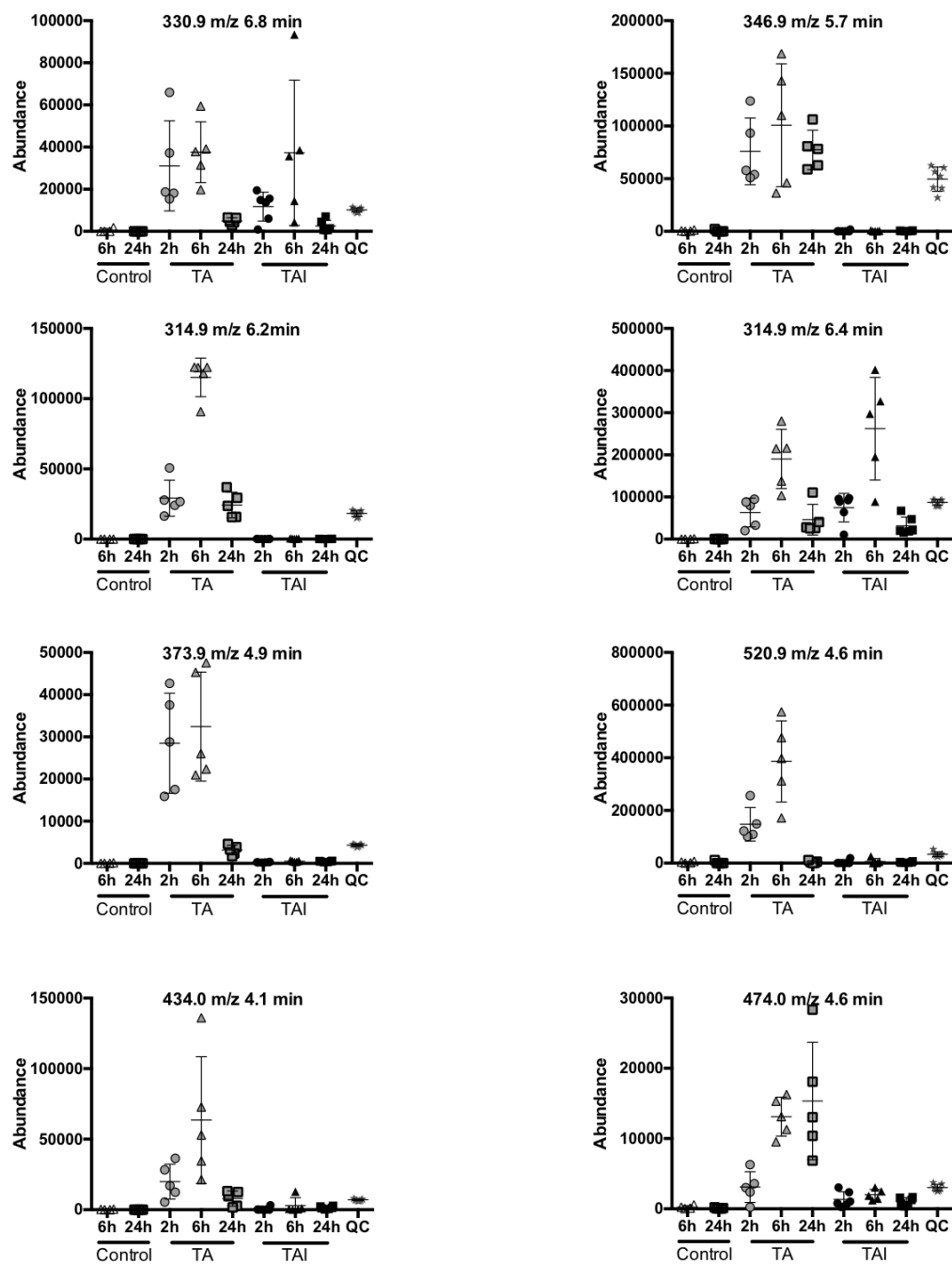


Figure 3.3-25 Abundance of selected TA metabolites in urine. Each symbol represents an animal, the line indicates the mean and error bars signify standard deviation.

3.3.5.6 TAI metabolites in urine

The OPLS-DA scores plot generated from UPLC-MS analysis of TAI and Control urine samples collected between 2-6h is shown in Figure 3.3-26, (1 predictive and 2 orthogonal component, cumulative $R^2X=0.788$, predictive component $R^2X= 0.569$, $Q^2= 0.98$, $Q^2Y=1$, C.V. ANOVA $p=0.009$). The S-plot was dominated by features corresponding to drug metabolites, which have been highlighted in the S-plot and shown in a representative extracted ion chromatogram.

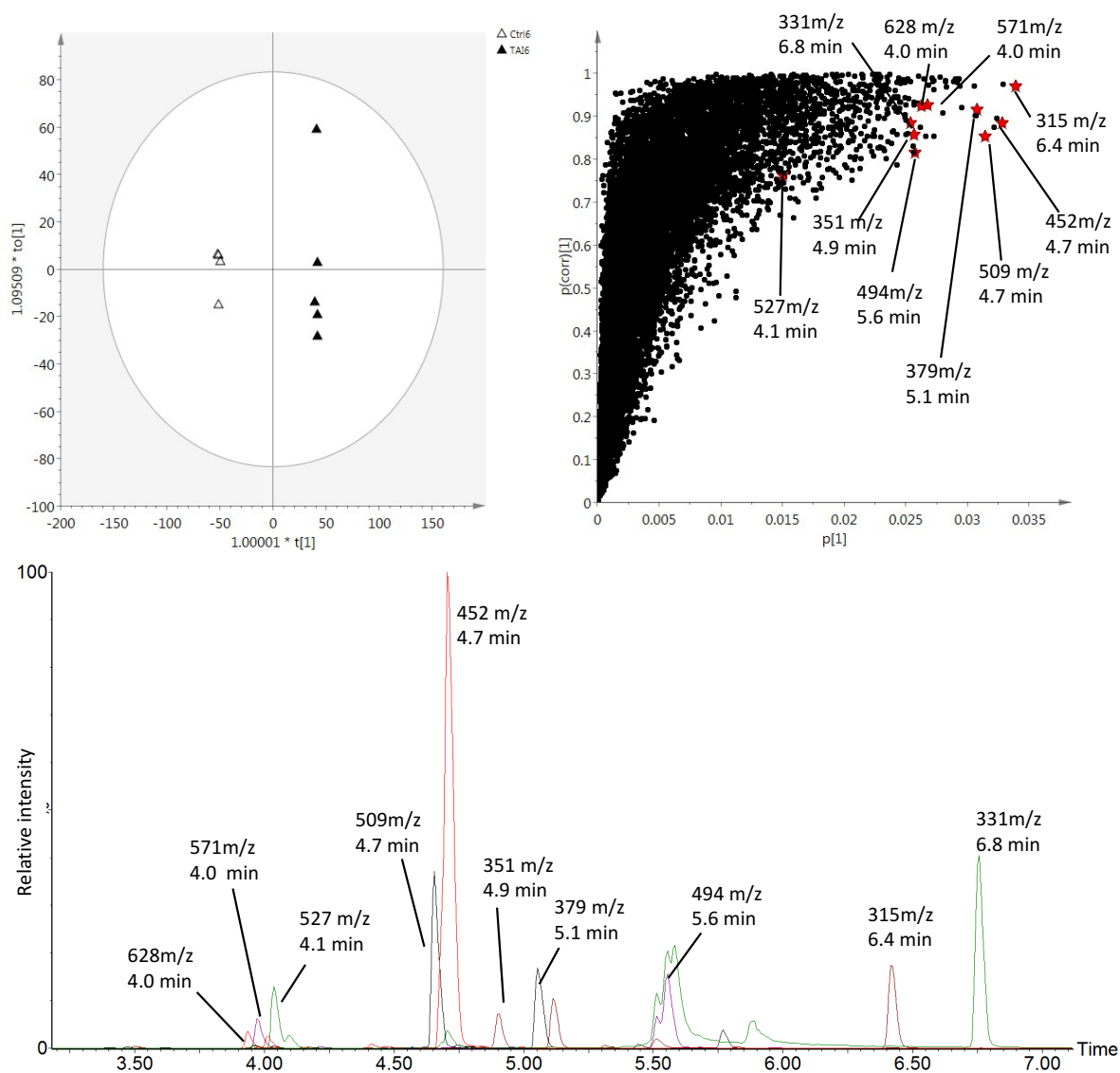


Figure 3.3-26. OPLS-DA, S-plots and extracted ion chromatograms of aqueous liver extracts from TAI treated animals.

There were a larger number of TAI metabolites detected in the urine of TAI treated animals than detected in the TA-treated animals. The predominant metabolites were glutathione related metabolites. These include a mercapturate, cysteinyl-glycine dihydro-TAI and cysteinyl-

dihydro TAI metabolites. The mercapturate was detected at 5.6min with a mass of 494.127m/z. MS/MS resulted in fragments of 331.0m/z, 247.0, 189.0, 164.1 and 122.1(Figure 3.3-27). The cysteinyl-glycine dihydro-TAI had a detected mass of 509.045m/z, and eluted at 6.6min. MS/MS analysis showed fragments of 331.0, 247.0, 189.0, 179.1, 162.0 (Figure 3.3-28). The cysteinyl-dihydro TAI was detected at 4.7min and had a mass of 452. Fragments were seen at 330.9, 247.0, 189.0, 122.0.

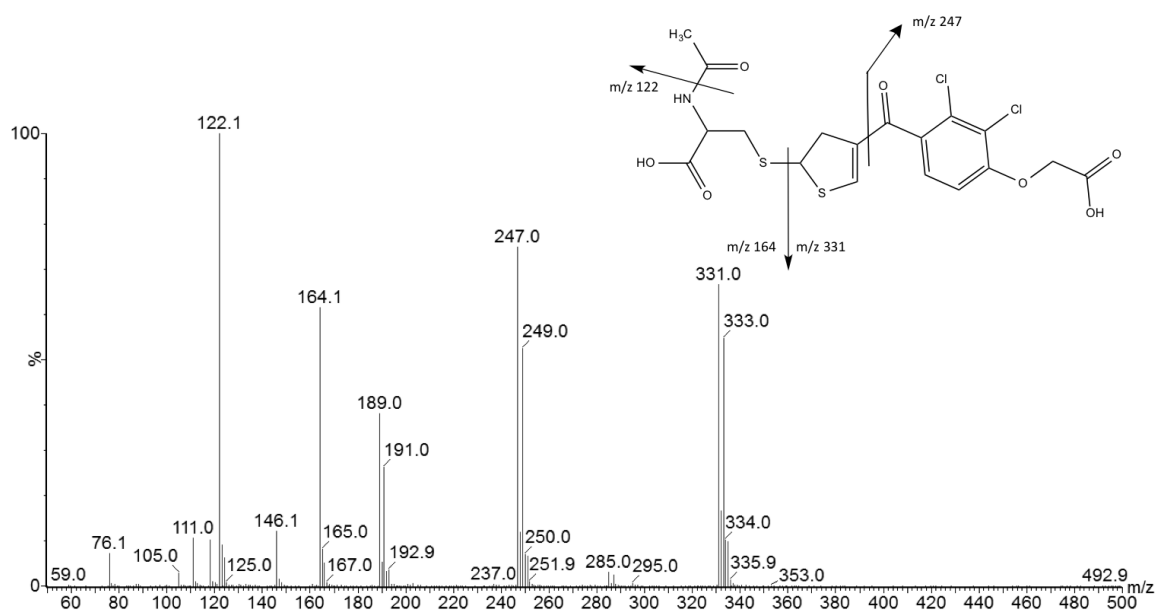


Figure 3.3-27 MS/MS spectra in ESI+ for ion 494m/z

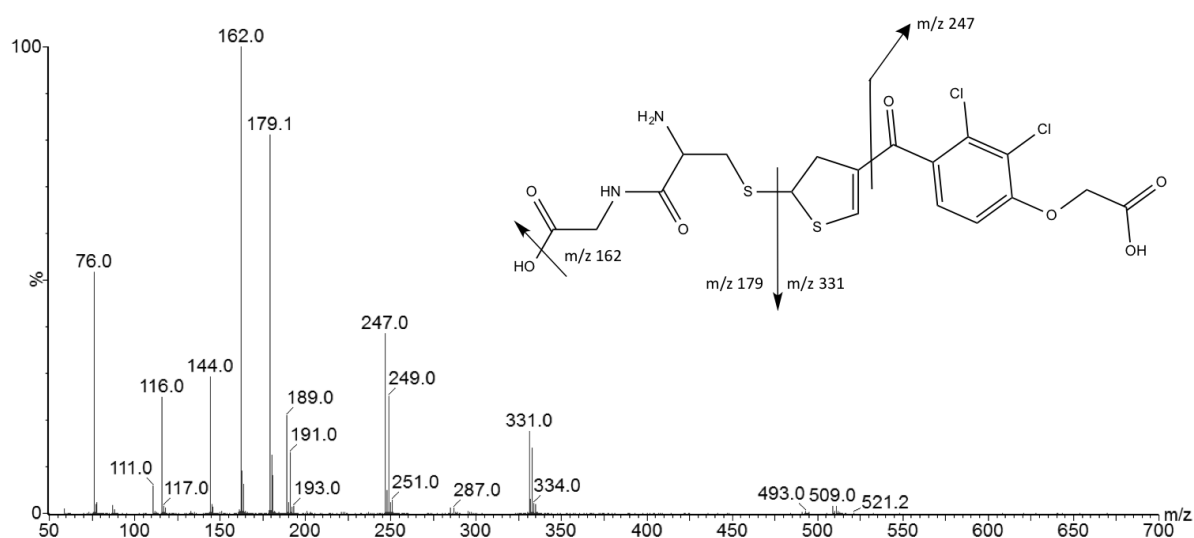


Figure 3.3-28 MS/MS spectra in ESI+ for ion 509m/z

Other dominant metabolites present in the S-plot were additional GSH-related metabolites which are likely to correspond to parts of di-GSH-TAI, as seen in the plasma. These included a metabolite with a mass of 628.0 3.976min (418.0, 298.1 and 247.0), 571m/z (418.0, 247.0, 189.0, 122.1m/z) and 527m/z (179.1, 162.0, 331.0). These shared fragmentation patterns with metabolites of the same mass detected from cell incubations (Rademacher et al., 2011). In addition, there was a metabolite with a mass of 628m/z and one with 602.981m/z (418.0, 247.0, 189.0, 122.1), as was detected in the plasma samples.

A reduced TAI, as with reduced TA, was detected at 6.447min, fragments of 280.0, 257.0, 221.0. The open-ring metabolite with mass 379m/z (361.0, 287.0, 247.0, 189.0) was detected at 4.8min. There were also a number of other metabolites eluting at a similar time, which may also be related open ring structures, such as the two metabolites with a mass of 351.0 at 4.9min and 5.2min. Both had fragments of 247.0, 189.0 and 131.0m/z. The abundance of the TAI metabolites is shown in Figure 3.3-29.

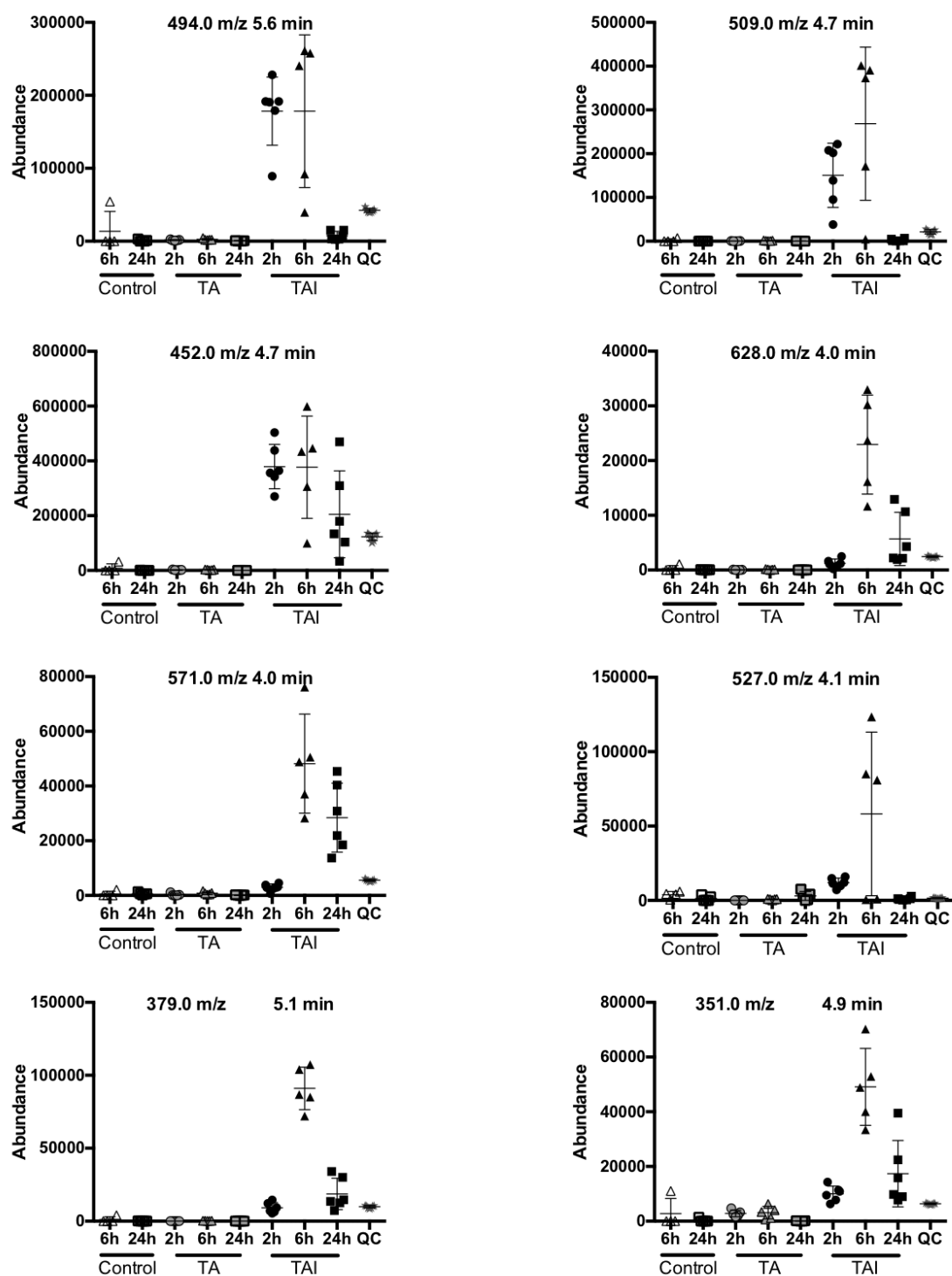


Figure 3.3-29 The abundance of eight TAI metabolites in urine. Each symbol represents an animal, the line indicates the mean and error bars signify standard deviation.

3.3.6 Summary tables of TA and TAI drug metabolite m/z and fragments

Table 3.3-1 Summary of TA metabolites

Metabolite Suggested ID	Detected m/z	Mass addition to TA	Fragments	Location detected*
Positive ESI				
Parent	330.96	-	111.0, 189.0, 247.0	L P U
Hydroxylated	346.95	+16	127.0, 189.0, 247.0	L P U
Cysteine conjugate	449.96	+119	-	L P
Glutamate conjugate	460.00	+129	414.0, 247.0, 189.0	P
Methylated TA Glucuronide conjugate (#)	520.97	+190	429.0, 372.0, 247.0, 189.0, 153.0	U
Unknown	473.07	+142	456.1, 247.0, 371.0, 127.1	U
Unknown (AA conjugate ?)	433.99	+103	417.0, 399.0, 371.0, 247.0, 189.0, 169.0, 151.0	U
Unknown (open ring?)	374.02	+43	327.0, 247.0, 189.0, 153.0	U
Negative ESI				
Reduction of =O to OH	330.96	+2	255.0, 273.0	U
Glucuronide, of reduced TA	506.97	+178	113.0, 131.0, 175.0, 193.0, 328.9	L, U
Taurine conjugate	435.95	+107	124.0, 270.9	P

*L= liver, P=plasma, U=urine # tentative assignment

Table 3.3-2 Summary of TAI metabolites

Metabolite Suggested ID	Detected m/z	Mass addition to TAI	Fragments	Location detected*
Positive ESI				
Parent	330.96	-	247.0, 189.0, 111	L P U
Dihydro-TAI +O	346.96	+16	127.0, 189.0, 247.0	P,U
Unknown	350.98	+20	246.97, 188.97, 131.03, 103.0	U
Open thiophene ring	378.98	+48	362.0, 287.0, 178.0	P U
Cysteine conjugate (#)	449.97	+119	360.93, 314.93, 246.97, 188.97, 120.03	L P
Dihydro-cysteiny conjugate	451.98	+121	331.0, 247.0, 189.0, 122.0	P
Dihydro n-acetyl cysteine conjugate	493.99	+163	331.0, 247.0, 189.0, 164.1, 122.1	U
Dihydro-cysteiny glycine conjugate	509.00	+178	331.0, 247.0, 179.1, 162.0,	U
Product of di-GSH	527.01	+196	491.00, 287.01, 241.01, 179.07, 162.05	U
Product of di-GSH	571.00	+240	418.01, 246.97, 188.97	P U
Product of di-GSH	602.98	+272	418.02, 273.02, 246.97, 188.97, 122.04	P
Product of di-GSH	628.02	+279	418.03, 298.07, 246.98, 177.05, 130.07	U
GSH conjugate	638.04	+307	509.0, 331.0, 308.0, 179.1, 162.0	L
Negative ESI				
Reduced TAI	331.96	+2	255.0, 273.0	U

*L= liver, P=plasma, U=urine # tentative assignment

3.4 DISCUSSION

The aim of this work has been to compare the xenobiotic profiles of TA and TAI in the rat. Male Sprague-Dawley rats (n=5/6 per group) were treated either with 250mg/kg TA, TAI, or vehicle, and urine, plasma and liver samples were collected at 2h, 6h and 24h post-dose. Clinical chemistry, ALT and histopathology data were collected alongside UPLC-MS and MS/MS profiles of urine, plasma and liver extracts.

As previously published by Coen et al. (2012), 250mg/kg of TAI, but not TA or vehicle, was found to result in a significant increase in ALT by 24h, and centrilobular necrosis in the liver. This was despite both TA and TAI having comparable pharmacological action as a diuretic, and the similar plasma concentrations and pharmacokinetics of TA and TAI observed in this study. This is broadly consistent with previous studies of the toxicity of TA in rodents, although toxicity has been reported at high (< 500mg/kg) and repeated doses of TA (Oker-Blom et al., 1980a), and following BSO induced GSH depletion (Nishiya et al., 2008b). There were no published reports on the toxicity of TAI in rats prior to this study, although it had previously been stated to be an intrinsic toxin (Dansette et al., 1991, Bonierbale et al., 1999, Mansuy, 1997).

LC gradients originally developed by others (Want et al., 2013, Want et al., 2010) for metabonomic profiling of urine and tissue extracts were chosen to enable the parallel acquisition of xenobiotic and endogenous metabolites. Xenobiotic metabolites were separated from endogenous metabolites, using multivariate statistical modelling and manual assessment of chlorine isotope patterns. This approach was largely effective, revealing a large number of TA and TAI metabolites both known and novel, although automated methods could also have been applied e.g. isotope pattern recognition software or neutral loss MS experiments.

3.4.1 TA metabolites

Consistent with the previously reported metabolism of TA, a hydroxylated TA metabolite (347m/z) was the dominant metabolite detected in liver, plasma and urine. Hydroxylation of TA on the thiophene ring is known to be CYP2C9/CYP2C11 mediated (Lecoeur et al., 1994), and was more recently confirmed to be via an epoxide intermediate (Rademacher, 2011b, Rademacher et al., 2012).

No TA-GSH adducts were found in this study, it also appears unlikely that the unidentified metabolites are GSH related as their MS/MS spectra do not appear consistent with the

expected GSH fragmentation. However, GSH-TA adducts are known to form in microsome incubations with TA (Belghazi et al., 2001, Lim et al., 2008, Rademacher, 2011b). Additionally, a GSH-TA conjugate has previously been detected in the bile of rats (Nishiya et al., 2006), which was unfortunately not collected or available for analysis here. Further MS/MS experiments could be performed such as neutral loss experiments which can be used to determine any precursor ions that lose a characteristic m/z during fragmentation (129 m/z in ESI+, 272 in ESI- for GSH-adducts).

Other metabolites observed, included two potential acyl glucuronic acid conjugates, one conjugated to a reduced TA (507 m/z in ESI-), the other a methylated TA (521 m/z ESI+). To confirm these identifications, they would need to be synthesised and run as standards, although this has not been performed, the fragmentation of the reduced TA-glucuronide in particular, was highly indicative of a glucuronide conjugate. In addition, the elevated glucuronic acid levels observed in plasma of TA treated animals (data presented in Chapter 4), may in part be indicative of the breakdown of the TA-glucuronide in the samples. Glucuronides were not detected in cell incubations by Rademacher (2011), and no reports can be found in the literature of TA-glucuronides *in vivo*. However, a TA-glucuronide was generated *in vitro* by Darnell et al. (2015), who were investigating the reactivity of different acyl glucuronides. The authors of that study suggested that the TA-glucuronide was unlikely to be contributing to toxicity, as it did not contribute to non-specific microsomal protein binding, unlike oxidative metabolism. This was also observed by Usui et al. (2009), in incubations with TA and NADPH and UDP-glucuronic acid (UDPGA), lower binding to microsomal proteins was observed than with just TA and NADPH.

In addition to the suspected reduced-TA-glucuronide, the reduced-TA was detected in the urine of both TA and TAI treated animals, observed as a metabolite with a neutral mass of 332 (+2Da) to native TA/TAI. This is thought to be due to the reduction of the keto group to an alcohol, and was also detected in cell cultures with TA/TAI by Rademacher (2011). This has previously been reported in *in vivo* studies of TA (Mansuy et al., 1984), and in drugs with a similar structure e.g. Tiaprofenic acid (Surgam Data Sheet), where it is also excreted as a glucuronide.

Several other conjugates have been preliminarily identified in this study that have not previously been reported; this demonstrates suitability of this approach for detecting new drug

metabolites. Several of these were provisionally identified as amino acid conjugates; cysteine, glutamine and taurine. Although it remains to be confirmed where they are conjugated to the TA molecule, they are most likely to be conjugated to the carboxylic acid, which would be formed via coenzyme-A (CoA) conjugation, which is a reactive metabolite (Knights et al., 2007). Additionally, one of the unidentified metabolites was 374m/z, which eluted at the same retention time as TAI metabolites that are suspected to be open thiophene ring metabolites. Thiophene ring opening has been implicated as another route for reactive metabolite formation in thiophene containing drugs (Gramec et al., 2014), and if TA was also found to produce open ring metabolites, this may provide an alternative possibility for a reactive metabolite formation in TA.

3.4.2 TAI metabolites

In stark contrast to TA, the dominant TAI metabolite observed in the liver was a TAI-GSH conjugate, and many of the metabolites in plasma and urine were glutathione related e.g. mercapturate and cysteinyl-glycine conjugates. This is reflective of a high reactive metabolite (S-oxide) burden generated by TAI metabolism, which is likely responsible for the intrinsic liver damage observed, possibly due to CRM binding to cellular macromolecules. The reactive metabolite that leads to TAI-GSH adducts is thought to be the S-oxide, although TAI does also form epoxides to form hydroxylated metabolites, although this is a minor route, in contrast to TA.

In addition to the TAI-GSH conjugate detected, the distinctive MS/MS fragments indicated several other GSH/thiol related TAI conjugates; this is the first report of these structures in TAI treated animals. However, di- and tri- GSH adducts have been seen in CYP2C9 supersome incubations with TAI and GSH, and products of di or tri-GSH adducts were also seen in HEPG2-2C9 cell incubations with TAI (Rademacher, 2011b). In that study, these metabolites were not detected in the presence of BSO (low GSH conditions), confirming they are products of di-GSH dihydro-TAI, not direct conjugation.

Although the dihydro-cysteinyl-TAI (452m/z) metabolite was previously detected in cell incubations where Rademacher (2011) found that the dihydro-TAI-cysteine was also detected in the absence of GSH, indicating that it was not (only) resulting from GSH conjugation, but could directly conjugate to TAI), the metabolite with mass 450m/z, seen during this study in

both TA and TAI treated animals in liver and plasma has not previously been reported. The mass addition of +119, is also suggestive of cysteine conjugation, although the site of conjugation is unknown.

Open ring metabolites have been reported in other thiophene containing xenobiotics including Suprofen, where it may be implicated in toxicity (O'Donnell et al., 2003). This is the first *in vivo* observation of open ring metabolite following TAI administration, and complements those detected in cell incubations of TAI (Rademacher, 2011).

In summary, the *in vivo* metabolism of TA and TAI is strikingly different. TA metabolism is dominated by CYP-mediated hydroxylation (via epoxide), with some glucuronide formation. In contrast, TAI is predominantly metabolised into a large number of glutathione related metabolites (via S-oxide). Very few metabolites were found to be common between TA and TAI, those that co-elute, include only the reduced TA/TAI, and the suspected cysteine and taurine conjugates. The evidence for a large CRM burden in TAI provides an explanation for the related liver damage, resulting from GSH depletion and reactive metabolites binding to proteins. However, which, if any, of the TA metabolites contribute to idiosyncratic toxicity is not known.

3.4.3 Limitations and future work

Glutathione conjugates of TA were not detected in this study, however, they have only previously been reported in the bile in the rat (Nishiya et al., 2006), which was not collected as part of this study. A further study comparing the bile of TA and TAI treated rats could confirm if TA-GSH conjugates are formed in this model, and whether they are preferentially excreted in the bile compared to the TAI-GSH conjugates, or as seems more likely, that TA produces far fewer GSH conjugates compared to TAI.

The metabolites detected both for TA and TAI were very similar to those detected in the cell incubations found by Rademacher (2011). However, very different sample treatment and LC-MS conditions were used, to further confirm these observations, samples from the present study could be analysed mirroring conditions used by Rademacher (2011). To further confirm the identification and enable the quantification of these metabolites, as they are not commercially available, the compounds would need to be synthesised for use as standards to enable the comparison of the retention time and fragmentation of each suspected metabolite.

RESULTS

A UPLC-MS BASED STUDY INTO THE METABOLIC IMPACT OF
TIENILIC ACID AND TIENILIC ACID ISOMER IN THE RAT

4.1 INTRODUCTION

4.1.1 Rationale and aims

Tienilic acid (TA) is an idiosyncratic hepatotoxin that caused over 300 cases of liver injury and 25 deaths in the US, prior to its withdrawal in 1980 (Zimmerman et al., 1984b). Due to the inherent rarity of idiosyncratic drug reactions such as this, safety issues often only arise after the drug has been marketed and is taken on a large scale by the general population (Kaplowitz, 2005). To reduce the clinical risks and financial costs associated with idiosyncratic DILI, further mechanistic understanding, and new approaches for predicting idiosyncratic DILI are required.

An emerging approach to study DILI is metabonomics, which involves the global profiling of small molecules in biological tissues and fluids. Metabonomics is thought to have great potential in the field of DILI biomarker discovery as it can be readily applied to accessible biofluids, such as urine and plasma, and is influenced by an individual's gut microbiota, diet, medications and other environmental factors which may influence DILI (Clarke and Haselden, 2008, Coen, 2010, Keun, 2006, Nicholson et al., 2002). Although overt liver injury is not detected in animal models in response to idiosyncratic toxins like TA, metabonomics may further our understanding of the metabolic impact of these toxins. This in turn could help develop hypotheses about which biological differences, or individual susceptibilities, contribute to idiosyncratic toxicity in humans.

One of the central limitations to metabonomic toxicology studies is differentiating metabolic alterations originating from pharmacological changes, from changes that are indicative of toxicity. In this study the metabolic impact of TA is compared alongside its regioisomer, Tienilic Acid Isomer (TAI), that has a shared pharmacology as a diuretic but is an intrinsic hepatotoxin. The unique alterations in response to either TA or TAI dosing, are therefore less likely to be linked to the pharmacological action of TA, and more likely to be linked to their differing metabolism, and subsequent toxicities.

In a parallel study, a nuclear magnetic resonance (NMR)-based approach was applied to compare the metabolic impact of TA and TAI (Coen et al., 2012). This NMR based approach was successful in differentiating the metabolic response of TA and TAI, however, there were relatively few metabolic changes found to be unique to TA using this approach. The unique metabolic change observed was an increase of hepatic hypotaurine at 2h, which has also been

reported in response to partial hepatectomy (Bollard et al., 2010, Brand et al., 1998, Sturman, 1980), and hypothesised to be related to liver regeneration. One of the potential reasons there were not more unique, and differentiating metabolites found in response to TA compared to TAI, may be the lesser sensitivity of NMR compared to techniques such as liquid chromatography (LC) coupled to mass spectrometry (MS).

LC-MS is one of the more emerging analytical approaches for metabonomic studies, due to its superior sensitivity and larger number of compound classes that can be detected compared to NMR (Want et al., 2007, Lu et al., 2008, Theodoridis et al., 2012) . However, as a less established methodology in metabonomics, there are still limitations to using an untargeted LC-MS approach, in particular with platform stability, reproducibility and critically, metabolite identification (Want et al., 2007). For this reason, targeted LC-MS assays are also used, where a select number of compounds from a given chemical class are monitored and can be quantified.

The aim of the work presented in this Chapter was to further characterise the metabolic impact of TA and TAI in the rat. This was achieved by profiling liver, plasma and urine, using both untargeted and targeted LC-MS approaches. The ultimate aim of studies such as this will be to improve the mechanistic understanding of idiosyncratic DILI, and to find new biomarkers that can be used to better predict idiosyncratic DILI.

4.1.2 Hypothesis

Metabonomics was applied in this Chapter primarily as a hypothesis generating tool, however, the broad hypothesis of this study was that TA and TAI would impact endogenous metabolism in divergent ways, and that differences in their systems level metabolic phenotypes may be indicative of their differing mechanisms of toxicity.

4.2 MATERIALS AND METHODS

4.2.1 Contribution of others

The animal study and sample collection were undertaken by collaborators at Michigan State University, U.S (Professor Roth). All sample preparation and UPLC-MS analyses and the related data processing were performed by the author at Imperial College, London. Sample preparation and application of an ion-pair chromatography-MS/MS method were conducted by the author whilst at Oncology iMed, AstraZeneca, under the supervision of Dr Filippos Michopoulos.

4.2.2 Summary of animal handling and sample collection

For full method details refer to Chapter 3 Materials and methods. Briefly, rats (n=5/6 per group) were dosed with TA, TAI or vehicle, before liver, plasma and urine were collected 2h, 6h, or 24h post-dose.

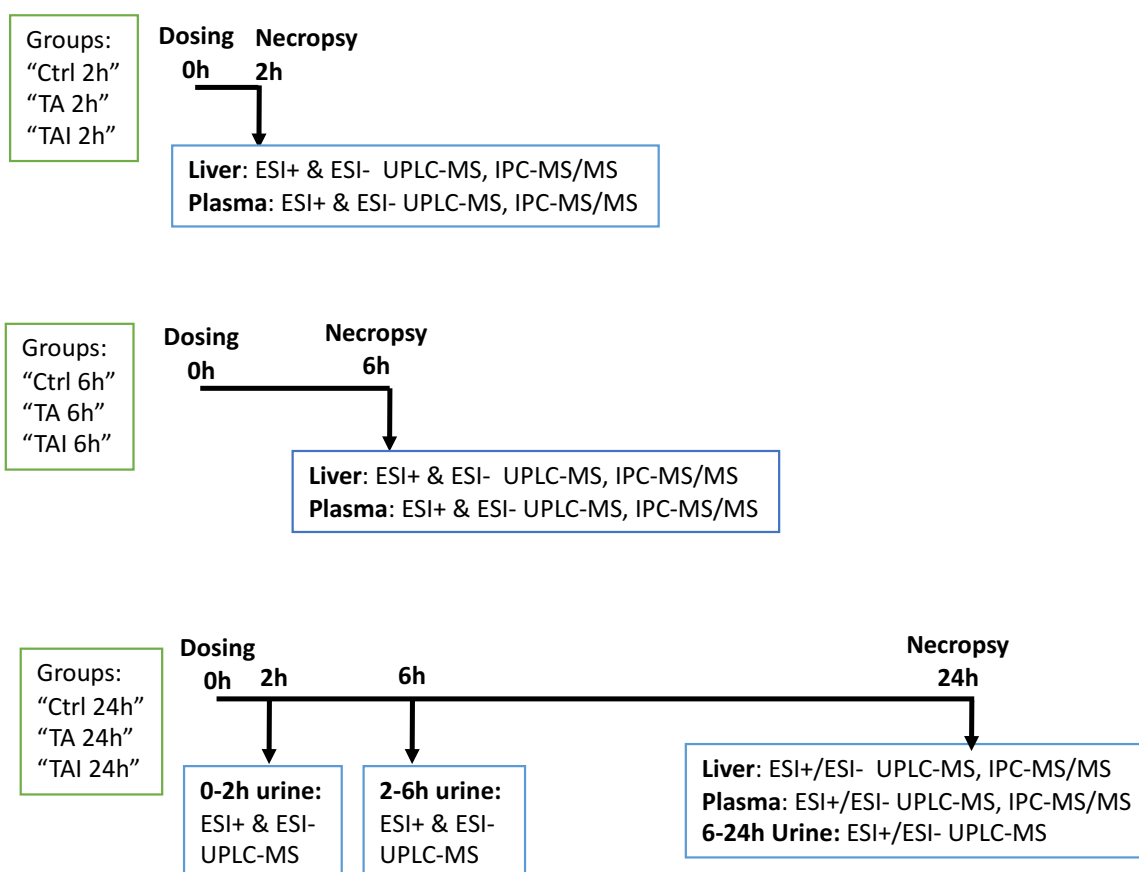


Figure 4.2-1 The study design, sample collection points and analyses performed.

4.2.3 Untargeted UPLC-MS analyses

The liver, plasma and urine data presented in the first half of this Chapter are a parallel analysis to those presented in Chapter 3. For details of the untargeted UPLC-MS method applied, refer back to Chapter 3 Methods for sample preparation and untargeted UPLC-MS analysis details. The same raw UPLC-MS data, collected in both electrospray ionisation positive and negative modes, were re-processed to optimise the extraction of information on the endogenous metabolites, as described below.

4.2.3.1 Data processing

Data were imported from MassLynx (Waters) to Progenesis (NonLinear Dynamics, Newcastle, UK), where peak picking, retention time alignment, and integration of peaks were performed. The selection of peaks for peak picking was restricted to the control samples. This means that only compounds present in control samples were integrated across the treatment groups and included in the metabolic profiles. This was required due to the large number of drug metabolites dominating the metabolic profiles if peak picking was to use a combined sample e.g. a composite quality control (QC) sample as a template. However, as a consequence it also means any endogenous metabolites that were *only* detected in response to drug treatment, were also excluded.

Data were normalised using ‘total compound normalisation’ (nonlinear.com, 2016) in Progenesis to adjust for any variation in sample concentrations or liver sample weights. Compounds were then excluded from further analyses if they were not detected in all but one composite QC or where the compound coefficient of variance in the QCs exceeded 30%. The composite QC was made up of all samples and re-injected at intervals throughout the run to monitor instrument and compound stability. This step was important to ensure compounds that were further studied, and considered as potential biomarkers, were reliably detected over the course of the experiment.

4.2.3.2 Metabolite selection, preliminary identification and statistical analyses

All compounds passing the QC criteria were included in multivariate analyses. To do so, data were exported from Progenesis and then imported to SIMCA (Umetrics). Principal component analysis (PCA) models, of log transformed and mean-centred data, were generated to provide an overview of the variation in the dataset and look for any outliers.

Metabolite identification is very time and resource consuming, and can therefore only realistically be limited to a subset of discriminating compounds for each data set, which can comprise of 1000s of compounds. Selecting the most ‘promising’ compounds is therefore an important step in maximising the value gained from each dataset analysed. Here, for each of the six data sets, fifteen ‘promising’ compounds were selected following the steps outlined in Table 4.2-1, along with the rationale for each step.

Table 4.2-1 Steps taken to select compounds for metabolite identification

Step	Data processing step	Rationale
1	Exclusion based on QC absence or CV >30%	To ensure any compounds of interest were reliably detected over the course of the run, and were varying due to experimental variables and not instrument/sample instability.
2	Exclude all compounds with a fold change greater than 2 in controls across time	A compound that is stable in control samples over time is more likely to be a more useful biomarker, as it is less likely to be impacted by factors such as the availability of food or the time of day a sample was collected.
3	Exclude compounds that do not have a minimum 2 fold difference between control and either treatment group at any time-point	Whilst larger changes are not necessarily more biologically significant, compounds that illicit a clear difference would likely make a more preferable/ reliable biomarker.
4	Exclude compounds with no database identification hits (further discussed below).	If no provisional compound identification could be found, based on search online databases for the calculated m/z, there are limited options for metabolite identification. Chose to prioritise time and resources on those with an improve chance of identification.
5	Select those with the greatest level of statistical significance (by t-test) across all time-points	Higher significance is indicative of lower intra-group variation, and are therefore more likely to be a consistent biomarker linked to phenotype of interest.
6	Exclude those compounds with poor peak shape, or very low intensity	Important step to ensure the compound is not an artefact or poorly integrated. This is performed last due to the time consuming nature of checking peaks.

Each of the fifteen compounds were then presented in dot plots, to show the variation across treatment groups and time. Further statistical analyses using two-tailed Mann-Whitney test, were performed to further determine statistical significance of the selected individual compounds. A non-parametric statistical approach was preferred as there were insufficient sample numbers to test for Gaussian distribution.

Provisional metabolite identifications were based on database searches, initially restricted to the Human Metabolome Database (HMDB) serum and urine metabolite databases (Psychogios

et al., 2011, Bouatra et al., 2013), before extending the search to the complete HMDB (Wishart et al., 2013), Lipidmaps (Sud et al., 2012) and Metlin databases (Smith et al., 2005). As only provisional identifications have been made from these data, information is provided to characterise the provisional identification, and allow some consideration of the strength of the provisional identifications presented. These include: the suggested adduct(s) present, the likely chemical formula calculated from the m/z , and the mass difference (ppm) and isotope similarity (%) between the unknown compound and the suggested identification (calculated in Progenesis).

4.2.4 Targeted IPC–MS/MS

To profile more polar metabolites from the same study, liver and plasma extracts were analysed using a targeted ion-pair chromatography (IPC)-MS/MS method, as detailed below.

4.2.4.1 Materials

Tributylamine (TBA), acetic acid, ammonium formate and all analytical standards, of the highest purity available, were purchased from Sigma–Aldrich. A full list of metabolites included in this assay and the origin of the analytical standards is given in (Michopoulos et al., 2014). Ultrapure water was obtained from a Purelab Ultra System from Elga (Bucks, UK). Methanol, acetonitrile and isopropanol used for sample extraction and analysis were of HPLC grade (Sigma–Aldrich, Gillingham, UK).

4.2.4.2 Quality control and sample preparation

For the preparation of plasma samples, 10 μ L of plasma was subjected to protein precipitation with the addition of 40 μ L cold (-20°C) MeOH/ACN 50/50 (v/v). Samples were briefly vortexed and kept for 10 min at -20°C , before the precipitated proteins were removed by centrifugation at 21 000 g, at 0°C , for 5min (5417R Eppendorf, Hamburg, Germany). 30 μ L of each clear supernatant were transferred to polypropylene HPLC vials. An additional 10 μ L aliquot of all samples were then combined to make two composite QC samples, which were vortexed, and 60 μ L of each placed into HPLC vials. The supernatants were dried at room temperature in a Savant SPD2010 SpeedVac (Thermo Fisher, UK) for approximately 30min. The plasma extracts of samples were then re-suspended in 60 μ L of ultra-pure water, one QC was re-suspended with 120 μ L of ultra-pure water, and the other QC was re-suspended in ultra-pure water containing a standards mix (5 μ M of all compounds to be monitored). Vials were vortexed, then

centrifuged at 3270g for 10 min at 4°C in an Allegra X12R centrifuge equipped with SX4750A swinging bucket rotor (Beckman Coulter).

For liver samples, tissue homogenisation and extraction was carried in a 2mL CK14 lysis kit on a Precellys 24 device equipped with Cryolys temperature control unit (Peqlab, Southampton, UK). Pre-weighed frozen tissue was extracted with 1mL MeOH/ACN/H₂O (40/40/20 v/v/v) / 100mg tissue, following a sequence of 2 × 30s shaking cycles (5 500 rpm) with a 20s pause in between each cycle. Each sample was centrifuged at 21,000 g, 4°C, for 5min (5417R Eppendorf, Hamburg, Germany). 10µL of each clear supernatant were transferred to polypropylene HPLC vials. An additional 10µL aliquot of all samples were then combined to make two composite QC samples, which were vortexed, and 60µL of each placed into HPLC vials. The supernatants were dried at room temperature in a Savant SPD2010 SpeedVac (Thermo Fisher, UK) for approximately 30min. The liver extracts were then re-suspended in 50µL of ultra-pure water, one QC was re-suspended with 300µL of ultra-pure water, and the other QC was re-suspended in ultra-pure water containing a standards mix (5µM of all compounds to be monitored). Vials were vortexed, then centrifuged at 3270g for 10 min at 4°C in an Allegra X12R centrifuge equipped with SX4750A swinging bucket rotor (Beckman Coulter).

Prior to the start of the main analytical run, ten injections (5µL) of the QC samples were performed to ensure adequate system conditioning. To monitor instrument stability, one QC sample was run after every ten sample injections through the run. Samples were run in a randomised order, generated using the random number function in Excel (Microsoft). To confirm metabolite retention times a test mixture containing 5µM of the compounds to be determined, in addition to the QC sample spiked with the standards mixture at a final concentration of 5µM, were analysed at the beginning and the end of the analytical batch.

4.2.4.3 Ion-pair chromatography (IPC)

The IPC separations were performed on an Acquity HSS T3 UPLC column (Waters Corp, 2.1 mm × 100 mm, 1.85µm particle size). Column temperature was maintained at 60±0.5°C during the analysis. 5µL of samples were injected, and elution was performed using a binary solvent system consisting of solvent A (H₂O, 10mM TBA, 15mM acetic acid) and solvent B (80% MeOH and 20% isopropanol). Chromatographic separation was achieved with a flow rate of 400µL/min and the following gradient profile: 0min, 0% B; 0.5min, 0% B; 4min, 5% B; 6min, 5%

B; 6.5min, 20% B; 8.5min, 20% B; 14min, 55% B; 15min, 100% B; 17min, 100% B; 18min, 0% B; 21min 0% B.

4.2.4.4 Mass spectrometry

All MS data was acquired on a QTRAP 4000 hybrid triple quadrupole linear ion trap mass spectrometer operating through Analyst 1.5.1 (Applied Biosystems/MDS Sciex, Warrington, UK) with the following settings: Turbo IonSpray voltage –3500 V, curtain gas 10 (arbitrary unit), temperature 550 °C, Gas 1 and 2 were at 60 and 50 (arbitrary unit) respectively and entrance potential –10 V. Data were acquired in negative ionization mode using the scheduled MRM transitions detail in Table 1.2-1 (Michopoulos et al., 2014).

Table 4.2-2 Details of compounds included in the IPC-MS/MS assay

Metabolite	Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Declustering potential	Collision energy	Collision exit potential
Acetoacetyl CoA	424.8	382.8	13.0	-35	-16	-4
Acetyl CoA	808.3	79.0	13.2	-85	-53	-5
Adenine	134.0	107.0	1.3	-70	-25	-5
Adipic acid	145.0	83.0	9.6	-35	-18	-5
ADP	426.1	79.0	10.7	-80	-88	-3
ADP ribose	558.1	346.1	10.3	-80	-33	-5
α-ketoglutaric acid	145.0	101.0	10.0	-35	-12	-7
AMP	346.2	79.0	8.4	-70	-62	-5
Arginine	173.1	131.1	0.4	-40	-19	-5
Asparagine	131.1	95.0	0.7	-31	-19	-5
Aspartic acid	132.0	88.0	2.7	-40	-19	-5
ATP	506.1	79.0	12.4	-90	-106	-1
Benzoic acid	121.0	77.0	10.7	-30	-19	-5
Butyryl CoA	418.0	79.0	14.2	-50	-80	-5
cAMP	328.0	134.0	9.0	-80	-40	-5
cGMP	344.0	150.0	8.5	-67	-39	-5
cis aconitic acid	173.0	85.0	10.8	-33	-18	-5
Citramalic acid	147.0	87.0	9.4	-50	-20	-5
Citric acid	191.0	87.0	10.9	-35	-26	-5
Citrulline	174.1	131.1	0.6	-30	-21	-5
CMP	322.0	97.0	6.1	-50	-39	-5
Coenzyme A	766.0	79.0	13.0	-150	-130	-5
Coumaric acid	163.1	119.1	9.5	-45	-21	-5
Creatine	130.0	88.0	0.6	-35	-14	-4
Creatinine	112.0	68.0	0.6	-55	-25	-4
Crotonyl CoA	834.0	79.0	13.9	-150	-90	-5
Cystine	239.0	120.0	0.6	-35	-17	-5
Cytosine	110.0	67.0	0.6	-30	-19	-5
dADP	410.0	79.0	11.0	-70	-85	-5
dAMP	330.1	79.0	8.6	-55	-60	-5
dATP	490.0	79.0	12.5	-75	-105	-5
dCDP	386.0	79.0	10.2	-50	-88	-5
dCMP	306.1	79.0	6.8	-45	-70	-5

Metabolite	Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Declustering potential	Collision energy	Collision exit potential
dCTP	466.0	79.0	12.1	-80	-105	-5
dGMP	346.2	79.0	8.2	-70	-62	-5
dinitrosalicylic acid	227.0	183.0	13.9	-50	-22	-5
dUMP	307.0	111.0	8.0	-55	-35	-5
dUTP	467.0	159.0	12.2	-60	-50	-9
FAD	784.5	97.0	12.2	-85	-53	-5
Fructose bisphosphate	339.0	97.0	10.7	-60	-28	-5
Ferulic acid	193.1	134.0	9.8	-46	-22	-5
Folic acid	440.0	311.0	10.7	-75	-32	-7
Fructose 1 phosphate	259.0	97.0	6.2	-55	-65	-5
Fructose 6 phosphate	259.0	169.0	5.1	-55	-17	-5
Fumaric acid	115.0	71.0	10.1	-31	-14	-5
G3P	185.0	79.0	10.2	-35	-44	-3
Galactose 1 phosphate	259.0	241.0	5.1	-55	-20	-5
GAP	169.0	97.0	10.1	-22	-16	-5
GBP	265.0	167.0	12.3	-40	-20	-5
GDP	442.0	79.0	10.3	-66	-87	-5
Glucosamine 6 phosphate	258.1	97.0	0.7	-35	-26	-5
Glucose 1 phosphate	259.0	241.0	5.3	-55	-20	-5
Glucose 6 phosphate	259.0	97.0	4.6	-55	-27	-5
Glucuronic acid	193.1	113.0	3.3	-31	-19	-5
Glutamic acid	146.0	102.0	2.3	-40	-19	-5
Glutamine	145.1	127.1	0.6	-45	-16	-5
Glutaric acid	131.1	87.0	9.2	-35	-19	-5
Glutathione ox	611.6	306.3	9.3	-80	-31	-5
Glutathione red	306.3	143.1	5.6	-50	-26	-5
Glyoxylic acid	91.0	73.0	10.1	-18	-13	-5
GMP	362.0	79.0	7.9	-50	-70	-5
GTP	522.1	79.0	12.2	-70	-105	-5
Guanine	150.1	133.1	1.0	-45	-20	-5
Guanosine	282.0	150.0	2.1	-80	-25	-5
Histidine	154.0	93.0	0.4	-45	-25	-4
Hydroxy phenyl propionic acid	165.1	121.1	11.9	-44	-21	-5
Hydroxy glutaric acid	147.0	85.0	9.5	-40	-22	-5
Hydroxy phenyl acetic acid	151.0	107.0	8.8	-30	-10	-5
Hydroxymethyl Glutaryl CoA	910.2	79.0	13.3	-150	-120	-5
ICA	160.1	116.1	12.5	-40	-20	-5
IMP	347.0	79.0	7.9	-55	-85	-5
Inosine	267.0	135.0	1.9	-80	-37	-10
Isobutyryl CoA	836.1	408.0	14.0	-155	-55	-5
Isocitric acid	191.0	73.0	11.1	-35	-32	-5
Isoleucine	130.0	84.0	1.2	-55	-18	-3
Itaconic acid	129.1	85.0	9.4	-33	-15	-5
Lactic acid	89.0	43.0	4.6	-40	-19	-1
Leucine	130.0	84.0	1.3	-55	-18	-3
Malic acid	133.0	115.0	9.4	-30	-16	-9
Maleic acid	115.0	71.0	8.7	-35	-10	-3
Malonic acid	103.0	59.0	8.5	-27	-14	-5
Malonyl CoA	425.5	404.0	13.2	-28	-12	-4

Metabolite	Q1 Mass (Da)	Q3 Mass (Da)	Time (min)	Declustering potential	Collision energy	Collision exit potential
Mannose 6 phosphate	259.0	79.0	4.8	-55	-70	-5
Mesaconic acid	129.0	85.0	10.0	-35	-12	-5
Methionine	148.0	47.0	0.9	-35	-25	-7
Methyl Malonyl CoA	432.5	410.5	13.4	-30	-10	-5
Methylxanthine	165.1	122.1	2.6	-47	-27	-5
NAD	662.3	540.1	7.0	-50	-22	-9
NADH	664.3	79.0	10.7	-110	-120	-3
NADP	742.2	620.1	10.6	-60	-22	-11
NADPH	744.3	79.0	12.4	-110	-118	-3
NAG	220.2	119.0	0.7	-44	-11	-5
Nicotinic acid	122.0	78.0	8.3	-35	-19	-5
Nitrophenol	138.1	108.0	10.5	-59	-20	-5
OH Butyryl CoA	852.0	79.0	13.2	-150	-90	-5
Orotic acid	155.0	111.0	5.8	-30	-18	-9
Pantothenic acid	218.0	88.0	8.5	-46	-20	-5
Phosphocreatine	210.0	79.0	9.2	-35	-35	-5
PEP	167.0	79.0	10.7	-35	-20	-5
PG	275.0	79.0	10.1	-60	-66	-5
Phenylalanine	164.1	147.1	2.5	-48	-19	-5
Phthalic acid	165.0	121.0	11.4	-40	-16	-5
Pimelic Acid	159.0	97.0	10.3	-40	-20	-5
Proline	114.0	68.0	0.6	-55	-18	-4
Propionyl CoA	822.0	79.0	13.6	-130	-120	-5
Phosphoserine	184.0	97.0	6.0	-35	-18	-5
Pyruvic acid	87.0	43.0	6.0	-30	-12	-1
Riboflavin	375.1	255.1	8.6	-55	-26	-5
Ribose 5 phosphate	229.0	97.0	5.2	-50	-22	-5
Ribulose 5 phosphate	229.0	97.0	5.4	-50	-22	-5
Salicylic acid	137.1	65.0	11.9	-48	-41	-5
Serine	104.0	74.0	0.6	-40	-18	-5
Shikimic acid	173.1	93.0	3.3	-40	-25	-5
Sorbitol/mannitol	181.0	89.0	0.6	-58	-19	-7
Succinate	117.0	73.0	8.7	-45	-16	-5
Succinyl CoA	866.2	79.0	13.3	-130	-120	-5
TDP	401.0	79.0	10.7	-70	-80	-5
Threonine	118.0	74.0	0.6	-45	-15	-5
Thymine	125.1	42.0	1.6	-35	-22	-5
TIA	186.1	142.1	12.1	-30	-29	-5
TMP	321.0	195.0	8.4	-55	-25	-5
Tryptophan	203.1	116.0	4.2	-50	-25	-5
TTP	481.0	159.0	12.3	-70	-48	-5
Tyrosine	180.0	163.0	1.1	-45	-20	-5
UDP	403.0	159.0	10.3	-60	-41	-5
UDP glucuronic acid	579.0	403.0	12.1	-60	-32	-5
UDP glucose	565.0	323.0	9.7	-80	-33	-5
UMP	323.0	97.0	7.4	-52	-33	-5
Uracil	111.0	42.0	0.9	-26	-30	-5
Uridine	243.0	110.0	1.3	-60	-25	-5
Valine	116.0	70.0	0.8	-70	-15	-5
Xylulose 5 phosphate	229.0	97.0	5.3	-50	-22	-5

4.2.4.5 Data processing

The raw spectrometric data were integrated with MultiQuan 2.0.2 (Applied Biosystems/MDS Sciex, Warrington, UK) and manual checking and adjustment of peak picking and integration were performed for all automatically selected peaks. The results were exported to Excel (Microsoft) where compounds were excluded from further analyses that were not detected in all but one QC, or where their coefficient of variance exceeded 30%. Data were normalised in R prior to statistical analyses to adjust for concentration differences in samples.

4.2.4.6 Statistical analyses

Data were imported to SIMCA (Umetrics) for multivariate analyses, where PCA and OPLS-DA models were generated. Statistical significance was determined using a two-tailed Mann-Whitney test performed in Prism (GraphPad).

4.3 RESULTS

4.3.1 Untargeted UPLC-MS analyses

4.3.1.1 Aqueous liver extracts- ESI- Mode

A PCA model was generated from ESI- analyses of aqueous liver extracts to obtain an overview of the data, 2462 compounds (features after deconvolution of adducts/fragments) were included in the model. The number of components were determined by selecting the model with the highest cumulative R^2 and Q^2 values. The model contained 7 components, and had a cumulative R^2X of 0.767, and $Q^2=0.569$. The scores plot is shown in Figure 4.3-1, displaying principal components (PC) 1 ($R^2X= 0.405$), and PC2 ($R^2X= 0.0897$). The tight clustering of QC samples indicates that the overall stability of the compounds included in the analysis was good.

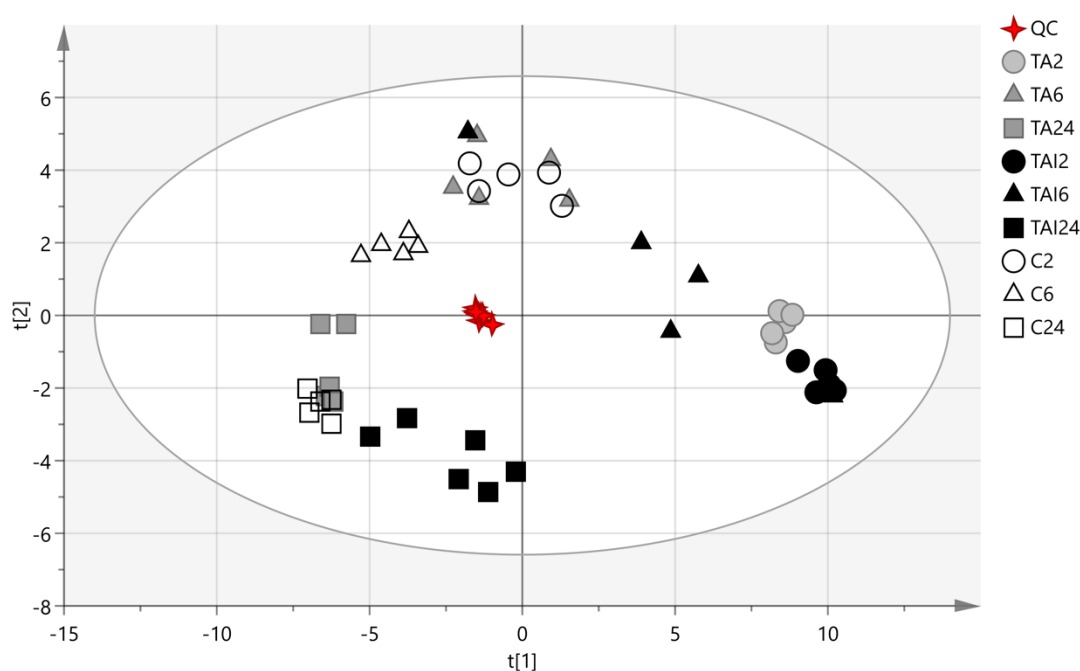


Figure 4.3-1. PCA scores plot from of ESI- UPLC-MS analyses of aqueous liver extracts. 7 components, R^2X (cum)= 0.767, Q^2 (cum)= 0.569, R^2X PC1= 0.405, R^2X PC2= 0.0897. The ellipse is Hotelling's T2 (95%). The key number refers to time-point (h) and drug treatment: TA- Tienilic Acid, TAI- Tienilic Acid Isomer, or QC- quality control samples.

The scores plot of PC1 and PC2 shows clear separation of classes and time-points. In particular, the 2h samples from TA and TAI treated animals are tightly clustered and situated closely together, but distinct from 2h control samples. While all the 24h samples share a similar multivariate space.

Fifteen of the most differentiating and 'promising' compounds, selected using the approach outlined in the methods section, are shown in Figure 4.3-8, and their preliminary identifications

of based on searching the compound databases using the detected m/z values, are presented in Table 4.3-1. As these compound identifications are not confirmed, they are named here by their retention time (min) and m/z or neutral mass (if it was possible to calculate from adducts formed) together with the provisional identification.

A unique alteration in TA dosed animals included the depletion of compound 0.74_210.0363n at 2h and 6h, which was provisionally identified as either galactaric or glucaric acid. In the 2h and 6h there was also a greater decrease detected in TA samples compared to TAI, in 0.62_196.0580n (galactonic or gluconic acid), and a greater increase in 0.61_193.0360m/z (provisionally identified as glucuronic acid or related compound). A far greater increase was also seen in TA samples at 2h in 6.37_144.0452m/z (1H-indole-3-carboxyaldehyde, a tryptophan metabolite). The only lasting alteration in TA samples was in 0.61_280.0360m/z (phospho-L-histidine).

There were four unique TAI specific alterations found using this approach, which were most distinct at the 24h time-point. These included 0.71_145.0368n (2-keto-glutamaric acid), 0.96_126.0196m/z (hydroxylaniline), 1.37_126.0196 (5-hydroxyisourate) and 6.84_192.0664m/z (methylhippuric acid/phenylacetic acid), refer to Figure 4.3-2 and Table 4.3-1 for further compound details.

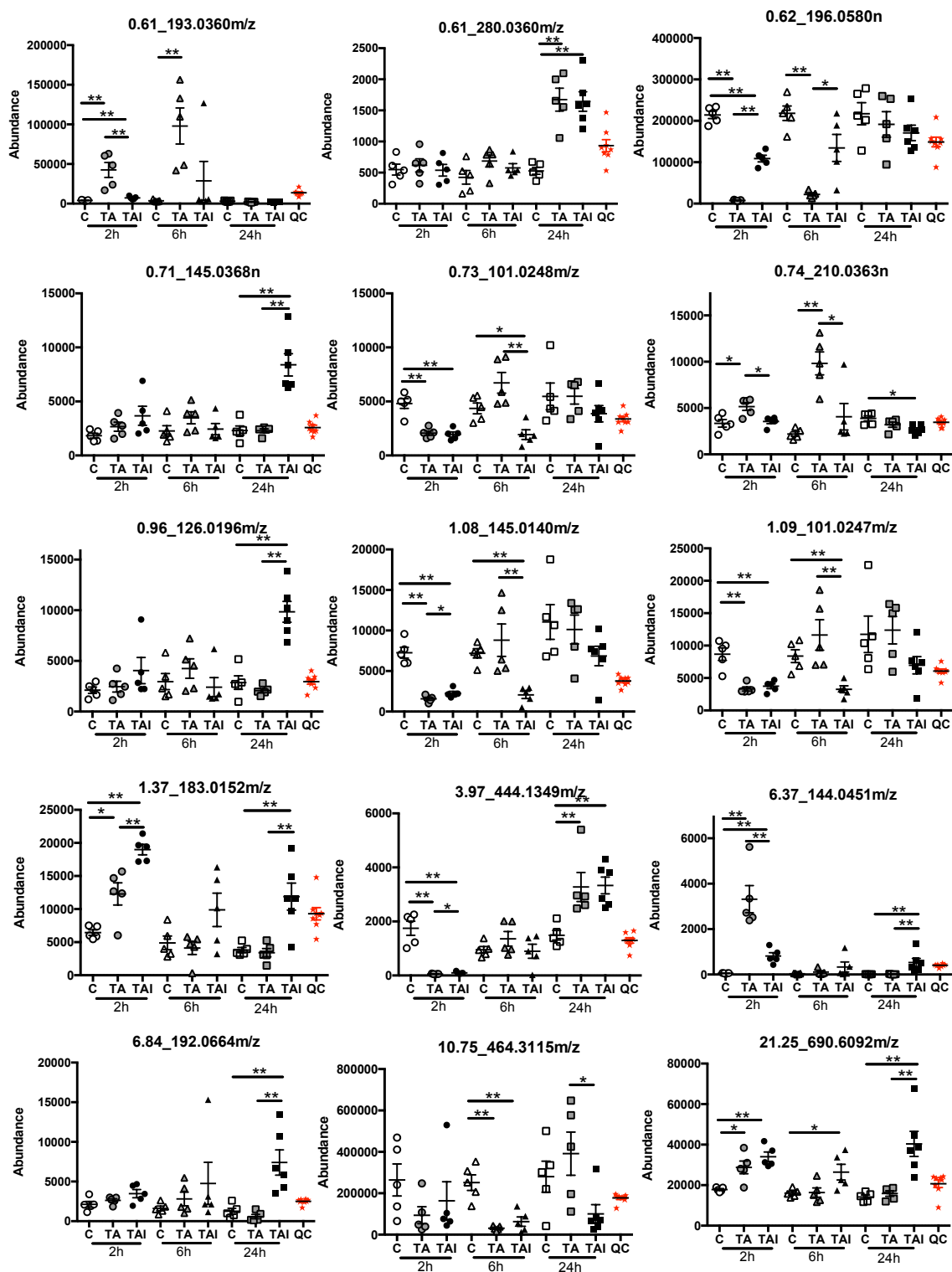


Figure 4.3-2 Selected compounds from aqueous liver extracts analysed by UPLS-MS in ESI-mode. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** < 0.01 . Bars show the mean and error bars represent $\pm S.E.M$.

Table 4.3-1 Details of selected compounds from ESI- analyses of aqueous liver extracts

Rt (min)	Mass	Suggested identification(s)*	Chemical formula	Mass difference (ppm)	Isotope similarity (%)	Group(s) significantly altered v control
0.61	193.0360m/z	Several, including glucuronic acid (M-H)	C ₆ H ₁₀ O ₇	3.18	95.60	TA & TAI 2h TA 6h
0.61	280.0360m/z	Phospho-L-histidine (M+FA-H)	C ₆ H ₁₀ N ₃ O ₅ P	8.38	94.28	TA & TAI 24h
0.62	196.0580n	Galactonic acid, gluconic, gulonic acid (M-H, M-H ₂ O-H)	C ₆ H ₁₂ O ₇	-1.74	96.70	TA & TAI 2h TA 6h
0.71	145.0368n	2-keto-glutaramic acid (M-H ₂ O-H, M+FA-H)	C ₅ H ₇ NO ₄	-5.02	98.21	TAI 24h
0.73	101.0248m/z	Acetoacetic acid, 2-ketobutyric acid, several others	C ₄ H ₆ O ₃	3.61	98.89	TA & TAI 2h
0.74	210.0363n	Galactaric acid, glucaric acid (M-H, M+Cl)	C ₆ H ₁₀ O ₈	-5.95	98.50	TA 6h
0.96	126.0196m/z	Hydroxyminaline (M-H) 2-keto-glutaramic acid (M-H ₂ O-H)	C ₅ H ₅ NO ₃	-0.65 -0.57	99.24 99.08	TAI 24h
1.08	145.0140m/z	Oxoglutaric acid (M-H)	C ₅ H ₆ O ₅	-1.84	98.11	TA & TAI 2h TAI 6h
1.09	101.0247m/z	Several including acetoacetic acid, 2-ketobutyric acid(M-H)	C ₄ H ₆ O ₃	3.06	97.82	TA & TAI 2h TAI 6h
1.37	183.0152m/z	5-hydroxyisourate (M-H)	C ₅ H ₄ N ₄ O ₄	-4.09	95.88	TAI 2h TAI 24h
3.97	444.1349m/z	Hexanoyl-adenosine-monophosphate (M-H)	C ₁₆ H ₂₄ N ₅ O ₈ P	13.26	91.83	TA & TAI 2h TA & TAI 24h
6.37	144.0452m/z	1H-indole-3-carboxyaldehyde (M-H)	C ₉ H ₇ NO	-2.56	90.18	TA & TAI 2h TAI 24h
6.84	192.0664m/z	Methylhippuric acid (M-H) Phenylacetyl glycine (M-H)	C ₁₀ H ₁₁ NO ₃	-1.25	96.62	TAI 24h
10.75	464.3115m/z	PE(P-18:0/0:0) (M-H) PC(P-15:0/0:0) (M-H)#	C ₂₃ H ₄₈ NO ₆ P	-6.73	97.68	TA & TAI 6h
21.25	690.6092m/z	n-(2r-hydroxytricosanoyl)-2s-amino-1,3s,4r-octadecanetriol (M+Na-2H)	C ₄₁ H ₈₃ NO ₅	11.07	92.30	TAI 2h TAI 24h

*Databases searched including HMDB v 3.0 and Lipidmaps. #could also be GCA seen in ESI+, # By Mann-Whitney test with p<0.01.

4.3.1.2 Aqueous liver extracts- ESI+ Mode

The PCA scores plot generated from ESI+ mode data of aqueous liver extracts (7 components, cumulative $R^2X = 0.665$, $Q^2 = 0.452$, 4080 compounds included in the model) is shown in Figure 4.3-3, displaying principal component one (PC1) ($R^2X = 0.313$) and PC2 ($R^2X = 0.0881$). The QCs on the scores plot show tight clustering, indicating good stability for the compounds included in the model. The slightly outlying QC is the first QC sample in the run; indicating that a larger number of conditioning QCs may have been optimal.

There is no clear pattern observed, however, the 2h TA and TAI samples are loosely clustered together and distinct from control 2h samples. Whereas by 24h, TA samples are clustered with control samples, distinct from TAI 24h samples.

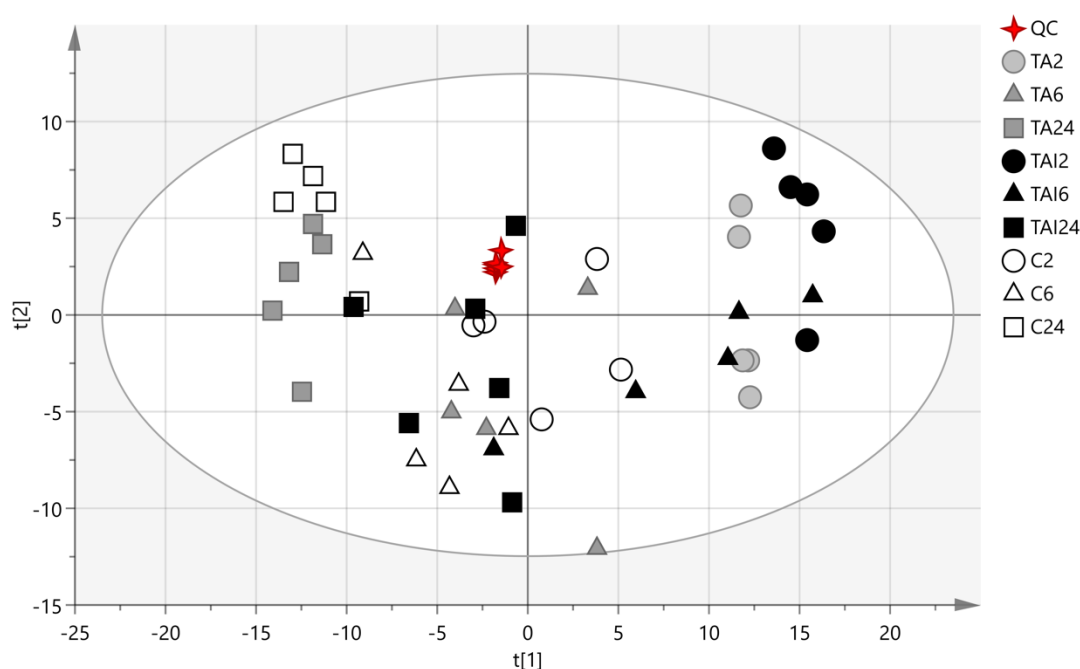


Figure 4.3-3 Scores plot from a PCA model of ESI+ UPLC-MS data of aqueous liver extracts. 7 components, $R^2X(\text{cum}) = 0.665$, $Q^2(\text{cum}) = 0.452$, R^2X principal component (PC) 1 = 0.313, R^2X of PC 2 = 0.0881. The ellipse is Hotelling's T^2 (95%). The key number refers to time-point (h), and drug treatment: TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples.

The fifteen selected compounds, are individually plotted in Figure 4.3-4. Table 4.3-2 shows the preliminary identifications. The compound 7.00_187.0640n, which was preliminarily identified as indoleacrylic acid (a tryptophan metabolite), was uniquely elevated in TA samples. Interestingly, although significant in both TA and TAI treated animals, at 2h there was also a significant elevation in 6.43_162.0554m/z, which was also provisionally identified as another tryptophan metabolite, indole-3-carboxylic acid. This compound was significantly more elevated in TA compared to TAI at 2h time-point. Two compounds remained significantly

altered in TA treated animals by 24h, these included a small elevation in 0.70_290.1366m/z, provisionally identified as ophthalmic acid, which was elevated to a greater extent at 6h in both TA and TAI. The other lasting decrease was detected in 11.10_314.2483n, provisionally identified as octadecanedioic acid.

There were seven unique alterations in TAI specific compounds at 24h. These included a depletion of 0.65_118.0885m/z, which had several potential identifications including betaine or valine, and elevations in six compounds, including 5.11_297.1460m/z, 5.65_311.1610m/z, 6.14_217.0981m/z, 6.43_162.0554m/z, 6.71_251.0499m/z and 6.91_193.0755n (refer to Table 4.3-2 for details of preliminary identifications).

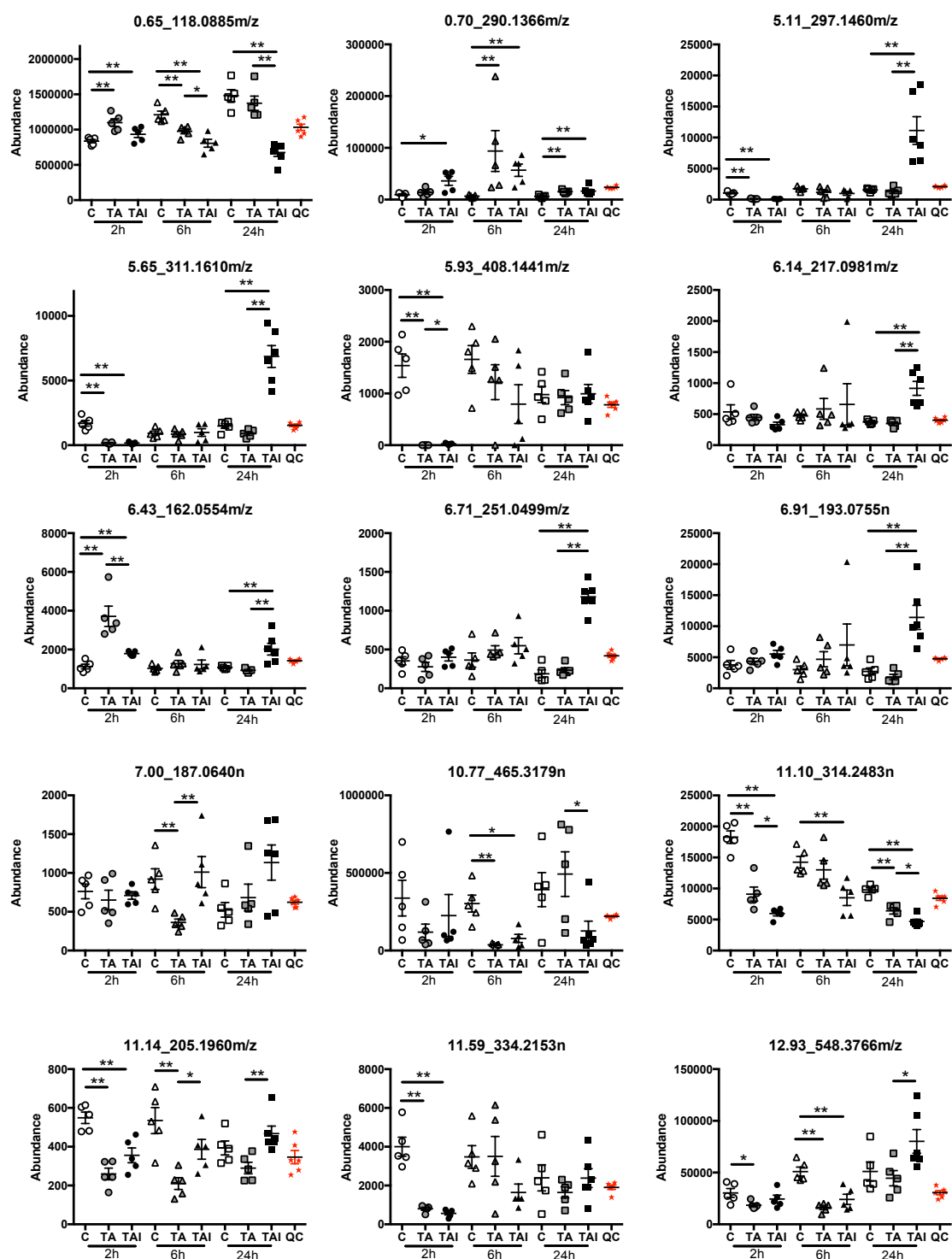


Figure 4.3-4 Dot plots of selected compounds from aqueous liver extracts analysed by UPLS-MS in ESI+ mode. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$. Bars show the mean and error bars represent $\pm$ S.E.M.

Table 4.3-2 Details of selected compounds from ESI+ analyses of aqueous liver extracts

Rt (min)	Mass	Suggested identification(s)	Chemical formula	Mass difference (ppm)	Isotope similarity (%)	Group(s) significantly [#] altered v control
0.65	118.0885m/z	Betaine, valine, many others (M+H)	C ₅ H ₁₁ NO ₂	19.06	98.48	TA & TAI 2h TA & TAI 6h TAI 24h
0.70	290.1366m/z	Ophthalmic acid (M+H)	C ₁₁ H ₁₉ N ₃ O ₆	6.68	93.88	TA & TAI 6h TA & TAI 24h
5.11	297.1460m/z	Tetradecanedioic acid (M+K)	C ₁₄ H ₂₆ O ₄	-1.12	95.57	TA & TAI 2h TAI 24h
		Alpha-Phenylacetyl-glutamine (M+CH ₃ OH+H)	C ₁₃ H ₁₆ N ₂ O ₄	5.60	96.01	
		Acetyl-formyl-Methoxykynurenamine (M+CH ₃ OH+H)	C ₁₃ H ₁₆ N ₂ O ₄	5.60	96.01	
5.65	311.1610m/z	Pantethine (M+CH ₃ OH+H)	C ₁₁ H ₂₂ N ₂ O ₄ S	-9.03	95.88	TA & TAI 2h TAI 24h
		Estriol, or related (M+Na)	C ₁₈ H ₂₄ O ₃	-2.63	88.96	
5.93	408.1441m/z	Hyaluronic acid (M+H-H ₂ O)	C ₁₆ H ₂₇ NO ₁₂	-13.98	86.44	TA & TAI 2h
6.14	217.0981m/z	5-Methoxytryptophan (M+H-H ₂ O)	C ₁₂ H ₁₄ N ₂ O ₃	3.92	97.74	TAI 24h
6.43	162.0554m/z	Indole-3-carboxylic acid (M+H)	C ₉ H ₇ NO ₂	2.59	89.69	TA & TAI 2h TAI 24h
6.71	251.0499m/z	Homocystine (M+H-H ₂ O)	C ₈ H ₁₆ N ₂ O ₄ S	-7.45	89.09	TAI 24h
6.91	193.0755n	Phenylacetyl-glycine (M+Na, M+K, M+H)	C ₁₀ H ₁₁ NO ₃	8.17	95.81	TAI 24h
		Methylhippuric acid (M+Na, M+K, M+H)	C ₁₀ H ₁₁ NO ₃			
7.00	187.0640n	Indoleacrylic acid (M+H, M+NH ₄)	C ₁₁ H ₉ NO ₂	3.79	98.90	TA 6h
10.77	465.31779n	Glycocholic acid (M+H, many)	C ₂₆ H ₄₃ NO ₆	18.97	99.33	TA 6h
11.10	314.2483n	Octadecanedioic acid, DHOME (M+Na, M+K, M+H-2H ₂ O)	C ₁₈ H ₃₄ O ₄	8.25	90.57	TA & TAI 2h TAI 6h TA & TAI 24h
11.14	205.1960m/z	Lipid (M+H), many	C ₁₅ H ₂₄	4.48	88.50	TA & TAI 2h TA 6h
11.59	334.2153n	Prostaglandin, or other lipid (M+Na, M+K, M+H-H ₂ O)	C ₂₀ H ₃₀ O ₄	2.72	88.72	TA & TAI 2h
12.93	548.3766m/z	LysoPC (20:2(11Z,14Z)) (M+H)	C ₂₈ H ₅₄ NO ₇ P	10.20	87.22	TA & TAI 6h
		PC (M+H)	C ₂₈ H ₅₄ NO ₇ P			

*Databases searched including HMDB 3.0, and Lipidmaps. # By Mann-Whitney test with p<0.01.

4.3.1.3 Plasma- ESI- Mode

The scores plot for the PCA model (7 components, $R^2X(\text{cum})=0.734$, $Q^2(\text{cum})=0.540$, $R^2X \text{ PC1}=0.225$, $R^2X \text{ PC2}=0.17$) of plasma analysed using untargeted UPLC-MS in ESI- is shown in Figure 4.3-2. The QC samples are well clustered, indicating good overall stability of the system. There were 2336 compounds included in the model.

There is some separation between control samples collected at different time-points, though all the samples are located in a similar multivariate space. The 2h TA and TAI samples are clustered together, but separate from the 2h control samples. The TA and TAI 6h samples show little clustering indicating the wider metabolic variability at this time-point. The 6h controls cluster with 24h control and TA samples. The TAI 24h samples are the most distinct group strongly separated from control and TA samples at 24h in PC2.

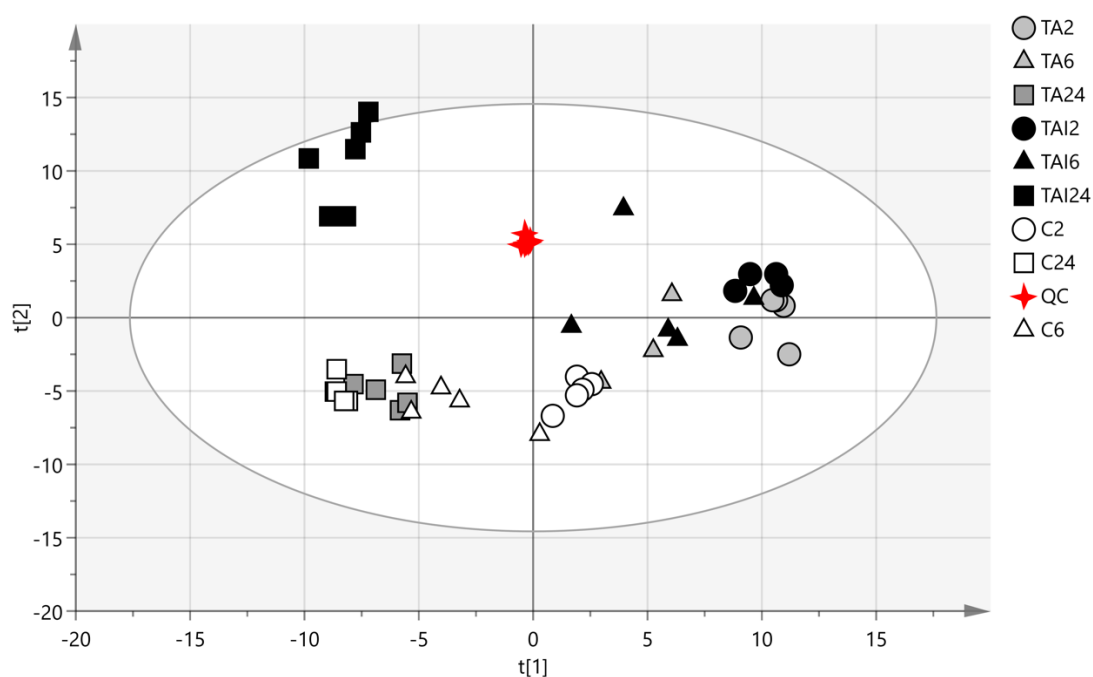


Figure 4.3-5 Scores plot from a PCA of ESI- UPLC-MS analyses of plasma. 7 components, $R^2X(\text{cum})=0.734$, $Q^2(\text{cum})=0.540$, $R^2X \text{ PC1}=0.225$, $R^2X \text{ PC2}=0.174$. The ellipse is Hotelling's T^2 (95%). The key number refers to time-point (h), and drug treatment: TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples.

Selected compounds from ESI- analyses of plasma are presented in Figure 4.3-6 and Table 4.3-3. At 24h there were TA specific elevations detected in 12.66_269.2497m/z (14-methylhexadecanoic acid, fatty acid) and 13.40_283.2648m/z (stearic acid, saturated fatty acid), which were both depleted in TAI samples at 24h. Additionally, 0.68_158.0445n,

(provisionally identified as allantoin) was significantly more elevated in the TA samples at 2h and 6h compared to TAI. Compounds that were increased significantly more in TAI samples included those provisionally identified as uridine (1.21_243.0633m/z) and pyroglutamic acid (1.62_128.0360m/z).

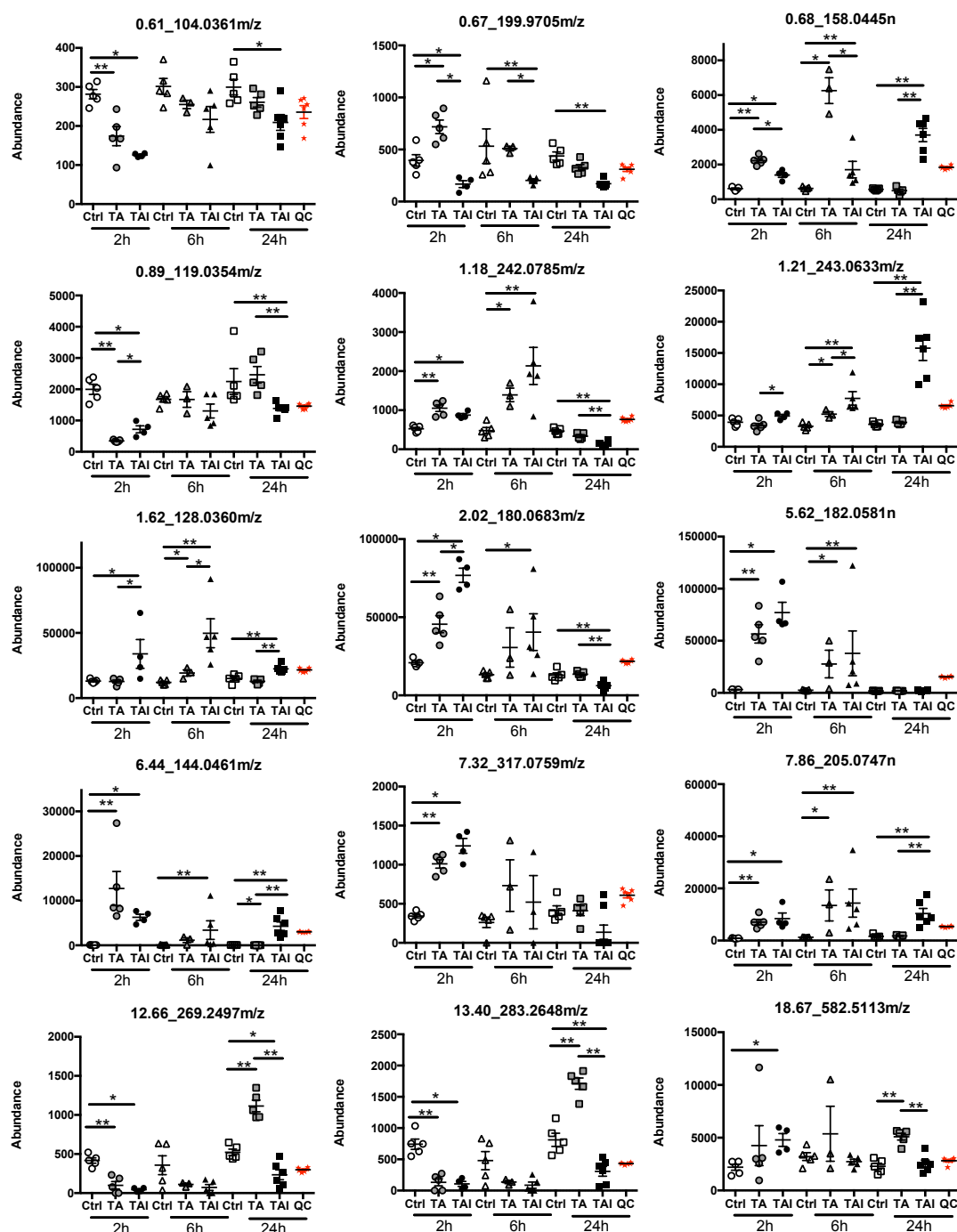


Figure 4.3-6 Selected compounds from plasma analysed by UPLS-MS in ESI- mode. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$. Bars show the mean and error bars represent $\pm$ S.E.M.

Table 4.3-3 Details of selected compounds from ESI- analyses of plasma

Rt (min)	Mass	Suggested identification(s)	Chemical formula	Mass difference (ppm)	Isotope similarity (%)	Group(s) significantly [#] altered v control
0.61	104.0361m/z	Serine (M-H)	C ₃ H ₇ NO ₃	7.93	97.34	TA 2h
0.67	199.9705m/z	Cysteine-S-sulphate (M-H)	C ₃ H ₇ NO ₅ S ₂	5.85	85.83	TAI 6h TAI 24h
0.68	158.0445n	Allantoin (M+Cl, M+K-2H)	C ₄ H ₆ N ₄ O ₃	3.36	95.67	TA 2h TAI 6h TAI 24h
0.89	119.0354m/z	Dihydroxybutyric acid (M-H)	C ₄ H ₈ O ₄	3.72	95.57	TA 2h TAI 2h
1.18	242.0785m/z	Cytidine (M-H)	C ₉ H ₁₃ N ₃ O ₅	0.92	88.57	TA 2h TAI 6h TAI 24h
1.21	243.0633m/z	Uridine (M-H)	C ₉ H ₁₂ N ₂ O ₆	4.13	92.95	TAI 6h TAI 24h
1.62		Pyroglutamic acid	C ₅ H ₇ NO ₃			TAI 6h TAI 24h
2.02	180.0683m/z	Tyrosine (M-H)	C ₉ H ₁₁ NO ₃	9.10	96.12	TA 2h TAI 24h
5.62	182.0581n	Several , including Hydroxyphenyllactic acid, homovanillic acid (M-H, M-H ₂ O-H)	C ₉ H ₁₀ O ₄	1.16	95.55	TA 2h TAI 6h
6.44	144.0461	1H-indole-3-carboxaldehyde (M-H)	C ₉ H ₇ NO	4.33	97.71	TA 2h TAI 6h TAI 24h
7.32	317.0759m/z	Hypoxanthine (2M+FA-H) Aspartyl-tyrosine (M+Na-2H)	C ₁₂ H ₁₂ N ₂ O ₃	2.53 1.38	96.34 93.92	TA 2h
7.86	205.0747n	Indolelactic acid (M-H, M+Na+Na-2H, M-H ₂ O+H)	C ₁₁ H ₁₁ NO ₃	3.77	96.17	TA 2h TAI 6h TAI 24h
12.66	269.2497m/z	14-methylhexadecanoic acid (M-H)	C ₁₇ H ₃₄ O ₂	3.97	89.57	TA 2h TA 24h
13.40	283.2648m/z	Stearic acid (M-H)	C ₁₈ H ₃₆ O ₂	1.89	93.22	TA 2h TA & TAI 24h
18.67	528.5510m/z	N-Palmitoyl sphingosine Ceramide (M+FA-H)	C ₃₄ H ₆₇ NO ₃	1.81	95.08	TA 24h

4.3.1.4 Plasma- ESI+ Mode

The PCA model computed for plasma analysed using ESI+ mode is shown in Figure 4.3-7 (6 components, cumulative $R^2X= 0.708$, $Q^2=0.564$, 3704 compounds). QC samples are well clustered indicating the stability of the system was good. Controls from all time-points grouped together, with some separation between each time point.

The 2h TA and TAI samples are grouped together, indicating their relative similarity. The 6h TA and TAI samples are located between controls and 2h TA/TAI time-points. The 24h TA samples show close clustering to the 24h control samples, whereas TAI are all grouped together separately, indicating the difference in composition from the control/TA samples at 24h.

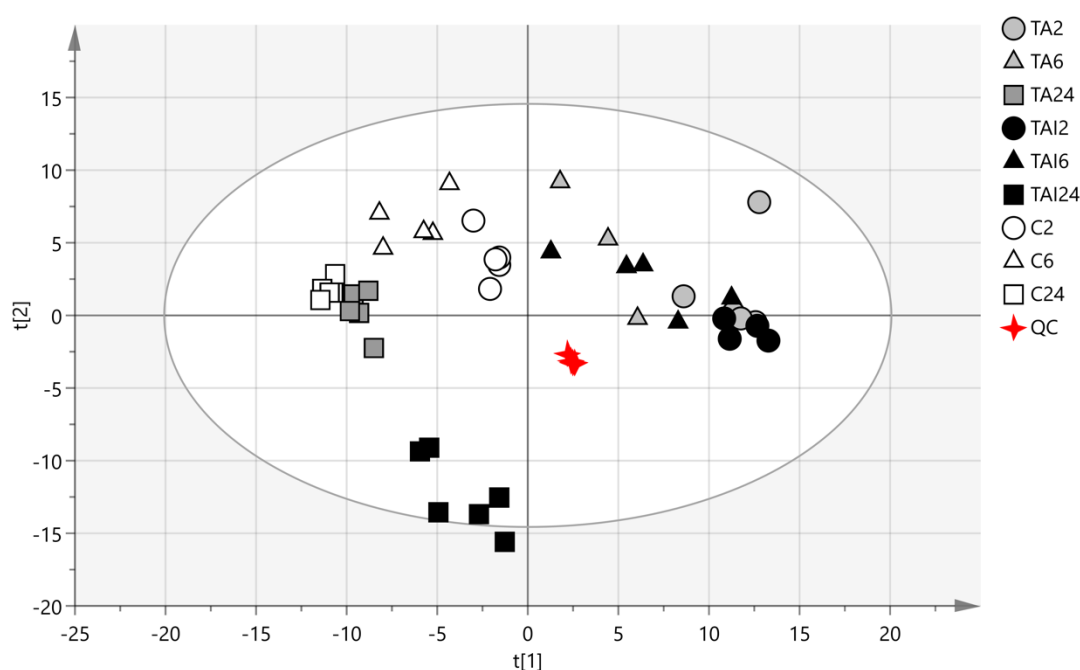


Figure 4.3-7 Scores plot from a PCA of ESI+ UPLC-MS analyses of plasma. 6 components, $R^2X(cum)=0.708$, $Q^2(cum)= 0.564$, $R^2XPC1= -0.305$, $R^2XPC2= 0.465$. The ellipse is Hotelling's T2 (95%). The key number refers to time-point (h), and drug treatment: TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples.

The 15 compounds selected from ESI+ plasma analyses are shown in Figure 4.3-8. Interestingly, 12.43_424.2840n (tetrahydroxycholanoic acid) was uniquely elevated in 24h TA samples. The most distinct, and unique changes observed in samples from TAI treated animals, were in 1.62_129.0437n (pyroglutamic acid or similar), 6.21_217.0997m/z (5-methoxytryptophan), 7.37_191.0602n (5-hydroxyindoleacetic acid), and 9.34_238.0865n (3,4,5-trimethoxycinnamic acid). Further details of the compound preliminary identifications can be found in Table 4.3-4. There was an elevation in both TA and TAI samples of 6.44_161.0488n (tryptophan metabolite,

Indole-3-carboxylic acid) and co-eluting compound, 6.44_189.0466n (tryptophan metabolite, kynurenic acid). These may be a fragment or adduct of one another as they closely correlate, however, it is unknown which is the parent compound, and presents an example of the difficulties involved in identifying metabolites based only on detected mass. There was a dramatic elevation in these compounds in TA samples at 2h, which returned to control levels by 6h whereas levels in TAI remained elevated, even increasing by 24h.

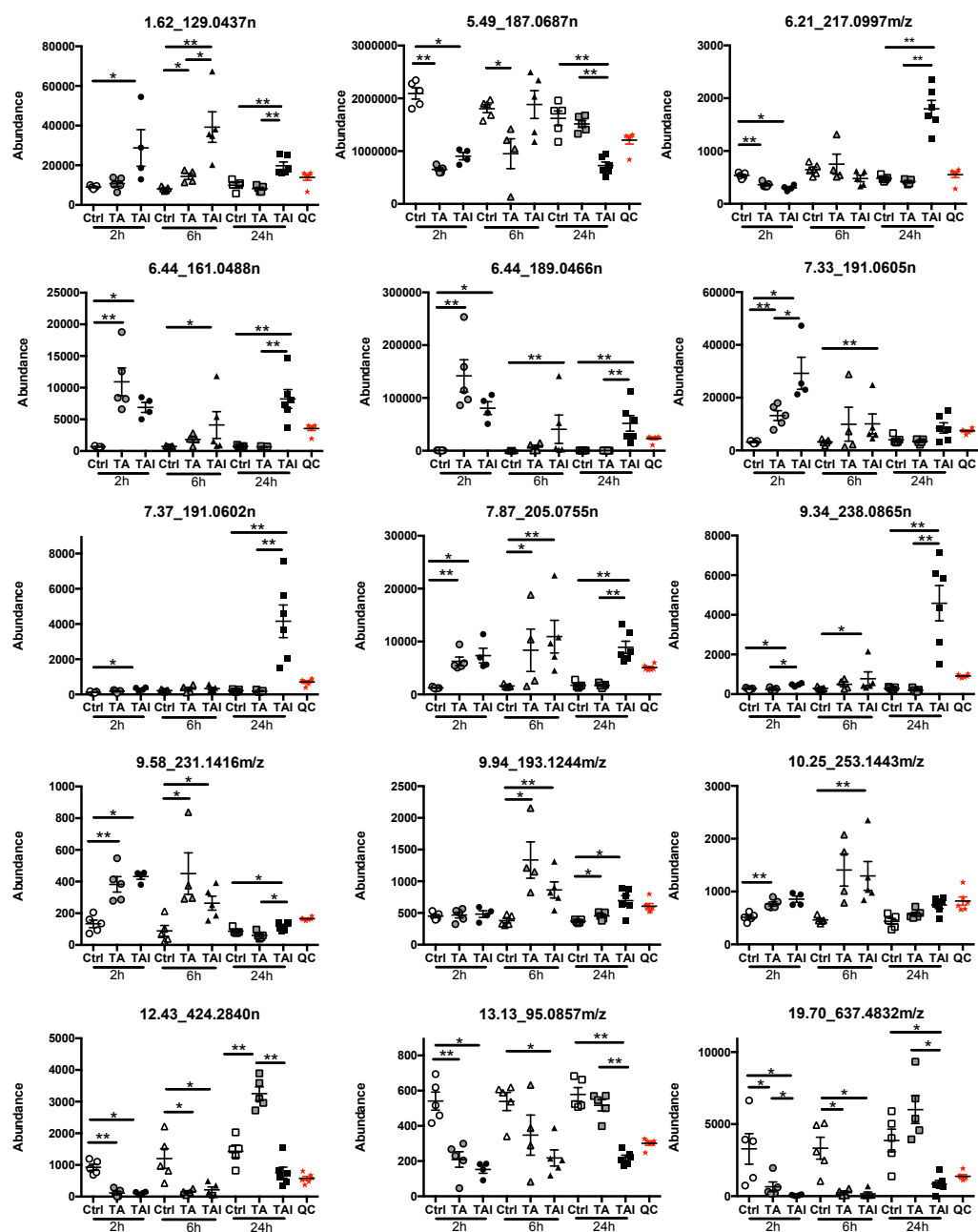


Figure 4.3-8 Selected compounds from plasma analysed by UPLS-MS in ESI+ mode. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compound names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$. Bars show the mean and error bars represent $\pm$ S.E.M.

Table 4.3-4 Details of selected compounds from ESI+ analyses of plasma

<i>Rt</i> (min)	Mass	Suggested identification(s)*	Chemical formula	Mass difference (ppm)	Isotope similarity (%)	Group(s) significantly altered [#] v control
1.62	129.0437n	Pyroglutamic acid (M+H, M+Na, M+K, 2M+K)	C ₅ H ₇ NO ₃	8.32	99.63	TAI 6h TAI 24h
5.49	187.0687n	Indoleacrylic acid (M+H, M+NH ₄ , M+H-H ₂ O)	C ₁₁ H ₉ NO ₂	28.65	99.20	TA 2h TAI 24h
6.21	217.0997m/z	5-Methoxytryptophan (M+H-H ₂ O)	C ₁₂ H ₁₄ N ₂ O ₃	11.08	94.95	TA 2h TAI 24h
6.44	161.0488n	Indole-3-carboxylic acid (M+H, M+H-H ₂ O)	C ₉ H ₇ NO ₂	6.73	94.89	TA 2h TAI 24h
6.44	189.0466n	Kynurenic acid (M+H, M+Na, M+H-H ₂ O)	C ₁₀ H ₇ NO ₃	21.04	96.19	TA & TAI 2h TAI 6h TAI 24h
7.33	191.0605n	5-hydroxyindoleacetic acid (M+H, M+Na, M+K, 2M+K)	C ₁₀ H ₉ NO ₃	11.69	99.36	TA 2h TAI 6h
7.37	191.0602n	5-hydroxyindoleacetic acid (M+Na, M+K)	C ₁₀ H ₉ NO ₃	10.20	96.98	TAI 24h
7.87	205.0755n	5-methoxyindoleacetate Indolelactic acid (M+ M+K, M+Na, M+H-H ₂ O)	C ₁₁ H ₁₁ NO ₃	7.64	93.44	TA 2h TAI 6h TAI 24h
9.34	238.0865n	3,4,5- trimethoxycinnamic acid (M+H-H ₂ O, M+Na)	C ₁₂ H ₁₄ O ₅	9.79	92.33	TAI 24h
9.58	231.1416m/z	Gamma-CEHC (M+H- H ₂ O)	C ₁₅ H ₂₀ O ₃	14.53	94.46	TA 2h
9.94	193.1244m/z	Traumatic acid (M+H- 2H ₂ O)	C ₁₂ H ₂₀ O ₄	8.99	90.83	TAI 6h
10.25	253.1443m/z	Dodecanedioic acid (M+Na) N-acetyl-b- glucosaminylamine (M+CH ₃ OH+H)	C ₁₂ H ₂₂ O ₄ C ₈ H ₁₆ N ₂ O ₅	14.13 22.13	91.81 94.98	TA 2h TAI 6h
12.43	424.2840n	Tetrahydroxycholanoic acid (M+H, M+H-H ₂ O, M+H-2H ₂ O)	C ₂₄ H ₄₀ O ₆	3.52	89.18	TA 2h TA 24h
13.13	95.0857m/z	Heptanoic acid (M+H- 2H ₂ O)	C ₇ H ₁₄ O ₂	1.66	94.05	TA 2h TAI 24h
19.70	637.4832m/z	DG (M+H)	C ₄₁ H ₆₄ O ₅	0.89	86.12	TAI 6h TAI 24h

*Databases searched including HMDB serum. # By Mann-Whitney test with p<0.01.

4.3.1.5 Urine ESI-

The PCA scores plot for urine profiles is shown in Figure 4.3-9 (7 components, R^2X 0.776, Q^2 0.633, 2648 compounds). The R^2X of PC1 was 0.459, and 0.0957 for PC2. Due to insufficient urine excretion in the control group between 0-2h, there were no control samples analysed from 0-2h collection. TAI and TA samples from 0-2h collection of urine cluster in a similar region of the plot, indicating their similarity, but the loose clustering indicates the wide variation within the groups. TA and TAI 2-6h are also clustered together, but separate from the control samples from 6h. By 24h both TA and TAI cluster in a similar region to the controls from both 6-24h and 2-6h.

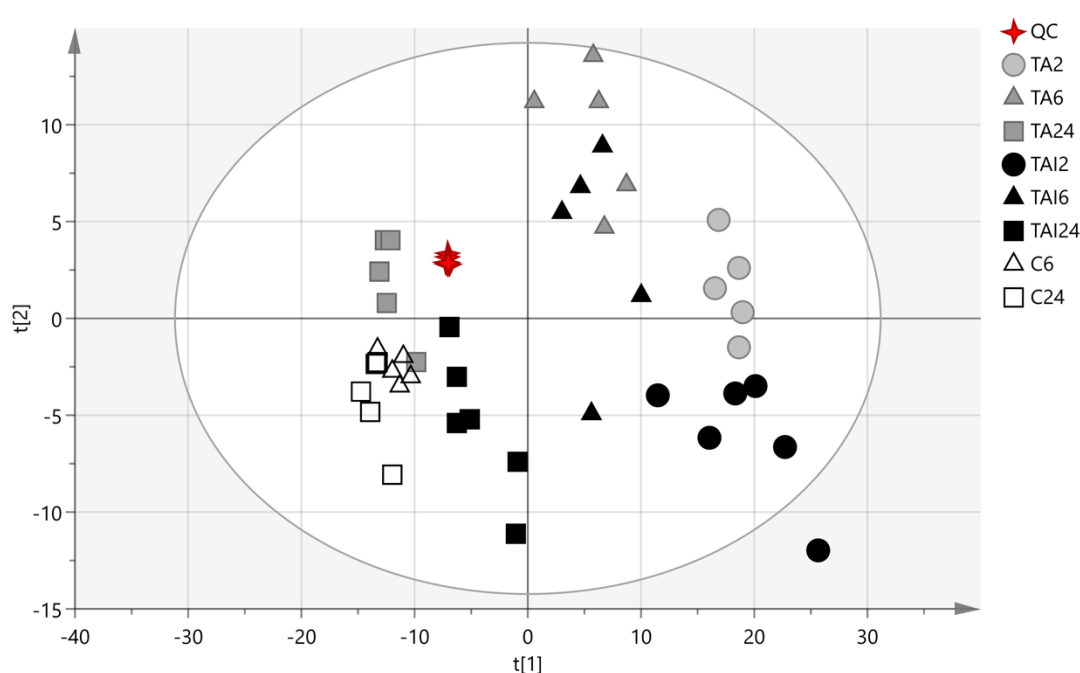


Figure 4.3-9 A PCA scores plot of ESI- UPLC-MS profiles of urine. 7 components, $R^2X(\text{cum}) = 0.776$, $Q^2(\text{cum}) = 0.633$, R^2X PC1 = 0.459, R^2X PC2 = 0.0957. The ellipse is Hotelling's T2 (95%). The key number refers to time-point (h), and drug treatment: TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples.

Selected compounds from ESI- analyses of urine are shown in Figure 4.3-10 and Bars show the mean and error bars represent $\pm$ S.E.M.

Table 4.3-5. At each time-point 2.73_84.04373 (amino-butyric acid/dimethylglycine) was found to be uniquely elevated compared to TAI. Additionally, 0.68_196.028m/z (tauropine or methylcysteine sulfoxide) and 3.49_145.0539m/z (indole-3-carboxaldehyde), although elevated in both TA and TAI samples, were altered to a greater extent in the TA samples.

From these selected compounds, a unique alteration found in the TAI samples was a dramatic elevation in 0.96_128.0363m/z (pyroglutamic acid) at the 6-24h collection.

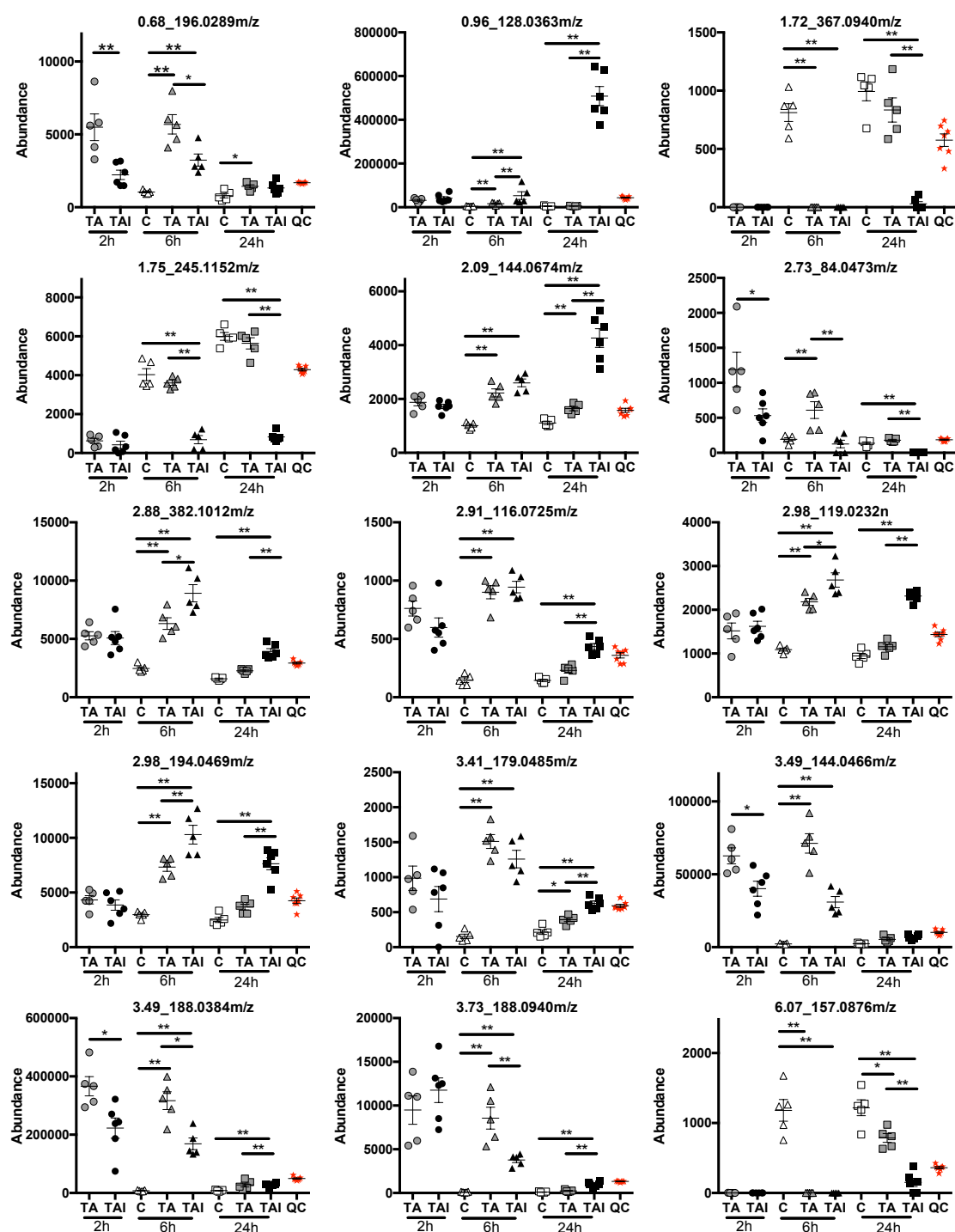


Figure 4.3-10 Selected compounds from ESI - UPLC-MS analysis of urine following TA or TAI treatment. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, *P< 0.05, **<0.01. Bars show the mean and error bars represent $\pm$ S.E.M.

Table 4.3-5 Suggested identifications for significant compounds found in urine analysed by ESI- UPLC-MS.

Rt (min)	Mass	Suggested identification(s)*	Chemical formula	Mass difference (ppm)	Isotope similarity (%)	Group(s) significantly [#] altered control	v
0.68	196.028m/z	Tauropine (M-H) Methylcysteine sulfoxide (M+FA-H)	C ₅ H ₁₁ NO ₅ S C ₄ H ₉ NO ₃ S	+2.10 +2.74	91.23 92.66	TA & TAI 6h	
0.96	128.0363m/z	Several including, pyroglutamic acid (M-H)	C ₅ H ₇ NO ₃	+7.65	93.69	TA & TAI 6h TAI 24h	
1.72	367.0940m/z	Several including indolecarboxylic acid (2M+FA-H)	C ₉ H ₇ NO ₂	+1.47	97.36	TA & TAI 6h TAI 24h	
1.75	245.1152m/z	Dipeptide (e.g. aspartyl- leucine M-H) ^a	C ₁₀ H ₁₈ N ₂ O ₅	+3.7	96.51	TAI 6h TAI 24h	
2.09	144.0674m/z	Allysine (M-H) Butyrylglycine (M-H)	C ₆ H ₁₁ NO ₃	+5.34 +5.34	96.71 96.71	TA & TAI 6h TA & TAI 24h	
2.73	84.0473m/z	Amino butyric acid, Dimethylglycine (M-H ₂ O-H)	C ₄ H ₉ NO ₂	+17.62 +17.62	95.71 95.71	TA 6h TAI 24h	
2.88	382.1012m/z	Succinyladenosine (M-H)	C ₁₄ H ₁₇ N ₅ O ₈	+1.92	94.26	TA & TAI 6h TAI 24h	
2.91	116.0725m/z	Many including valine, betaine (M-H)	C ₅ H ₁₁ NO ₂	+6.51	98.87	TA & TAI 6h TAI 24h	
2.98	119.0232n	Aminomalonic acid (M- H ₂ O-H, M+FA-H)	C ₃ H ₅ NO ₄	+11.60	95.44	TA & TAI 6h TAI 24h	
2.98	194.0469m/z	Alpha- hydroxyhippuric acid (M-H)	C ₉ H ₉ NO ₄	+5.46	97.50	TA & TAI 6h TAI 24h	
3.41	179.0485m/z	Nicotinuric acid (M-H)	C ₈ H ₈ N ₂ O ₃	+3.46	96.29	TA & TAI 6h TAI 24h	
3.49	145.0539n	Indole-3-carboxaldehyde (M-H, M+FA-H)	C ₉ H ₇ NO	+7.57	99.37	TA & TAI 6h TAI 24h	
3.49	188.0384m/z	Kynurenic acid (M-H)	C ₁₀ H ₇ NO ₃	+16.23	96.07	TA & TAI 6h TAI 24h	
3.73	188.0940m/z	Proline betaine (M+FA-H)	C ₇ H ₁₃ NO ₂	+8.13	91.86	TA & TAI 6h TAI 24h	
6.07	157.0876m/z	4- hydroxycyclohexylacetic acid (M-H)	C ₈ H ₁₄ O ₃	+3.52	94.11	TA & TAI 6h TAI 24h	

*Search based on HMDB urine metabolite database. # By Mann-Whitney test with $p < 0.01$. ^a no hits from urine database extended to HMDB.

4.3.1.6 Urine ESI+

The scores plot for the first two components of a PCA model of urine samples analysed in ESI+ mode is shown in Figure 4.3-12 (7 components, R^2X 0.740, Q^2 0.559, 1438 compounds). The R^2X of PC1 was 0.305, and 0.135 for PC2. The QCs clustered well, with one slight outlier, indicating further conditioning may have been optimal.

There were no 0-2h control samples analysed due to insufficient sample size. The 2-6h and 6-24h control group urine samples grouped closely together indicating little variation over time. The 0-2h and 2-6h TA and TAI samples group in the same region. The 2-6h and 6-24h control samples and the 6-24h TA samples group together. The TAI 6-24h samples remain distinct from both control/TA samples from the 6-24h collection.

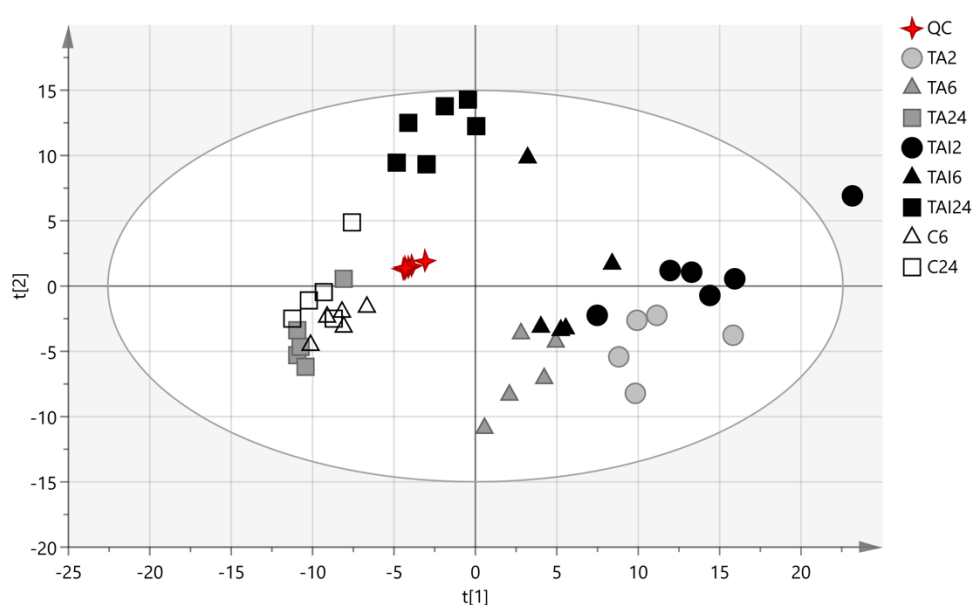


Figure 4.3-11 Scores plot from an unsupervised PCA of ESI+ UPLC-MS analyses of urine. 7 components, $R^2X(cum)$ = 0.740, $Q^2(cum)$ = 0.559, R^2X PC1 = 0.305, R^2X PC2 = 0.135. The ellipse is Hotelling's T^2 (95%). The key number refers to time-point (h), and drug treatment: TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples.

Selected compounds are presented in Figure 4.4-12 and Table 4.3-6. The only compound uniquely altered in the urine of TA treated animals was 3.06_120.0460m/z which was preliminarily identified as aminobenzoic acid, or could be a fragment of 3.06_195.0772 (aminohippuric acid). The majority of the selected compounds were unique elevations in TAI samples from 6-24h, these included: 0.51_162.1141m/z (carnitine), 0.57_70.0649m/z (4-aminobutyraldehyde), 0.99_130.0510m/z (pyroglutamic acid), 1.24_126.0650m/z (methylcytosine), and 1.41_181.0751n (tyrosine).

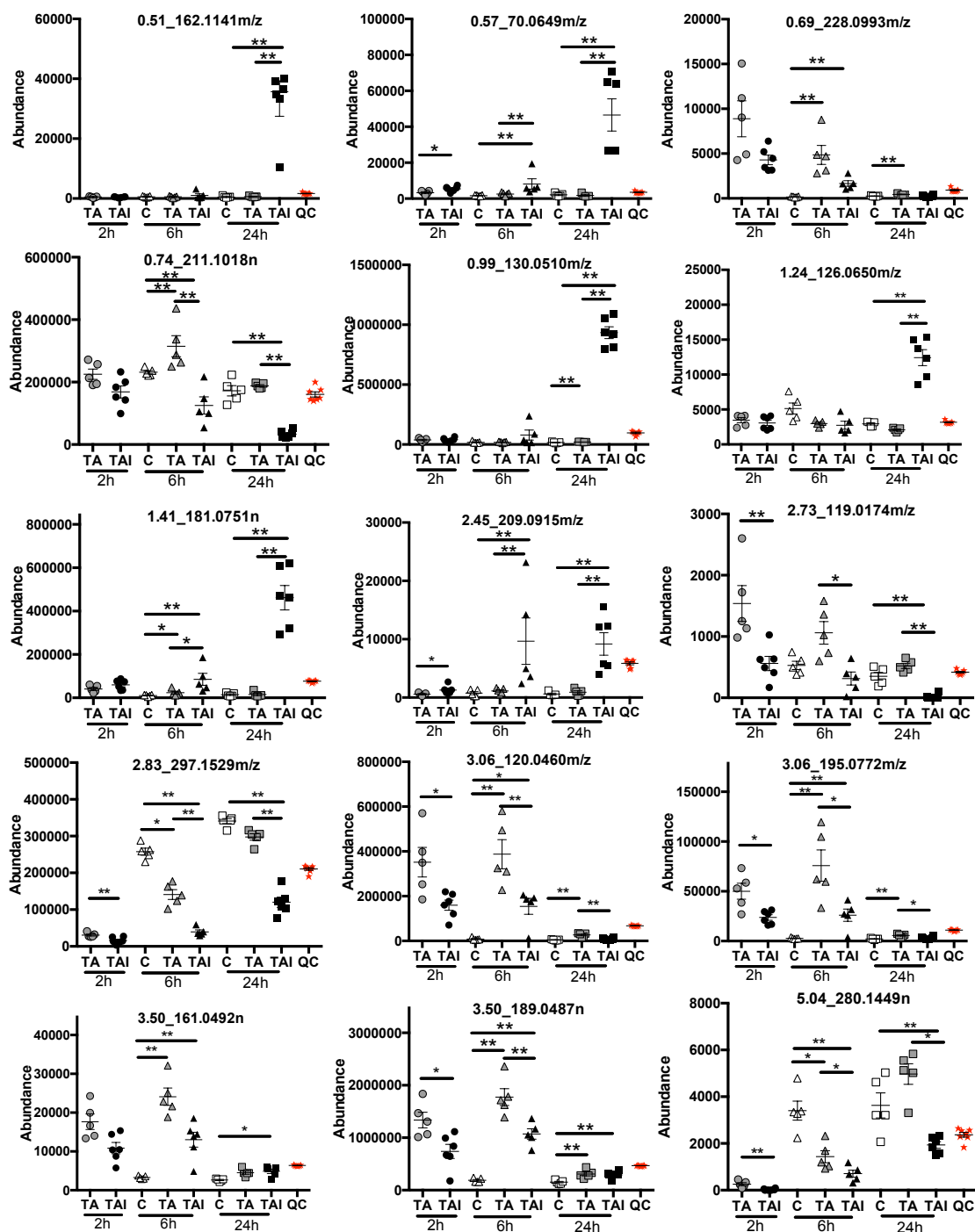


Figure 4.3-12 Selected compounds from ESI + UPLC-MS analysis of urine following TA or TAI treatment. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** < 0.01 . Bars show the mean and error bars represent $\pm$ S.E.M.

Table 4.3-6 Details of selected compounds from ESI+ analyses of urine

Rt (min)	Mass	Suggested identification(s)*	Chemical formula	Mass difference (ppm)	Isotope similarity (%)	Group(s) significantly altered [#] v control
0.51	162.1141m/z	Carnitine (M+H)	C ₇ H ₁₅ NO ₃	+9.92	99.69	TAI 24h
0.57	70.0649m/z	4-aminobutyraldehyde (M+H-H ₂ O)	C ₄ H ₉ NO	-2.72	98.58	TAI 6h TAI 24h
0.69	228.0993m/z	Deoxycytidine (M+H)	C ₉ H ₁₃ N ₃ O ₄	+6.05	88.79	TA & TAI 6h TA 24h
0.74	211.1018n	2-Methoxy-3-methyl- 9H-carbazole (M+H, M+Na, M+H-H ₂ O) 3-methoxytyrosine	C ₆ H ₁₁ N ₃ O	+10.04	95.12	TA & TAI 6h TAI 24h
0.99	130.0510m/z	Many including pyroglutamic acid (M+H)	C ₅ H ₇ NO ₃	+8.63	97.29	TAI 24h
1.24	126.0650m/z	Methylcytosine (M+H)	C ₅ H ₇ N ₃ O	-9.39	99.42	TA & TAI 24h
1.41	181.0751n 136.0768m/z 123.0458m/z	Tyrosine (M+H, M+Na)	C ₉ H ₁₁ NO ₃	+6.76	98.49	TAI 6h TAI 24h
2.45	209.0915m/z	Kynurenine (M+H)	C ₁₀ H ₁₂ N ₂ O ₃	-2.70	88.46	TAI 6h TAI 24h
2.73	119.0174m/z	S-methyl D- thioglycerate (M+H- H ₂ O)	C ₄ H ₈ O ₃ S	+9.37	93.20	TAI 24h
2.83	297.1529m/z	Mevalonic acid (2M+H)	C ₆ H ₁₂ O ₄	-4.88	90.98	TAI 6h TAI 24h
3.06	120.0460m/z	Several including, 2- aminobenzoic acid (M+H-H ₂ O)	C ₇ H ₇ NO ₂	+11.87	98.83	TA 6h TA 24h
3.06	195.0772m/z	4-Aminohippuric acid (M+H)	C ₉ H ₁₀ N ₂ O ₃	+4.09	67.49	TA & TAI 6h TA 24h
3.50	161.0492n	2-Indolecarboxylic acid/ Indole-3-carboxylic acid (M+H, M+H-H ₂ O)	C ₉ H ₇ NO ₂	+9.64	89.01	TA & TAI 6h TA 24h
3.50	189.0487n	Kynurenic acid (M+H, M+Na, M+H-H ₂ O)	C ₁₀ H ₇ NO ₃	+32.04	99.25	TA & TAI 6h TA & TAI 24h
5.04	280.1449n	Valyl-tyrosine ^a , Feruloyl-2- hydroxyputrescine (M+H, M+Na)	C ₁₄ H ₂₀ N ₂ O ₄	+9.18	96.18	TAI 6h

*Search based on HMDB urine metabolite database,⁹ no hits from urine database extended to HMDB 3.0. # By Mann-Whitney test with $p < 0.01$.

4.3.1.7 Overview of unique alterations from untargeted UPLC-MS analyses

Of the ninety metabolites selected as the most differentiating across the six analyses, several were uniquely altered in TA treated animals, these are summarised in Table 4.3-7. A far larger number of compounds were found that were uniquely altered in response to TAI, these are detailed in Table 4.3-8.

Table 4.3-7 Unique alterations detected in response to TA

Compound name	Suggested identification(s)	Tissue detected/ESI mode	Time-point
7.00_187.0640m/z	Indoleacrylic acid	Liver +	6h
0.61_193.0360m/z	Glucuronic acid (several others)	Liver -	2h, 6h
0.62_196.0580n	Galactonic acid, gluconic acid, gulonic acid	Liver -	2h, 6h
0.74_210.0363n	Galactaric acid, glucaric acid	Liver -	6h
9.58_231.1416m/z	Gamma-CEHC (vitamin E metabolite)	Plasma +	2h
12.43_424.2840n	Tetrahydroxycholanoic acid (bile acid)	Plasma +	24h
12.66_269.2497m/z	14-methylhexadecanoic acid	Plasma -	24h
13.40_283.2648m/z	Stearic acid	Plasma -	24h
18.67_528.5510m/z	N-Palmitoyl sphingosine (ceramide)	Plasma -	24h
0.74_211.1018n	2-Methoxy-3-methyl-9H-carbazole 3-methoxytyrosine	Urine +	6h
3.06_195.0772m/z	Aminobenzoic acid 4-Aminohippuric acid (acyl glycine)	Urine +	6h, 24h

Table 4.3-8 Unique alterations in response to TAI

Compound ID	Suggested identification	Tissue detected/ESI mode	Time-point
5.11_297.1460m/z	Tetradecanedioic acid, Alpha-Phenylacetyl-glutamine, Acetyl-formyl methoxykynurenamine	Liver +	24h
5.65_311.1610m/z	Pantethine , Estriol (or related)	Liver +	24h
6.14_217.0981m/z	5-Methoxytryptophan	Liver +	24h
6.71_251.0499m/z	Homocystine	Liver +	24h
6.91_193.0755n	Methylhippuric acid, Phenylacetyl-glycine (fatty acid metabolites)	Liver +	24h
0.71_145.0368n	2-keto-glutaramic acid	Liver -	24h
0.96_126.0196m/z	2-keto-glutaramic acid	Liver -	24h
6.84_192.0664m/z	Methylhippuric acid, Phenylacetyl-glycine (acyl glycines/ fatty acid metabolites)	Liver -	24h
21.25_690.6092m/z	n-(2r-hydroxytricosanoyl)-2s-amino-1,3s,4r-octadecanetriol (ceramide)	Liver -	24h
1.62_129.0437n	Pyroglutamic acid (& several others)	Plasma +	6h, 24h
6.21_217.0997m/z	5-Methoxytryptophan	Plasma +	24h
7.37_191.0602n	5-hydroxyindoleacetic acid (product of serotonin)	Plasma +	24h
9.34_238.0865n	3,4,5- trimethoxycinnamic acid	Plasma +	24h
1.21_243.0633m/z	Uridine	Plasma -	6h, 24h
1.62_128.0360m/z	Pyroglutamic acid (& several others)	Plasma -	24h
13.40_283.2648m/z	Stearic acid	Plasma -	24h
0.51_162.1141m/z	Carnitine	Urine +	24h
0.57_70.0649m/z	4-aminobutyraldehyde (metabolite putrescine)	Urine +	6h, 24h
0.74_2211.1018m/z	2-Methoxy-3-methyl-9H-carbazole, 3-methoxytyrosine	Urine +	6h, 24h
0.99_130.0510m/z	Pyroglutamic acid (& several others)	Urine +	24h
1.24_126.0650m/z	Methylcytosine	Urine +	24h
1.41_181.0751n	Tyrosine	Urine +	6h, 24h
2.45_209.0915m/z	Kynurenine	Urine +	6h, 24h
0.96_128.0363m/z	Pyroglutamic acid (& several others)	Urine -	6h, 24h

4.3.2 Targeted IPC-MS/MS assay

Numerous metabolites involved in important cellular processes, especially energy and amino acid metabolism, are very polar metabolites. Reversed-phase chromatography approaches, such as the method used in the first half of this Chapter, do not retain polar metabolites well, meaning they elute shortly after the solvent front and are more likely to suffer from ion-suppression, or be excluded from analysis. A common approach chosen to profile more polar metabolites by LC-MS is with the use of hydrophilic interaction chromatography (HILIC). However, as demonstrated in the first part of this Chapter, a major bottleneck or limitation of an untargeted UPLC-MS approach is the difficulty of accurately identifying differentiating metabolite. To avoid this problem, a recently developed targeted ion-pair chromatography (IPC)-MS/MS approach was chosen to enable the profiling of polar metabolites in this study. The assay enabled the simultaneous profiling of over one hundred polar metabolites, and had the additional benefit of including several metabolites provisionally identified using the untargeted approach (e.g. glucuronic acid, ophthalmic acid, pyroglutamic acid, kynurenic acid, uridine, serine), allowing confirmation or exclusion of those identifications, as well as others often perturbed in drug metabolism (e.g. oxidised and reduced glutathione).

4.3.2.1 Targeted IPC-MS/MS assay applied to liver extracts

A total of 85 metabolites were detected in liver extracts using the IPC method, of those, 61 met QC criteria and were included in further analyses. A scores plot of a PCA model of all samples and QCs (2 components, $R^2X=0.825$, $Q^2=0.267$, R^2X PC1=0.414, R^2X PC2=0.118) is shown in Figure 4.3-13. Samples from TA and TAI treated animals at 2h and 6h share a similar trajectory, distinct from control animals. By 24h TA samples grouped with control animals, whilst TAI 24h samples are the most distinct group.

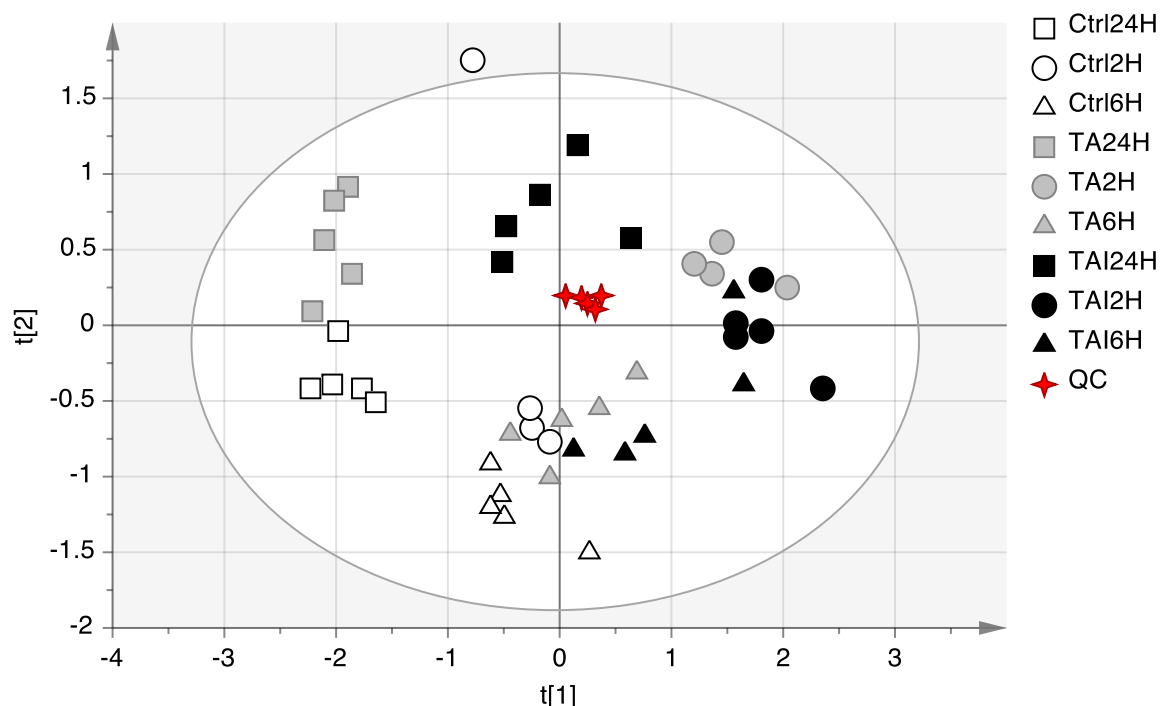


Figure 4.3-13 PCA scored plot of liver analysed by IPC-MS/MS. 2 components, $R^2X=0.825$, $Q^2=0.267$, R^2X PC1=0.414, R^2X PC2=0.118.

OPLS-DA models between TA and control, and TAI and control animals were generated for the 2h time-point. The s-plot from the 2h model is shown in Figure 4.3-14, where the most differentiating metabolites shown to be depleted in TA compared to controls where sorbitol/mannitol, GSSG, UDP glucose, glucose-6-phosphate, lactate and hydroxyglutaric acid. These were also statistically significant ($p<0.05$) by Mann-Whitney test, except GSH and glucose-6-P (Figure 4.3-19). Metabolites found to be most discriminating and elevated in TA group relative to controls included kynurenic acid, glucuronic acid, kynurenine, citrulline, proline and mannose-6-phosphate, these were all statistically significant by Mann-Whitney test at 2h.

The s-plot from 2h model between TAI and Ctrl is shown in Figure 4.3-15, the most differentiating metabolites higher in the control samples were the same as TA, with the addition of uridine monophosphate (these were all statistically significant by Mann-Whitney test). The most differentiating compounds elevated in the TAI samples were kynurenic acid, ophthalmic acid, quinolinic acid, cystine, creatine, glucuronic acid, proline, NAG, glutarate and tyrosine.

To visualize and explore the differences between TA and TAI, an SUS plot was generated, which plots the $p(\text{corr})$ of TA v C model against the $p(\text{corr})$ of TAI v C model (appendix). It showed the majority of metabolic changes were common between the two drugs. Interestingly, although not dominant in either OPLS-DA model, a depletion in tryptophan and uridine was found to be unique to the TA model. Both tryptophan and uridine were significant ($p < 0.05$) by Mann-Whitney test, whereas there was no significant depletion seen in the TAI treated animals at this time-point.

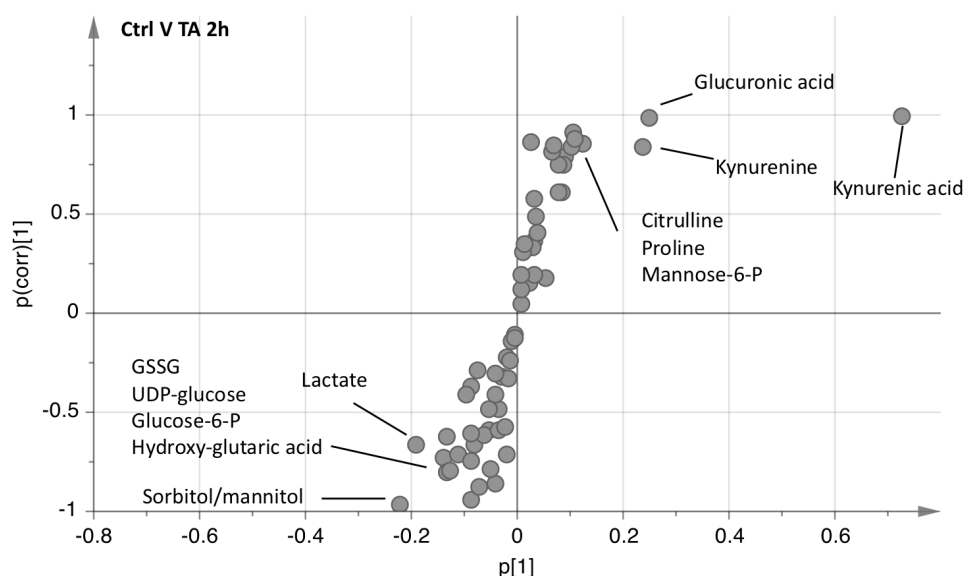


Figure 4.3-14 S-plot generated from OPLS-DA between TA ($n=5$) and control ($n=4$) liver samples collected at 2h post-dose. 1predictive+2 orthogonal component, R^2X 0.890, R^2Y 0.997, Q^2 0.989, predictive component R^2X 0.475, CV ANOVA $p=0.003$.

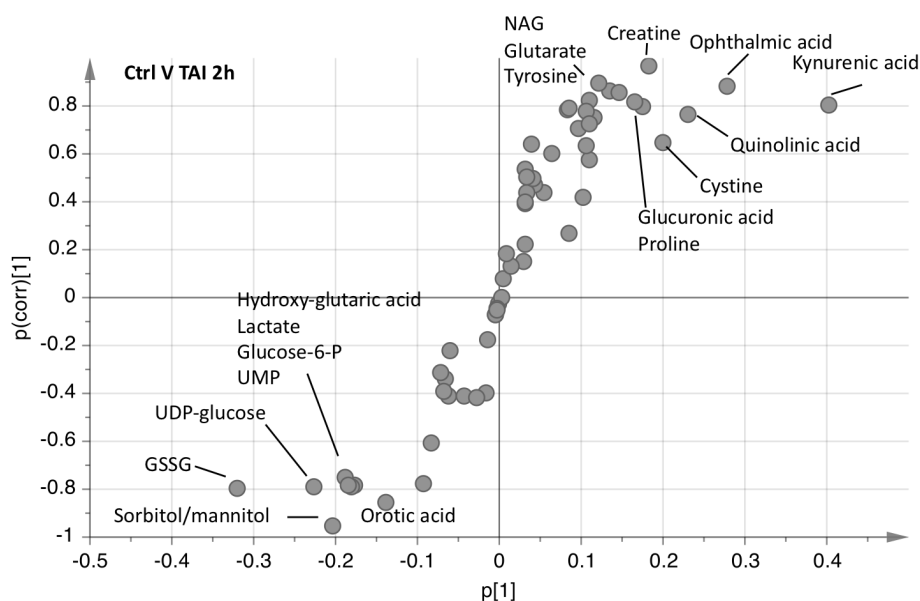


Figure 4.3-15 S-plot generated from OPLS-DA between TAI ($n=5$) and control ($n=4$) liver samples collected at 2h post-dose. 1predictive+3 orthogonal components, $R^2X=0.872$, $R^2Y=0.999$, $Q^2=0.987$, predictive component $R^2X=0.486$, CV ANOVA $p=0.04$.

The only metabolite correlated to controls/depleted in TA, in the TA v C model at 6h was cystine ($p < 0.01$ by Mann-Whitney test), whereas ophthalmic acid, glucuronic acid, creatine, UMP and GSSG were correlated to TA (all sig by Mann-Whitney test). In the 6h TAI and control model, the most differentiating compounds relating to control included lactate, sorbitol/mannitol and glucose-6-P, whilst ophthalmic acid, creatine, citrulline, creatinine and kynurenic acid and were most correlated to the TAI group.

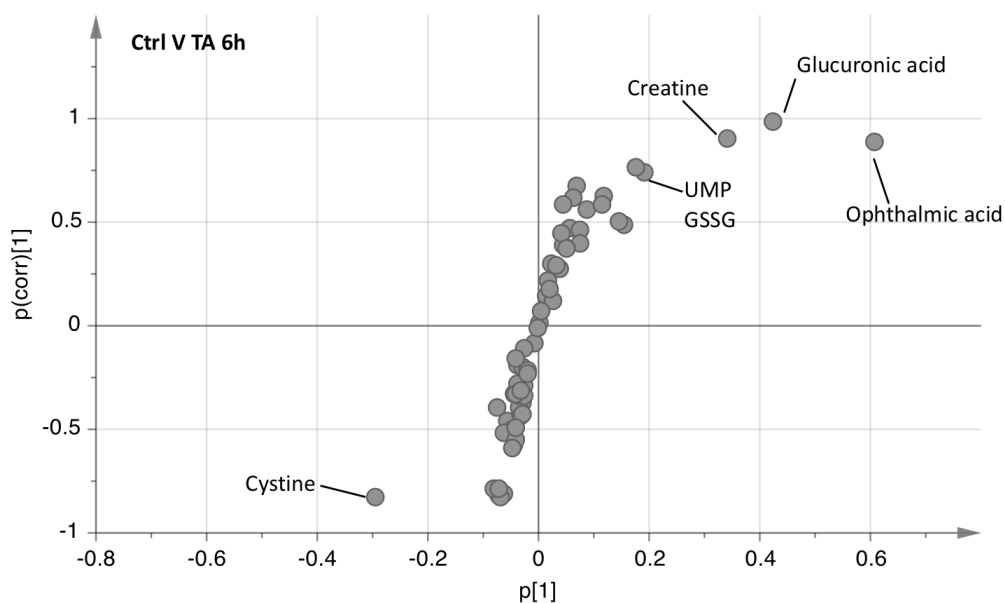


Figure 4.3-16 S-plot generated from OPLS-DA between TA ($n=5$) and control ($n=5$) liver samples collected at 6h post-dose. 1predictive+2 orthogonal component, R^2X 0.786, R^2Y 0.992, Q^2 0.96, predictive component R^2X 0.491, C.V. ANOVA 0.007.

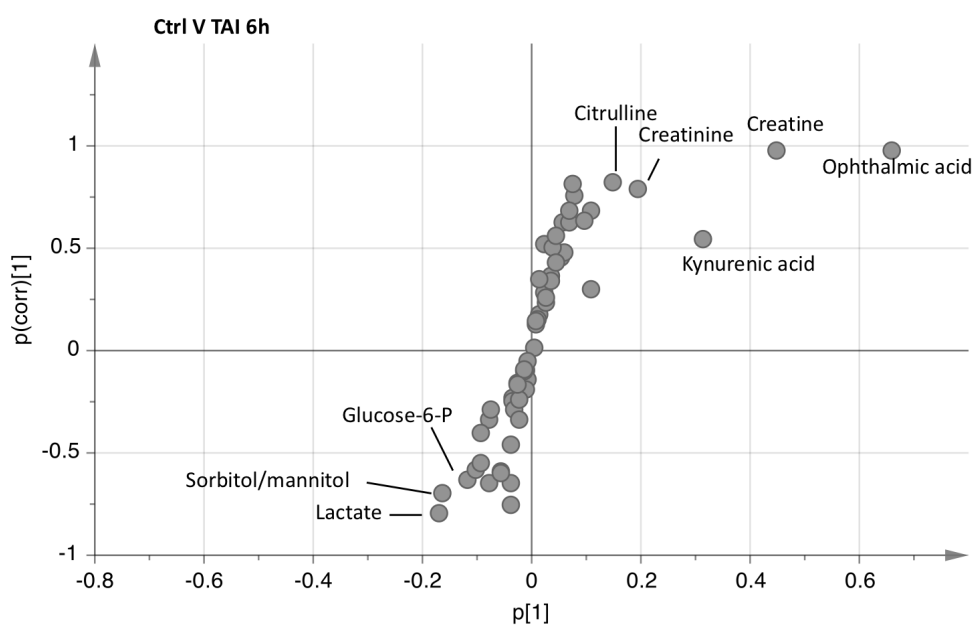


Figure 4.3-17 S-plot generated from OPLS-DA between TAI ($n=5$) and control ($n=5$) liver samples collected at 6h post-dose. 1predictive+3 orthogonal component, R^2X 0.851, R^2Y 1, Q^2 0.984, predictive component R^2X 0.473, CV ANOVA 0.008

An SUS plot generated between the 6h models shows a large number of compounds that are inversely correlated between the models, for example tryptophan which is depleted in the TA samples, but elevated in the TAI samples. Uridine is also inversely correlated between the models, however, it was elevated in TA samples and depleted in the TAI samples.

A valid model could not be generated between the control and TA liver samples from 24h time-point. In contrast, a strong and statistically valid OPLS-DA model was generated between TAI and control samples. At 24h glucose-1-phosphate, glucose-6-phosphate, glutamine, UMP, inosine, and AMP were correlated to control samples (depleted in TAI samples). Creatine, ophthalmic acid, proline, citrulline, serine, uracil, threonine, orotic acid, NAG, and glutamate were all elevated in the TAI group.

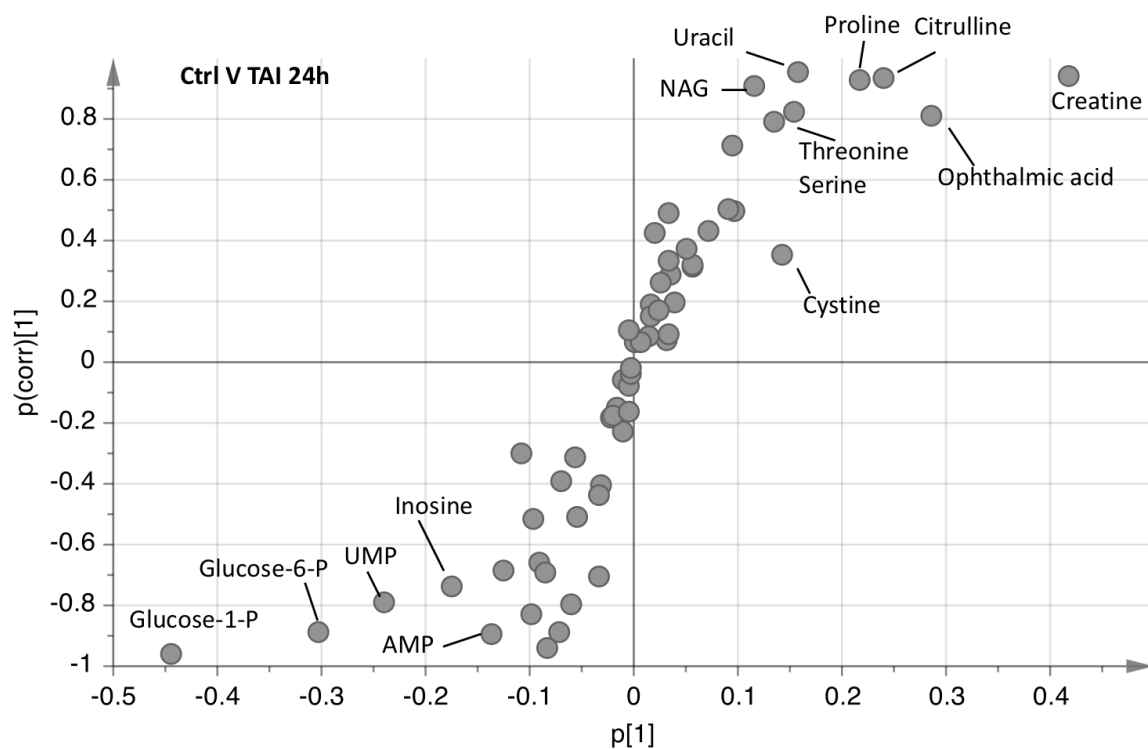


Figure 4.3-18 S-plot generated from OPLS-DA between TAI (n=5) and control (n=5) liver samples collected at 24h post-dose. 1predictive+1 orthogonal component, R^2X 0.730, R^2Y 0.963, Q^2 0.921, predictive component R^2X 0.49, C.V. ANOVA 0.006.

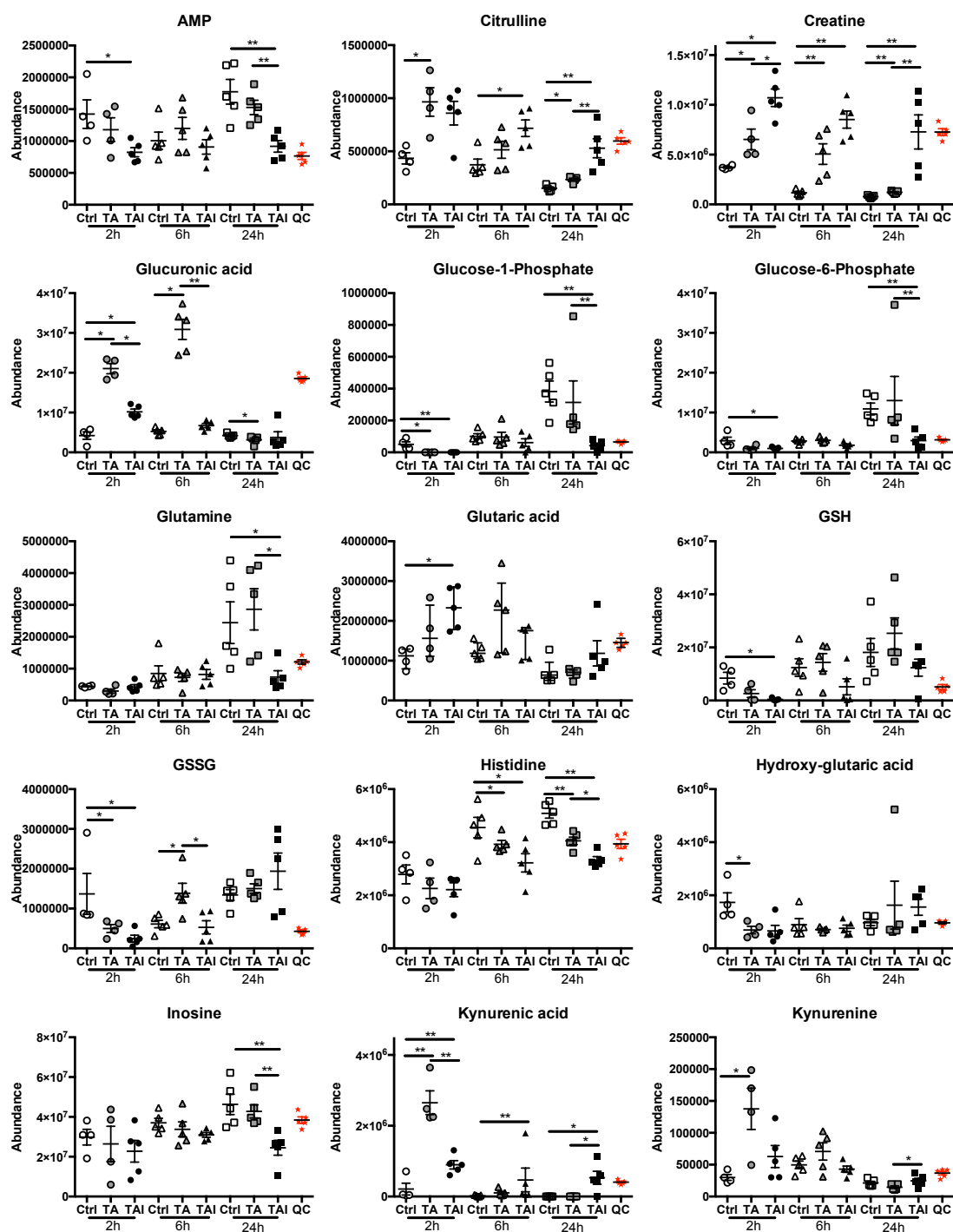


Figure 4.3-19 Compounds found to be differentiating in OPLS-DA models of ion-pair LC-MS analysis of aqueous liver extracts. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$. Bars show the mean and error bars represent $\pm$ S.E.M.

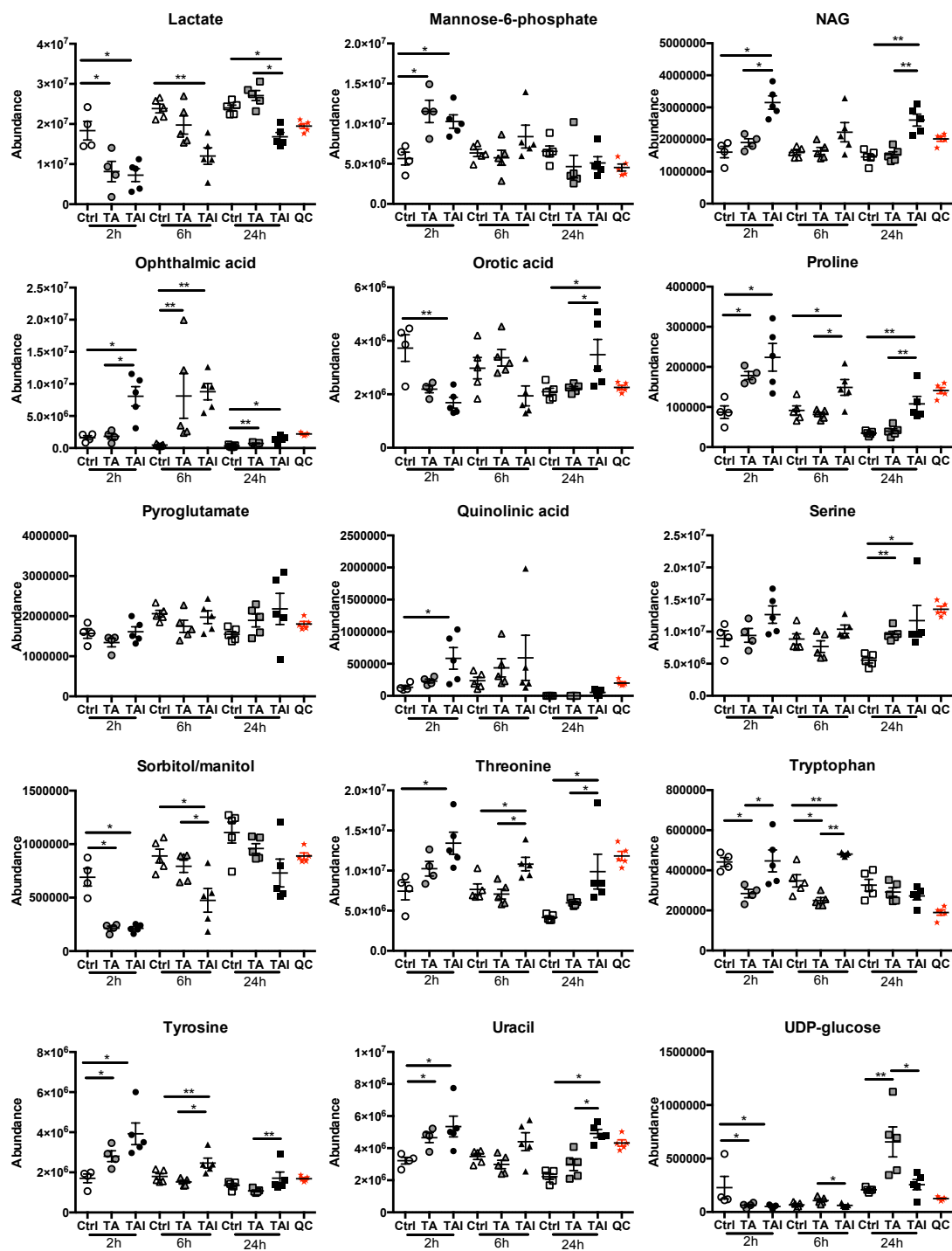


Figure 4.3-20 Compounds found to be differentiating in OPLS-DA models of ion-pair LC-MS analysis of aqueous liver extracts. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$. Bars show the mean and error bars represent $\pm$ S.E.M.

4.3.2.2 Targeted IPC-MS/MS assay applied to plasma

There were 42 metabolites detected in plasma that met QC standards. A PCA model was generated from all samples and QCs (5 components, $R^2X=0.855$, $Q^2=0.621$). The scores plot of PC1 and PC2 is shown in Figure 4.3-21. The control samples from all time-points cluster together TA/TAI samples from 2h and 6h share a similar trajectory, distinct from control animals. By 24h TA samples grouped with samples from control animals, in contrast TAI 24h remained separate.

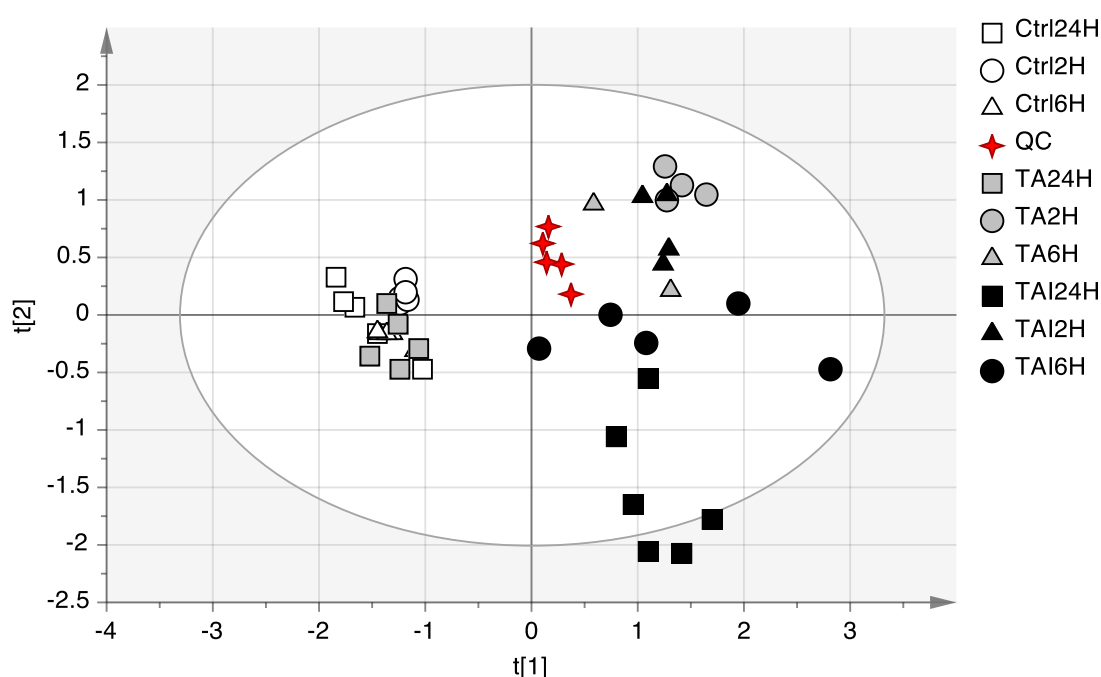


Figure 4.3-21 PCA scores plot from IPC-MS/MS of plasma. 5 components, R^2X 0.855, Q^2 0.621.

S-plots from OPLS-DA models between control and TA 2h samples, and control and TAI 2h samples are shown in Figure 4.3-22 and Figure 4.3-23. The most differentiating metabolites shown to be depleted in TA compared to controls were arginine, aspartate, glutamate, quinolinic acid and tryptophan. The most differentiating metabolites elevated in TA samples were found to be kynurenic acid, glucuronic acid followed by kynurenine, TIA, uracil, hydroxyl-phenyl-acetic acid and tyrosine. In the TAI s-plot the same metabolites were correlated to controls/depleted in TAI samples, with the addition of proline, and the absence of quinolinic acid correlation. Similar metabolites were also correlated to the TAI samples, however, glucuronic acid was altered to a lower magnitude and uracil, pyroglutamic acid and ophthalmic acid were also elevated in TAI samples.

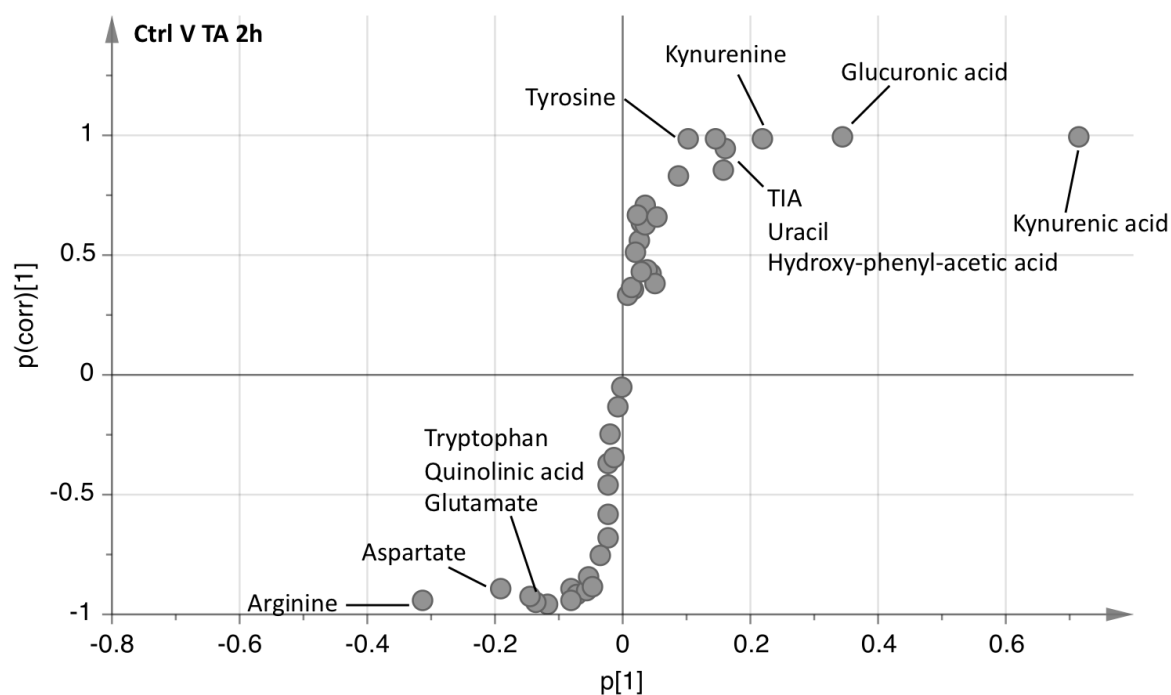


Figure 4.3-22 S-plot generated from OPLS-DA between TA (n=4) and control (n=5) plasma samples collected at 2h post-dose. 1predictive+0 orthogonal component, R^2X 0.890, R^2Y 0.998, Q^2 0.997, C.V. ANOVA $4e-8$.

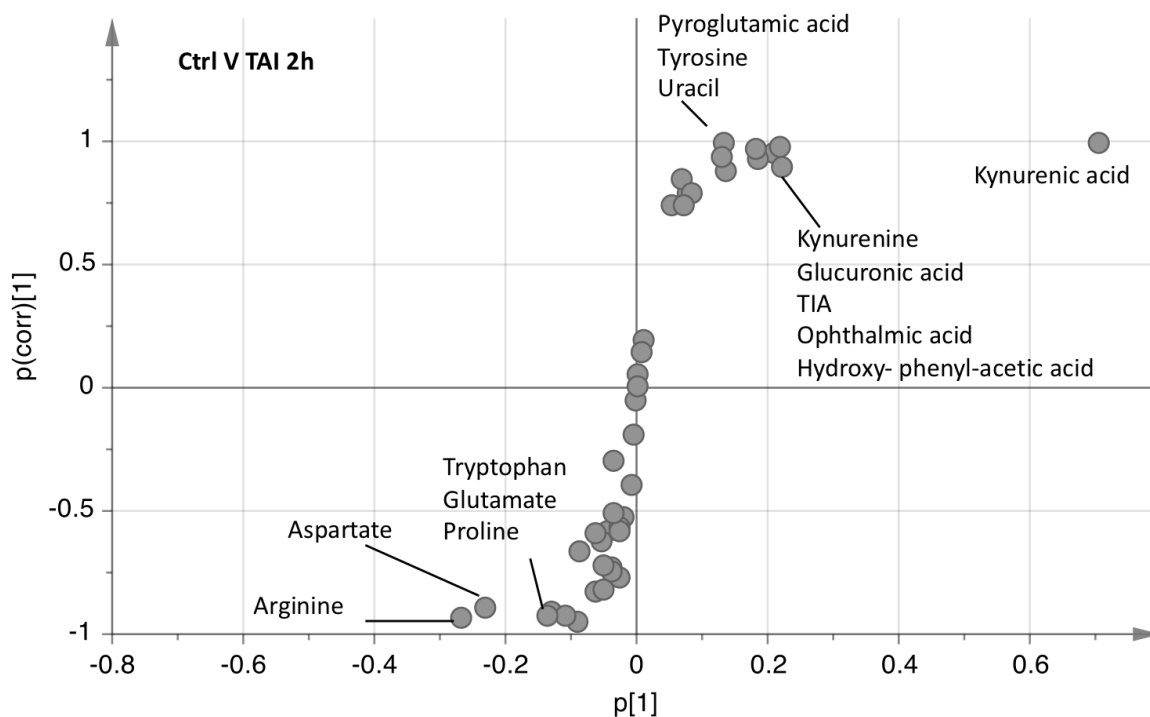


Figure 4.3-23 S-plot generated from OPLS-DA between TAI (n=4) and control (n=5) plasma samples collected at 2h post-dose. 1predictive+0 orthogonal component, R^2X 0.859, R^2Y 0.993, Q^2 0.99, ANOVA $1e-8$.

To visualize and explore the differences between TA and TAI, and SUS plot was generated, which plots the p(corr) of TA v C model against the p(corr) of TAI v C model. At this time-point the majority of responses were positively correlated, there were only weak negative correlations and no unique correlations between the models found.

There were insufficient TA samples (n=2) to calculate significance at 6h. The S-plot from the OPLS-DA model generated between TAI and control samples is shown in Figure 4.3-24. In TAI samples there were elevations in ophthalmic acid, kynurenic acid, creatine, pyroglutamic acid, uracil, glucuronic acid, hydroxyl-phenyl-acetic acid, glucuronic acid, kynurenine and alpha-ketobutyrate. There were also significant decreases in arginine, cystine, proline, and histidine.

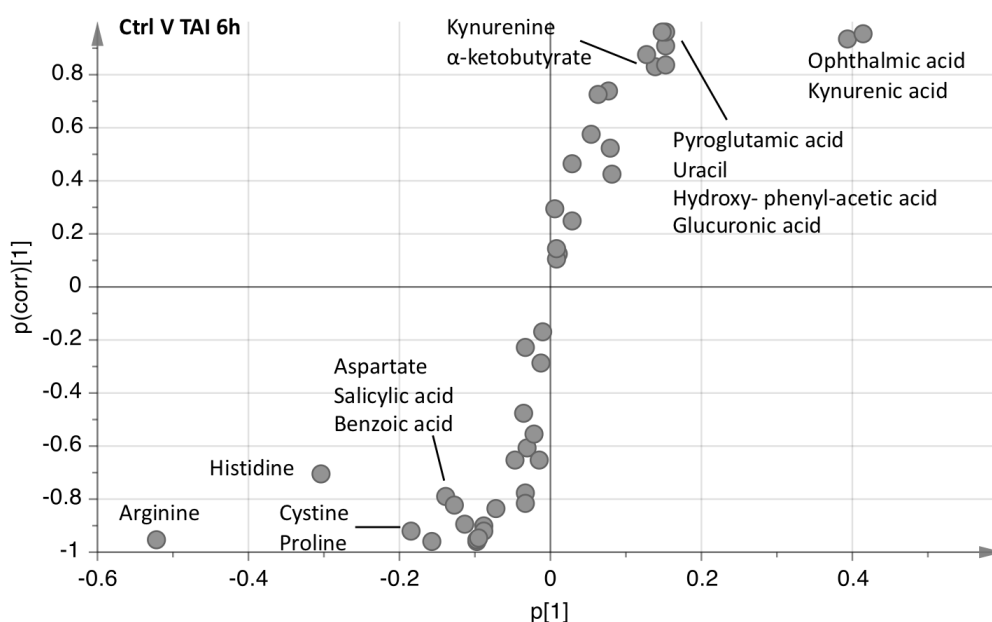


Figure 4.3-24 S-plot generated from OPLS-DA between TAI (n=5) and control (n=5) plasma samples collected at 6h post-dose. 1 predictive + 0 orthogonal component, R^2X 0.721, R^2Y 0.847, Q^2 0.83, ANOVA 0.003

A statistically valid OPLS-DA model between TA and control samples could not be generated at the 24h time-point. However, the S-plot from a strong and valid OPLS-DA model between the TAI and control samples is shown in Figure 4.3-25.

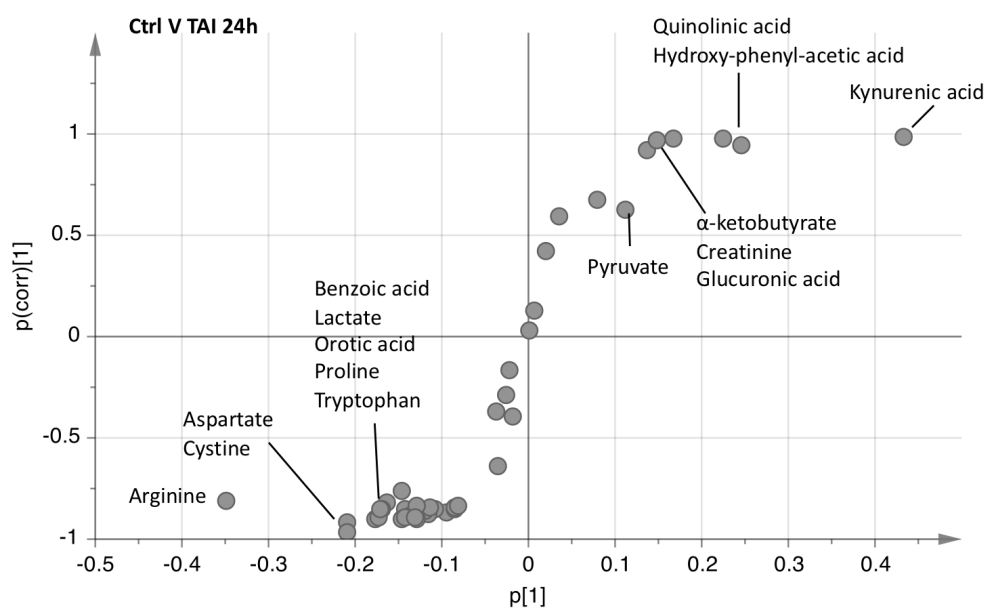


Figure 4.3-25 S-plot generated from OPLS-DA between TAI (n=6) and control (n=5) plasma samples collected at 24h post-dose1predictive+1 orthogonal component, R^2X 0.848, R^2Y 0.982, Q^2 0.939, ANOVA 0.0008

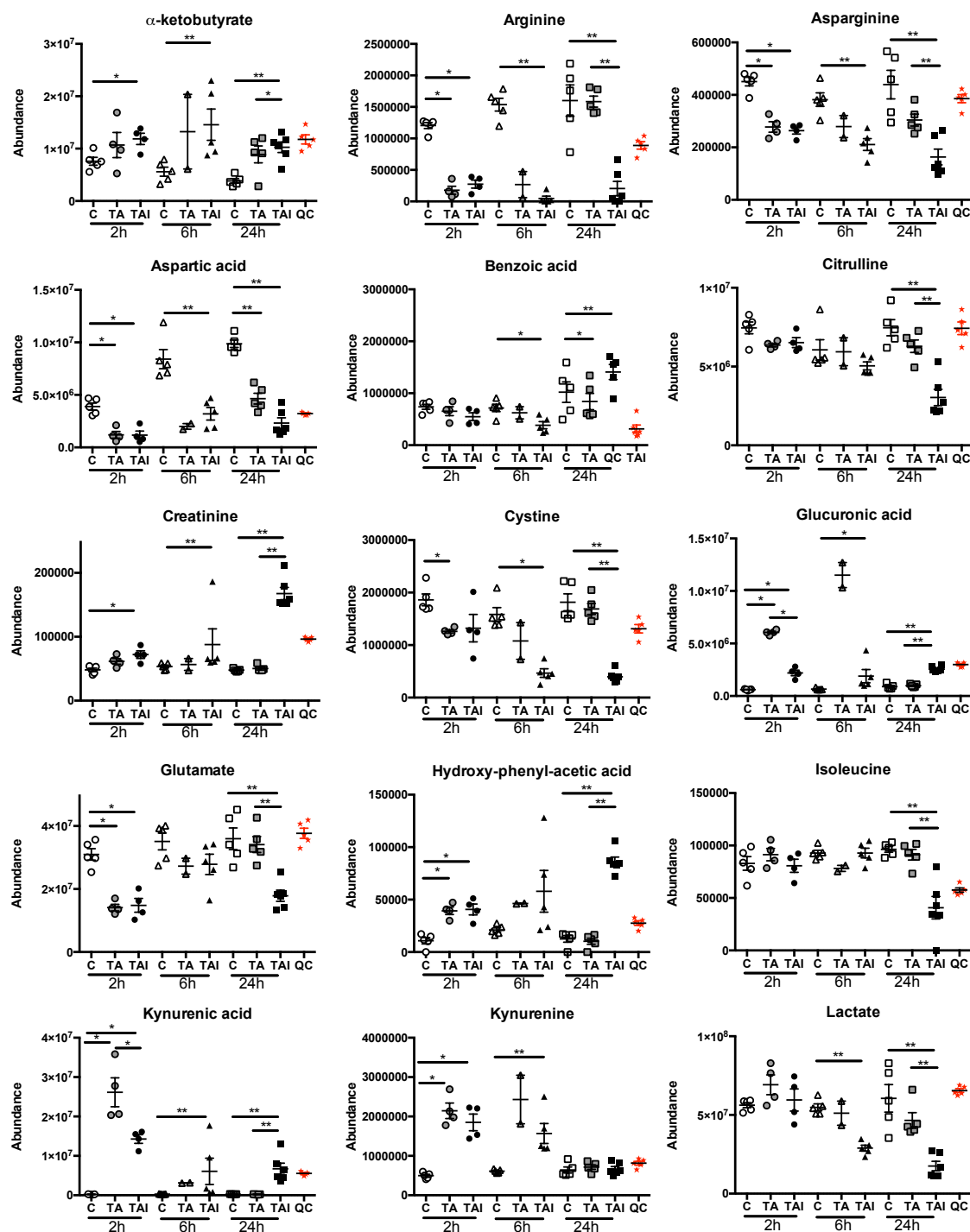


Figure 4.3-26 Selected significant compounds from ion-pair LC-MS analysis of plasma extracts following TA or TAI treatment. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$. Bars show the mean and error bars represent $\pm$ S.E.M.

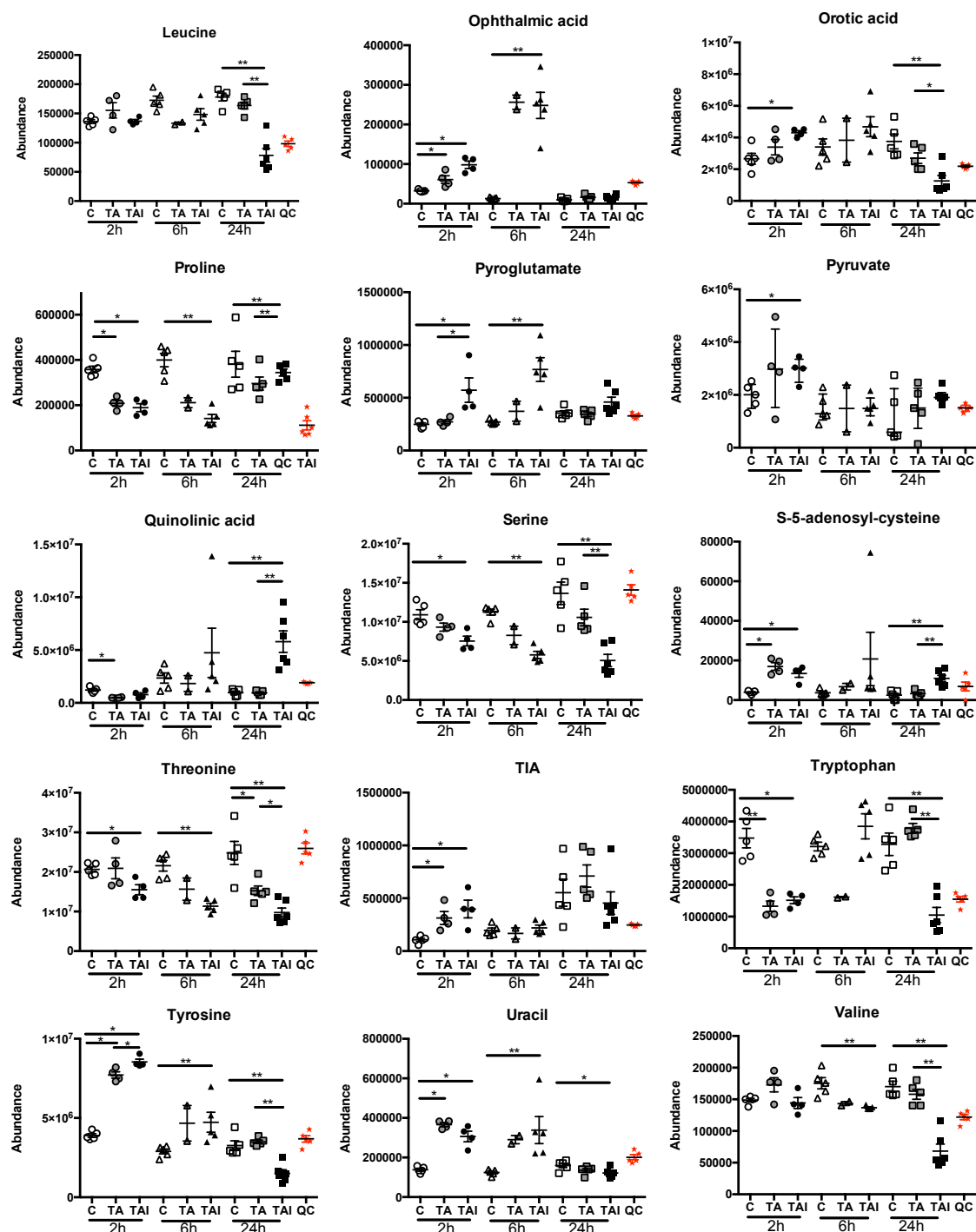


Figure 4.3-27 Selected significant compounds from ion-pair LC-MS analysis of plasma extracts following TA or TAI treatment. C- control/vehicle, TA- Tienilic Acid, TAI- Tienilic Acid Isomer, QC- quality control samples. Compounds names are retention time (min) and mass (either m/z or neutral). Significance was calculated using a Mann-Whitney test, * $P < 0.05$, ** $P < 0.01$. Bars show the mean and error bars represent $\pm$ S.E.M.

4.3.2.3 The ratio of kynurenine to tryptophan

The ratio of kynurenine to tryptophan in serum has previously reported to correlate to indoleamine 2,3-dioxygenase (IDO) activity: the enzyme that is involved in the metabolism of tryptophan (Yeung et al., 2015). To assess this relationship in the present study the peak area ratio of kynurenine and tryptophan was calculated from the IPC-MS/MS hepatic and plasma analyses (Figure 4.3-28).

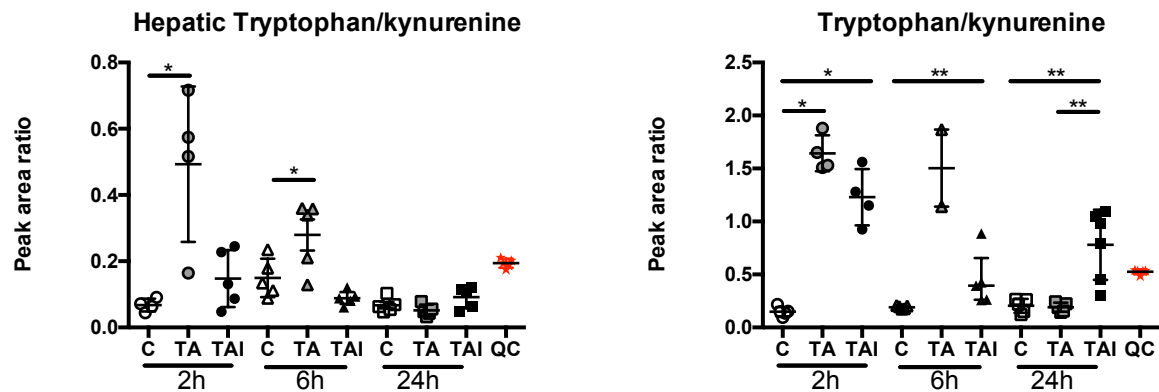


Figure 4.3-28 The ratio of plasma kynurenine and tryptophan abundance (peak area from IPC-MS/MS analyses). Statistical significance was calculated using Mann Whitney test, * $p > 0.05$, ** $p > 0.01$. Dots are the individual animals, bars show the mean and error bars represent $\pm$ S.E.M.

4.3.2.4 Plasma ophthalmic acid correlation with hepatic GSH

The correlation between plasma ophthalmic acid and hepatic GSH was assessed by calculating the Spearman's correlation coefficient (Figure 4.3-29). There was a weak but significant ($r = -0.4670$, $p = 0.00027$) correlation between plasma ophthalmic acid and hepatic glutathione detected in this study.

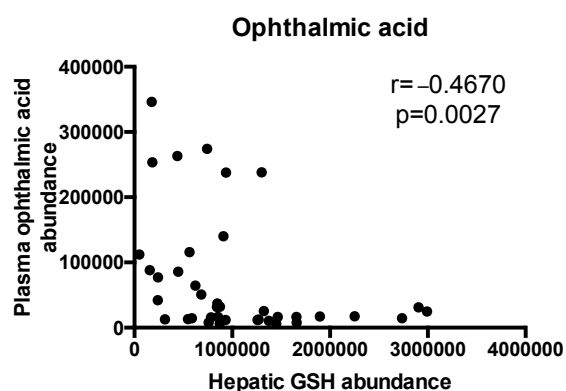


Figure 4.3-29 The correlation of plasma ophthalmic acid with hepatic glutathione. Spearman's rank correlation coefficient is " r ", and " p " indicates statistical significance.

4.4 DISCUSSION

There is a pressing need to broaden our understanding of DILI, and find new and more sensitive biomarkers of DILI in preclinical animal models which translate to clinical studies. The aim of the present study was to compare the metabolic impact of the idiosyncratic hepatotoxin Tienilic acid (TA) to the intrinsic hepatotoxin Tienilic Acid Isomer (TAI).

4.4.1 Summary of untargeted data

Overall, the metabolic alterations captured using this untargeted UPLC-MS approach were similar between TA and TAI dosing at 2h in liver, plasma and urine. This was seen by the similar clustering of TA and TAI 2h samples in the majority of PCA scores plots. By the latest time-point, 24h, in the majority of data sets TA had regrouped with controls indicating a return to homeostasis. In contrast, TAI samples from 24h were usually distinct from the TA and control group samples, indicative of unrecovered metabolic perturbations/phenotype. This corresponds well with the evidence of toxicity found in clinical chemistry and histopathology analyses of TAI treated animals (Chapter 3).

This was also observed in the majority of the metabolites selected as most significant (determined by t-test and fold change) and promising (had a preliminary database match, good peak shape etc.), showed a similar response in both TA and TAI compared to controls. This supports the observation from the PCA that TA/TAI at 2h shared 'multivariate space' and overall they were metabolically similar. This could be a result of their shared pharmacology, as uricosuric diuretics, or resulting from a similar initial stress response due to metabolising the drug, which was ultimately reversible in TA, but not in TAI. Interestingly, there were also several unique metabolic responses to both TA and TAI treatment, and as any metabolic changes related to the common pharmacological action should be observed in response to both, these unique differences may be more likely to correlate to their differing mechanisms of drug metabolism and toxicity.

Clustering of control groups was less well defined in the liver, with greater variation found in the controls over the different time-points. These changes may relate to the impact of withdrawing food prior to the study commencing, which was subsequently returned after dosing allowing for the replenishment of some metabolites. Similar time-point (food) related metabolic changes were previously observed by histopathology scoring of hepatic glycogen,

and in hepatic betaine determined by NMR in these animals (Coen et al., 2012). Hepatic glycogen and betaine were found to be depleted in all groups at the start of the study, and were replenished in the control and TA groups, but not the TAI group, by 24h. Although the impact of diet related metabolism may be mechanistically interesting, or important, these are less likely to be suitable biomarkers given their variability depending on food intake. To avoid focusing identification efforts on these compounds in the untargeted data presented in this Chapter, the compounds of interest were preferentially selected based on their stability in controls over time.

Several of these TA specific changes were preliminary identified as relating to tryptophan metabolites, for example in the 2h liver samples compounds suspected to be indole-3-carboxylic acid and 1H-indole-3-carboxaldehyde were uniquely elevated. These were elevated in TAI treated animals only to a lesser degree and not until 24h. Both of these were also in the top fifteen most significant compounds selected in urine, where they were detected at a greater intensity in TA treated animals, compared to TAI.

Some of the other most striking and unique alterations were observed in ESI- analysis of the liver, where compounds provisionally identified as glucuronic acid (oxidation product of glucose and major metabolite for 'phase 2' conjugation reactions of drug metabolites) and galactonic acid (oxidation of galactose) were elevated, and a depletion in the compound provisionally identified as gluconic acid (oxidation of glucose). All of these changes were resolved by 24h, indicating they were transient alterations. Interestingly, there were four lasting metabolic changes observed in plasma, that were also unique to TA. These included a metabolite preliminary identified as tetrahydroxycholeic acid (a bile acid), 14-methylhexadecanoic acid (a fatty acid), stearic acid (a saturated fatty acid) and N-palmitoyl sphingosine (a ceramide).

Although it is not possible to make firm biological interpretations prior to the confirmation of the metabolite identifications, which have so far only been tentatively assigned based on their m/z, guide choice of subsequent targeted assay. These preliminary identifications could be further confirmed/ excluded by analysing commercially available standards to compare the retention times, and MS/MS fragmentation. Additionally, the unique and lasting lipid changes suggest that further exploration of the impact of TA on lipids may be of interest. Although not explored in this thesis an UPLC-MS lipidomic analysis was performed on liver extracts and also

indicates numerous lasting TA specific changes (data not shown).

4.4.2 A targeted approach

One of the key limitations of this untargeted LC-MS approach was the lack of a streamlined route for metabolite identification. An alternative approach for LC-MS based metabonomics is the use of more targeted assays for a select number of metabolites/metabolic pathways. In the second half of this Chapter a targeted assay was applied to re-analyse the liver and plasma samples from this study using ion-pair LC-MS. An advantage of this assay was that it included several metabolites involved in tryptophan metabolism (a metabolic pathway implicated in the untargeted analyses). The assay also included many compounds linked to energy and glutathione metabolism; which are often altered in response to xenobiotic challenge, and again several of these were preliminarily identified from the untargeted approach e.g. ophthalmic acid, pyroglutamic acid.

4.4.2.1 TA and TAI impact tryptophan metabolism in different ways

Several tryptophan metabolites were found to be altered in response to both TA and TAI using the IPC-MS/MS method. This complements the provisional identification of several of these metabolites, and others linked to tryptophan metabolism, that were revealed using the untargeted approach. Tryptophan is an essential amino acid required for protein synthesis, and is the precursor for the neurotransmitters serotonin and melatonin. However, the vast majority (95%) of free tryptophan is metabolised down the kynurenine pathway (Yeung et al., 2015).

Tryptophan is converted to kynurenine by hepatic tryptophan 2,3-dioxygenase (TDO) and the more widely expressed indoleamine 2,3- dioxygenase (IDO)(Yeung et al., 2015). As TDO is predominantly located in the liver and is responsible for basal tryptophan metabolism, the ratio of kynurenine to tryptophan is reported to be a marker of IDO upregulation. IDO can be upregulated in response to inflammatory cytokines (Wirthgen and Hoeflich, 2015). Kynurenine can itself interact with receptors such as aryl hydrocarbon receptor (AHR) to modulate cell function (Wang et al., 2010, Opitz et al., 2011), or it be further metabolised, predominantly to quinolinic acid or kynurenic acid. Quinolinic acid is a precursor of the important co-factor nicotinamide dinucleotide (NAD), and kynurenic acid acts as an immune suppressant (Moffett and Namboodiri, 2003).

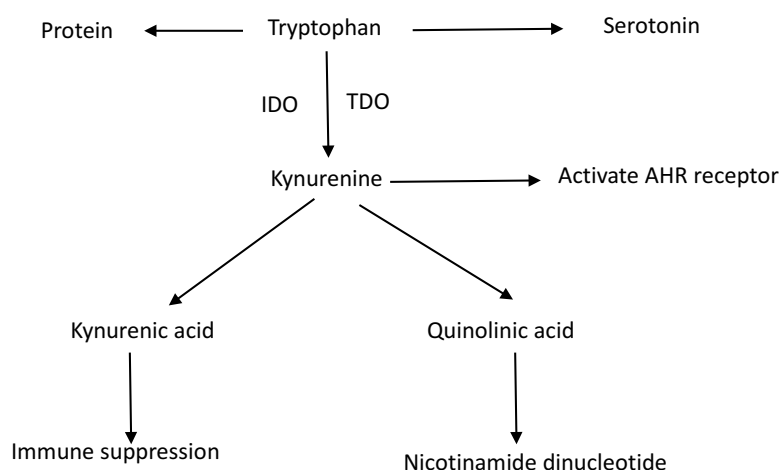


Figure 4.4-1 Key tryptophan metabolism pathways. AHR- aryl hydrocarbon receptor TDO- tryptophan 2,3-IDO- indoleamine 2,3- dioxygenase.

In the present study, plasma tryptophan was depleted, whilst kynurenine was elevated in response to both TA and TAI. The ratio of kynurenine to tryptophan was elevated to a similar extent in the animals treated with TA and TAI. In contrast, hepatic tryptophan was only depleted in response to TA not TAI, so although kynurenine was elevated to a similar extent between the treatment groups, the ratio of kynurenine to tryptophan was uniquely elevated in the liver of TA not TAI animals. In both plasma and liver, kynurenic acid was elevated to a greater degree in response to TA, whilst hepatic and plasma quinolinic acid was uniquely upregulated in response to TAI. Overall, both TA and TAI alter tryptophan metabolism, TA seems to promote greater kynurenic acid production, whilst TAI produces a greater amount of quinolinic acid. Further, more mechanistic, work would be required to determine the importance of kynurenine and its metabolites in response to toxicity.

The differential modulation of tryptophan metabolism could be indicative of the differing degrees of cell stress induced by TA and TAI; a less severe stress promoting immune tolerance, whereas a more severe stress requiring more NAD. Interestingly, decreased tryptophan and elevated kynurenic acid have previously been reported in a study looking for shared biomarkers of hepatotoxicity in rats dosed with one of eight model hepatotoxins (alpha-Naphthylisothiocyanate, Amineptine, Cyclosporine A, Erythromycin, Glibenclamide, Methylene Dianiline, Phalloidin or Tetracycline) (Buness et al., 2014). In addition, kynurenine and its metabolites have been studied for their role of tumour immune evasion in cancer, and have been associated with numerous neurological disorders, and in maternal foetal tolerance (Yeung et al., 2015).

4.4.2.2 TA and TAI both impact uronic acid pathway

Both TA and TAI treated animals had depletion of metabolites in the uronic acid pathway; involved in the production of glucuronic acid, which was significantly elevated in plasma of both TA and TAI. These included significant depletions in glucose-6-phosphate, glucose-1-phosphate and UDP-glucose, which are the precursors of UDP-glucuronate (not detected), involved in glucuronide formation. This is likely to be adaptive/protective response to increase the excretion of xenobiotics. Interestingly, there was a significantly greater elevation in hepatic and plasma glucuronic acid detected in TA compared to TAI samples, and this complements the TA-glucuronic acids reported in Chapter 3, that were not observed in response to TAI.

4.4.2.3 TAI dosing led a greater perturbation in energy metabolism than TA

Hepatic glucose-6-phosphate, and sorbitol/mannitol were depleted to a greater extent and for longer in TAI samples compared to TA. This complements the more severe depletion of glucose and glycogen detected using NMR (Coen et al., 2012), and is indicative of greater energy burden created by TAI. Depleted lactate, and disturbance in amino acid metabolism could be linked to disturbance in this energy metabolism. NMR analyses also detected elevations in 3-hydroxybutyrate, indicative of a compensatory shift to lipolysis, the fatty acids detected in untargeted UPLC-MS could be indicative of lipolysis. The majority of these amino acid and energy related changes were resolved by 24h in TA plasma and liver samples, however by 24h dramatic changes in amino acid metabolism was still evident in TAI samples indicating of toxic/unrecovered phenotype.

4.4.2.4 TA and TAI alter metabolites indicative of oxidative stress

In both TA and TAI, the abundance of GSSG (and GSH, excluded for analysis as QCs were not stable) was depleted in the liver. This has previously been seen by NMR in these animals (Coen et al., 2012), and in other studies of TA in the rat (Nishya et al., 2008). In addition, two metabolites previously linked to oxidative stress were found to be elevated; ophthalmic acid and pyroglutamic acid.

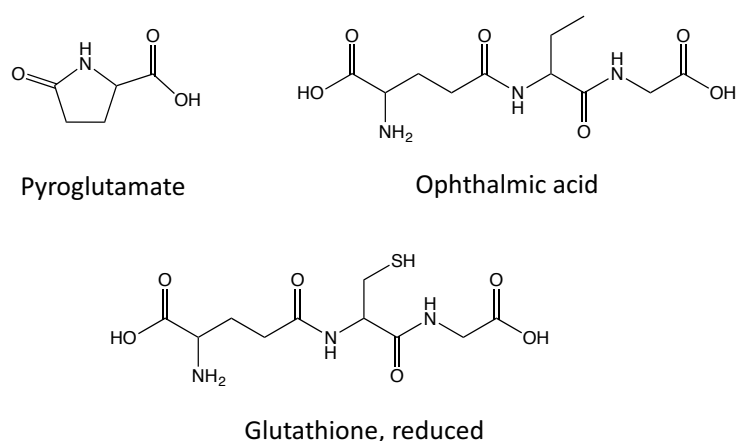


Figure 4.4-2 Structures of glutathione, pyroglutamic acid and ophthalmic acid

Pyroglutamic acid is an amino acid derivative involved in glutathione synthesis. In this study it was only found to be elevated in response to TAI. Pyroglutamic acid has previously been reported to be elevated in response to other hepatotoxins including bromobenzene (Waters et al., 2006) and paracetamol in the rat (Sun et al., 2008, Ghauri et al., 1993). Clinically pyroglutamic acid has also been linked to several cases of APAP induced toxicity (Beger et al., 2010, Fenves et al., 2006), demonstrating its potential as a biomarker. There was no correlation between hepatic glutathione and plasma pyroglutamic acid found in this study.

Ophthalmic acid (OA) was elevated in both TA and TAI treated animals at 6h in liver and plasma. OA is a non-sulphur containing analogue of glutathione (Figure 4.4-2) produced in a parallel pathway where the cysteine is replaced by 2-aminobutyrate (Figure 4.4-3) (Soga et al., 2006). The function of OA is largely unknown, however, it is thought of as a biomarker of oxidative stress as its biosynthesis is induced by the same enzymes as GSH, including glutamate-cysteine ligase (GCL) which is upregulated in response to depleted GSH (Soga et al., 2006)(Figure 4.4-3). The upregulation of OA is therefore indicative of GCL upregulation. Interestingly, GCL upregulation has previously been reported in response to TA in the rat (Nishiya et al., 2008b).

OA was first linked to hepatic GSH depletion in the mouse in response to APAP dosing (Soga et al., 2006). More recently it was found at a higher concentration in non-survivors of APAP overdose than survivors, indicative of its potential as a translatable biomarker (Kaur et al., 2015). The elevation in ophthalmic acid in plasma samples at 6h, supporting the suggestion of others that circulating ophthalmic acid could be a useful biomarker of hepatic oxidative stress, however, only a moderate correlation was found between plasma OA and hepatic GSH in this study.

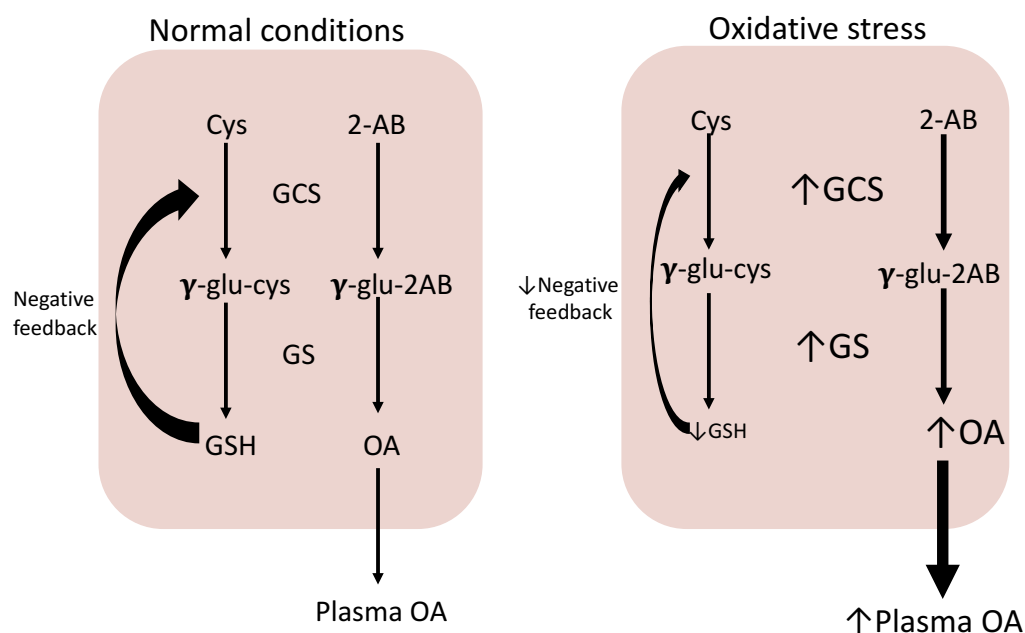


Figure 4.4-3 Biosynthesis of glutathione and ophthalmic acid under normal and oxidative stress conditions (Dello et al., 2013)

4.4.3 Key limitations and future work

One of the limitations of the animal study was the lack of information collected about the amount of food consumed by the treatment groups. Information about the food consumption could have helped determine if some of the metabolic changes were more likely due to differences in food intake. In addition, due to the low animal number and low sample volumes, there was insufficient sample volume to perform all analyses with sufficient animals in a group, this restricted data interpretation at certain groups.

The first part of this Chapter focused on the use of a global/untargeted LC-MS based metabonomic approach. Whilst there is a large volume of data produced, this is both an advantage and limitation due to the difficulties surrounding metabolite identification. Due to the time and resource consuming nature of metabolite identification, selecting the most biologically significant compounds is an important step. Overall, the univariate approach effectively applied here isolated approximately ninety compounds that were deemed the most promising, and became the focus of further characterisation. However, this approach was predominantly chosen due to the built-in function in Progenesis at the time of analysis, and alternative methods, such as the multivariate approach could also have been further explored.

The untargeted metabolite data presented in this chapter are greatly limited by the fact no confirmational experiments were performed, however as many of the suggested compound identifications are commercially available, these could be used in confirmational studies. In

future studies it would be helpful to run analyses applying MSe mode, where high and low collision energies are applied in the collision cell, enabling fragmentation data at the time of acquisition. Newer UPLC-MS analysis software (e.g. within Progenesis) can use this fragmentation data in the initial analysis, and should enable more accurate preliminary identifications to be obtained.

Although numerous metabolic changes have been demonstrated in this work, the biological significance or contribution to DILI is unproven in this model, and further mechanistic studies will be required. To being to explore some of these metabolic changes further, a quantitative assay is developed in the next Chapter to quantify ophthalmic acid, pyroglutamic acid and related metabolite. These metabolites were chosen to focus on due to their previous association with DILI, including some reports of translatability to humans (Fenves et al., 2006, Kaur et al., 2015).

RESULTS

THE DEVELOPMENT AND APPLICATION OF A TARGETED UPLC-MS/MS ASSAY TO SIMULTANEOUSLY QUANTIFY OPHTHALMIC ACID, PYROGLUTAMIC ACID AND RELATED METABOLITES IN PLASMA

5.1 INTRODUCTION

5.1.1 Rationale and aims

Glutathione (GSH) is an important antioxidant for maintaining redox balance in cells, and this is especially important in the liver as it is the major site of drug metabolism. In particular, hepatic GSH is vital for the neutralisation of reactive electrophilic drug metabolites, and its depletion can lead to drug induced liver injury (DILI) (Kalgutkar and Soglia, 2005). In Chapter 4, it was shown that Tienilic Acid Isomer (TAI) depletes hepatic GSH to a greater extent, and was slower to replenish GSH compared to Tienilic Acid (TA). This is supported by the other observations from Chapter 3, and summarised in Table 1, that only rats treated with TAI, had elevated ALT activity, evidence of reactive metabolite formation, and liver necrosis (first published by Coen et al., 2012).

As hepatic GSH depletion is commonly observed in response to CRM formation and resultant toxicity, biomarkers associated with the perturbation of hepatic glutathione homeostasis, such as pyroglutamic acid (Ghauri et al., 1993, Waters et al., 2006) and ophthalmic acid (Soga et al., 2006), have previously been proposed as translatable biomarkers for hepatic oxidative stress and DILI (Geenen et al., 2012, Stahl et al., 2015). Interestingly, in Chapter 4, plasma ophthalmic acid was found to be elevated in response to both TA and TAI at 6h, whereas plasma pyroglutamic acid was elevated only in response to TAI at 2h and 6h.

Table 5.1-1 Summary of key differences following TA and TAI dosing in the rat

Tienilic Acid Idiosyncratic toxin	Tienilic Acid Isomer Intrinsic toxin
No elevated ALT activity	Elevated ALT activity by 24h
No liver necrosis observed	Liver necrosis by 24h
Major metabolite is an hydroxylated-TA	Major metabolites are glutathione conjugates
Depletion in hepatic glutathione replenished by 6h	Depletion in hepatic glutathione replenished by 24h
Little perturbation in endogenous metabolism by 24h= homeostasis/ recovery	Perturbation in endogenous metabolism accelerating by 24h= toxic endpoint
Transient elevation in plasma ophthalmic acid (OA)	Elevation in plasma OA and pyroglutamic acid

Although associated with drug treatment, pyroglutamic acid did not correlate to hepatic GSH and similarly ophthalmic acid was only weakly correlated to hepatic GSH. Glutathione metabolism is a part of a complex system and influenced by multiple factors, therefore it may be the case, as has previously been suggested (Stahl et al., 2015), that hepatic glutathione status is more accurately determined using the concentration of multiple metabolites. In an *in vitro* model of APAP induced GSH depletion, both ophthalmic acid and pyroglutamic acid concentrations were dependent on methionine availability and only the simultaneous measurement of methionine, pyroglutamic acid, and ophthalmic acid could enable the accurate inference of the glutathione content (Geenen et al., 2012).

To further explore the impact of xenobiotics on circulating compounds related to hepatic glutathione, a method to simultaneously quantify metabolites linked to glutathione metabolism was required. As glutathione production has previously been shown to be dependent on the availability of the Sulphur containing amino acids cysteine/cystine and methionine (Lu, 1998), pathways of interest include the gamma-glutamyl cycle, the transsulfuration pathway and methionine/methylation cycle (Figure 1).

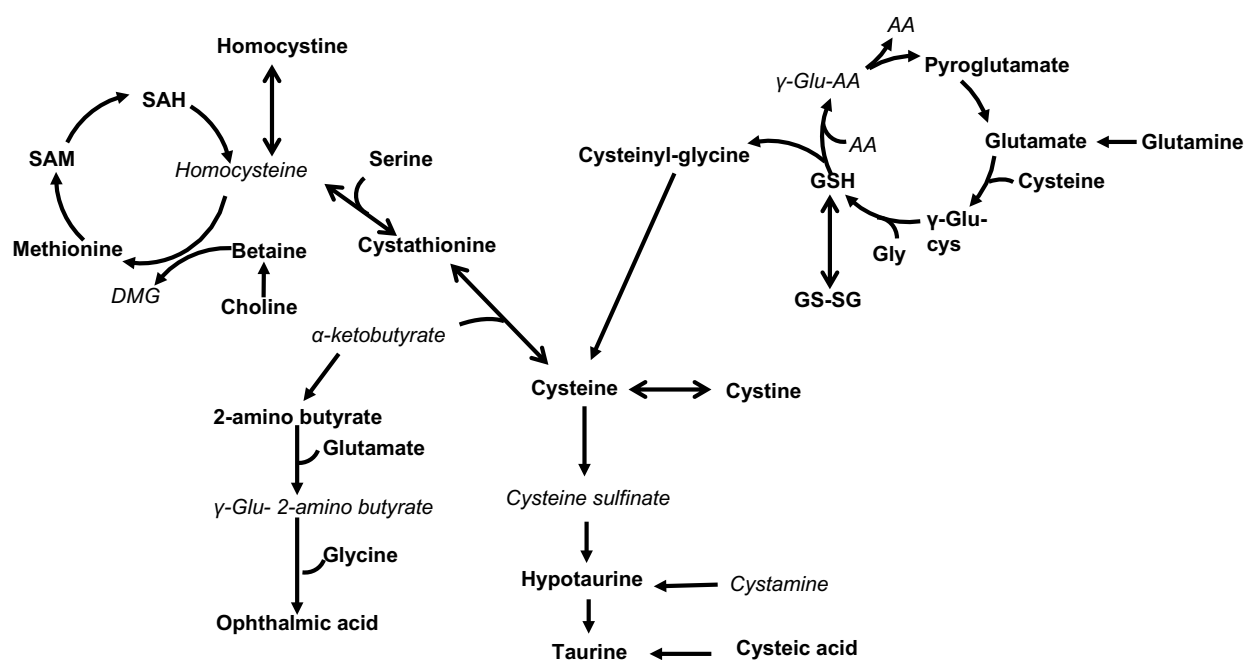


Figure 5.1-1 Metabolic pathways linked to glutathione metabolism and related sulphur containing amino acid metabolism. Metabolites not included in the assay are in italic font.

Although several methods exist to quantify aspects of these pathways, for example for pyroglutamic acid (Geenen et al., 2011a) and ophthalmic acid (Geenen et al., 2011b), a single, high-throughput, quantitative method to cover this broad range of metabolites was

notavailable. Therefore, the aim of work presented in this Chapter was to develop a targeted UPLC-MS/MS assay to simultaneously quantify ophthalmic acid, pyroglutamic acid and other endogenous metabolites linked to GSH metabolism in plasma. This includes twenty-four metabolites selected from the gammaglutamyl cycle and sulphur amino acid metabolism (Figure 5.1-1). To ensure the accuracy of the method, it was assessed following the FDA Guidance for Bioanalytical Method Validation (FDA, 2001, FDA, 2015, FDA, 2013), including an assessment of assay selectivity, accuracy, precision, carryover, and stability. The assay was then applied to plasma samples collected from rats dosed with TA, TAI or vehicle.

5.2 MATERIALS AND METHODS

5.2.1 Contribution of others

The animal study and sample collection were undertaken by collaborators at Michigan State University, U.S. All analytical sample preparation, UPLC-MS/MS analysis, validation experiments and data analysis were performed by the author at Imperial International Phenotype Training Centre.

5.2.2 Animal handling and sample collection

For detailed methods of Animals, TA/TAI, TA/TAI administration and Sample Collection, refer to Chapter 3 Material and Methods, p 57. Briefly, male Sprague-Dawley rats (n=46 total: n=5 per group, excluding TAI 24h n=6) were treated with equimolar doses of 250mg/kg TA, TAI or vehicle. Rats were euthanized, and plasma obtained at necropsy at 2,6 and 24h post-dose.

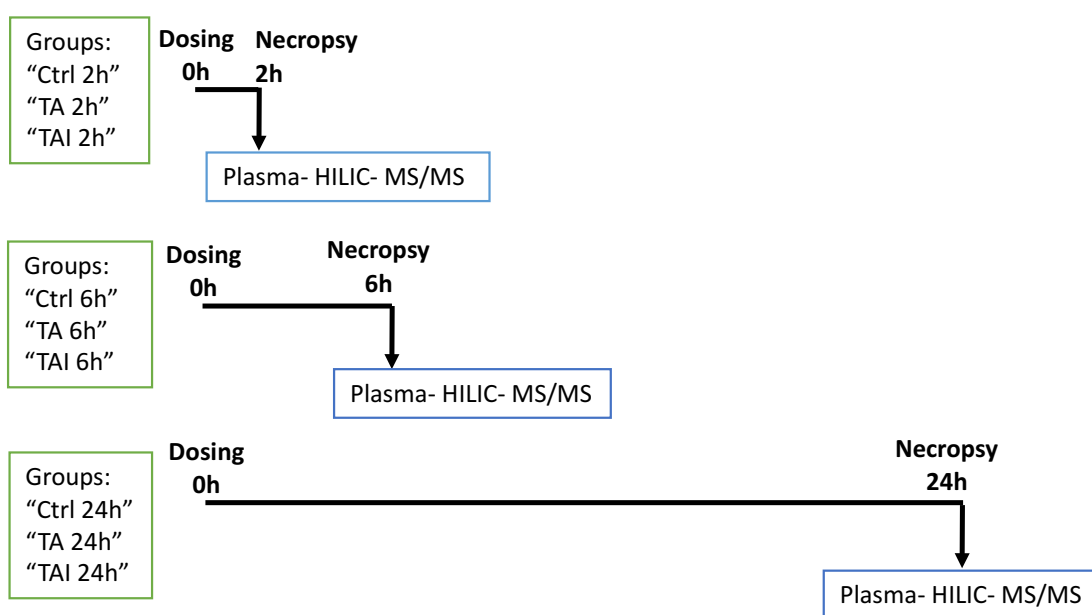


Figure 5.2-1 A figure depicting the study design, and sample collection points of plasma analysed using the quantitative HILIC-MS/MS method.

5.2.3 Chemical Standards and Reagents

All unlabelled standards were purchased from Sigma Aldrich (Gillingham, UK), except for ophthalmic acid which was purchased from Bachem AG (Bubendorf, Switzerland). DL-glutamic acid-d3, L-glutamine-d5, and DL-serine-d3, were purchased from Cambridge Isotope Laboratories (MA, USA). DL-Methionine-¹³C¹⁵N was purchased from QMX Laboratories (Essex, UK). Choline-1,1,2,2-d4 Bromide and 2-Aminoethane-d4-sulfinic Acid (hypotaurine-d4) were purchased from Qmx Laboratories Ltd, CND isotopes. D, L-cystathionine-d4, glutathione disulfide-¹³C₄¹⁵N₂, 5-oxo-DL-proline-d5, and taurine-d4 were from Toronto Research Chemicals Inc. (Ontario, Canada).

Optima grade water was obtained from Fisher Scientific (Leicester, UK). LC-MS grade acetonitrile and formic acid were purchased from Sigma Aldrich (Gillingham, UK).

5.2.4 Preparation of calibration standards, quality control and plasma samples

Due to the natural occurrence of these metabolites in plasma, any endogenous metabolite present would interfere with quantification. Therefore, the calibration curve and QC samples were prepared in solvents (H₂O and CH₃CN with 0.2%FA).

5.2.4.1 Calibration, QC and internal standard stock preparation

For each validation exercise, two combined stock solutions, labelled A and B, were made containing each compound at 5x the calibration curve ULOQ concentration, dissolved in H₂O +0.2%FA. Stock A was used in the preparation of the calibration standards and stock B was used in the preparation of the QC samples. Compounds were assigned either a 'high', 'mid' or 'low' ULOQ concentration, as defined in (Table 5.2-1), depending on the expected biological range required and/or the analytical range of the method.

Table 5.2-1 The final concentration of the ULOQ for each compound

High (20,000ng/mL)	Mid (2000ng/mL)	Low (1000ng/mL)
Glutamine	Cysteine	2-aminobutyric acid
Glutathione, oxidized (GSSG)	Cystine	Betaine
Glycine	Cysteinyl-Glycine	Choline
Serine	Glutamic acid	Cystathionine
Taurine	Homoserine	Cysteic acid
	Hypotaurine	Glutathione, reduced (GSH)
	Methionine	γ-Glutamyl-Cysteinyl
	Pyroglutamic acid	Homocystine
		Ophthalmic acid
		S-adenosyl-homocystine
		S-adenosyl-methionine

A combined internal standard stock solution, containing all the internal standards at either a 'high', 'mid' or 'low' concentration, was made up in CH₃CN + 0.2%FA. The stock solution concentrations were 1.25x the final concentrations shown in Table 5.2-2.

Table 5.2-2 Concentrations of Stable Isotope Labelled Internal Standards in all final solutions

High (4000ng/mL)	Mid (400 ng/mL)	Low (200ng/mL)
Glutamine-d5	Cystine-d6	Choline-d4
Glycine-d5	Glutamic acid-d3	Cystathionine-d4
GSSG-13C415N2	Hypotaurine-d4	
Serine-d3	Methionine-13C15N	
Taurine-d4	Pyroglutamic acid-d5	

Stock solutions for the seven-point calibration curve were prepared from combined stock A by appropriate dilution with 0.2% FA in H₂O, to 5x the final concentrations shown in Table 5.2-3. Stock QC samples, including an upper limit of quantification QC (ULOQQC), high QC (HQC), mid QC (MQC), low QC (LQC) and lowest limit QC (LLOQQC), were prepared by diluting stock B with 0.2% FA in H₂O, to x5 final concentrations shown in Table 5.2-4.

5.2.4.2 Calibration curve and QC preparation for LC-MS

To produce the final calibration curve and QC samples, the stock solutions were diluted 1 in 5 (100 µL: 400 µL) with the internal standard stock solution. To replicate the plasma preparation procedure, the QC and calibration samples, were kept at -20°C for 20 min and centrifuged at

10,000g for 10 min. Samples were then transferred to Waters Maximum Recovery glass vials for analysis. The final concentrations for the standard curve points and QCs are seen in Table 5.2-3 and Table 5.2-4.

Table 5.2-3 Final concentration range of calibration curves

Standard Curve	1	2	3	4	5	6	7
High- Final conc. (ng/mL)	20,000	10,000	5,000	2,000	1,000	500	200
Mid- Final conc. (ng/mL)	2,000	1,000	500	200	100	50	20
Low- Final conc. (ng/mL)	1,000	500	250	100	50	25	10

Table 5.2-4 Final concentrations of Quality Control (QC) samples

QC	LLOQQC	LQC	MQC	HQC	ULOQQC
High- Final conc. (ng/mL)	200	600	2,000	8,000	20,000
Mid- Final conc. (ng/mL)	20	60	200	800	2,000
Low- Final conc. (ng/mL)	10	30	100	400	1,000

Each calibration curve also consisted of a double blank and a single blank, which were water processed with no IS or analyte, and water prepared with IS but no analyte, respectively.

5.2.4.3 Plasma sample preparation for LC-MS

Plasma samples stored at -80 °C were left to thaw at 4°C. A 20 µL aliquot of plasma was diluted with 80 µL of the internal standard stock solution (SIL in acetonitrile + 0.2% FA in CH₃CN) and briefly vortexed. Samples were kept at -20°C for 20 min to precipitate proteins, before being centrifuged at 10,000g for 10 min. The supernatant placed in Waters Total Recovery vials for analysis.

5.2.5 Chromatography

An ACQUITY Chromatography system (Waters Corporation, MA, USA) was employed, comprising on a Binary Solvent Manager, an automated temperature controlled Sample Manager and a heated Column Manager. Chromatographic separation was achieved using a

BEH amide 2.1 x 150 mm, 1.8 μ m column (Waters, Milford, MA, USA). An injection volume of 2 μ L was used, on full loop mode with a 2 μ L loop installed. Mobile phase A consisted of 0.2 % formic acid (FA) in water and mobile phase B was 0.2 % FA in acetonitrile (CH₃CN). A column temperature of 45 °C was maintained, and a flow rate of 0.6 mL/min. The 15min mobile phase gradient is shown in Table 5.2-5. The weak and the strong washes were 100% CH₃CN + 0.2% FA and 95:5 H₂O/CH₃CN (v/v) respectively.

Table 5.2-5 UPLC mobile phase gradient

Time (min)	Flow rate (mL/min)	% A	% B	Curve
0.0	0.6	16	84	6
4.0	0.6	16	84	6
8.0	0.6	60	40	6
10.0	0.6	60	40	6
10.1	0.6	16	84	6
14.70	0.6	16	84	6

A = Water + 0.2 % formic acid (v/v)

B = Acetonitrile +0.2 % formic acid (v/v)

5.2.6 Mass Spectrometry

The mass spectrometry was performed on a Xevo tandem quadrupole (TQ)-S mass spectrometer (Waters Corporation, Manchester, UK), operated in electrospray ionisation (ESI) positive ion mode. Both full scan MS and MS/MS modes were employed. Nitrogen was used as the desolvation gas and argon was used as the collision gas. The following generic source conditions were used: capillary voltage, 3.0 kV; source offset, 50 V; desolvation temperature, 550°C; source temperature, 150 °C, desolvation gas flow, 1000 L/h; cone gas flow, 150 L/h; nebuliser gas, 7.0 bar; collision gas, 0.15 mL/min.

The compound specific MS parameters, detailed in Table 5.2-6, include: cone voltage, collision energy, and ion transitions (where possible one fragment mass was selected for quantification and another for identification). These were determined by the direct infusion, at 10-20 μ L/min, of 100-500 ng/mL solutions of each analyte, combined with an LC flow of 50:50 H₂O/CH₃CN at 0.2 mL/min.

Table 5.2-6 Mass spectrometry acquisition parameters for each transition including cone voltage (CV) and collision energy (CE) listed in alphabetical order

Compound	Parent (m/z)	Fragment (m/z) Quantification Identification	Window (min)	Dwell time (s)	CV (V)	CE (eV)
2-amino-butyric acid	103.866	57.892 -	1-2.5	0.004	50	12
Betaine	118.068	58.89 42.16	0.5-2.5	0.004	110 56	22 74
Choline	104.132	60.028 45.002	0-2	0.004	90 90	20 16
Cystathionine	223.077	134.01 87.972	5.5-7.5	0.013	40 40	12 24
Cysteic acid	169.937	105.981 42.641	5-7	0.013	30	18 24
Cysteine	122.192	75.984 104.976	1-3	0.004	24	12 8
Cysteinyl-glycine	178.987	75.952 58.98	0.5-2.5	0.004	22	14 24
Cystine	241.14	74.046 152.049	5.75-7.75	0.013	60	24 12
γ-glutamyl-cysteine	251.077	121.977 84.008	1.5-3.5	0.004	22	10 22
Glutamic acid	148.098	84.071 102.032	1.5-3.5	0.004	60	16 12
Glutamine	147.037	84.038 56.074	2.2-4.2	0.004	42	14 22
Glutathione, reduced	308.097	179.005 75.991	2-4	0.004	14	12 24
Glutathione, oxidised	613.287	355.054 230.996	6-14.7	0.013	40	22 30
Glycine	76.038	30.042 48.078	1-3	0.004	30	4 6
Homocystine	269.19	136.028 87.97	5-7	0.013	10	10 30
Homoserine	119.96	73.812 55.863	1.5-3.5	0.004	28	20 25
Hypotaurine	109.927	29.987 45.077	2.75-4.75	0.004	34	8 14
Methionine	150.05	104.048 56.025	0.3-2.3	0.004	50	10 16
Ophthalmic acid	290.13	58.102 161.072	2-4	0.004	10	16 12
Pyroglutamic acid	130.072	83.962 55.992	0-2	0.004	36	17 20
S-adenosyl-homocysteine	385.113	TIC (249.98 & 87.97) 87.97	4.5-6	0.016	30	12 40
s-adenosyl-methionine	399.113	249.99 135.955	5.5-14.7	0.013	46	14 32
Serine	105.968	70.007 42.102	2.5-4.5	0.004	30	13 17
Taurine	126.054	44.079 107.969	1.75-3.75	0.004	42	30 25

“-“ indicates that only one transition was obtained

Table 5.2-7 Mass spectrometry acquisition parameters for each SIL transition including cone voltage (CV) and collision energy (CE) listed in alphabetical order

Compound	Parent	Fragment Quantification Identification	Window (min)	Dwell time (s)	CV (V)	CE (eV)
Choline-d ₄	108.196	60.258 49.079	0-2	0.004	58	35 35
Cystathionine-d ₄	227.013	137.836 91.81	5.5-7.5	0.013	4	14 26
Cystine-d ₆	246.949	122.837 154.763	5.75-7.75	0.013	20	40 30
DL-Glutamic acid-d ₃	150.977	132.852 104.892	1.5-3.5	0.004	32	27 33
L-Glutamine-d ₅	151.968	134.819 88.119	2.2-4.2	0.004	2	30 40
Glycine-d ₅	77.968	31.875 49.885	1-3	0.004	28	8 6
GSSG - ¹³ C ₄ ¹⁵ N ₂	619.377	TIC (inc 361.09, 490.15) 231.059	6-14.7	0.013	40	22 14 30
Hypotaurine-d ₄	114.132	32.135 49.083	2.75-4.75	0.004	38	10 14
DL-Methionine- ¹³ C ¹⁵ N	155.977	59.892 108.854	0.3-2.3	0.004	30	40 35
Pyroglutamic acid-d ₅	135.136	89.03 61.06	0-2	0.004	28	14 22
DL-Serine-d ₃	108.95	62.842 44.886	2.5-4.5	0.004	32	10 16
Taurine-d ₄	130.118	48.169 -	1.75-3.75	0.004	32	25

“-” indicates that only one transition was obtained

5.2.6.1 Allocation of Internal Standards

The compounds analysed in this study are listed in Table 5.2-8. Where available, stable isotope labelled (SIL) analogues were acquired. Where no SIL analogues were available, compounds were evaluated for quantification using the closest eluting SIL, as a surrogate IS. For compounds where there was no appropriate surrogate SIL, no IS was used. The allocation of internal standards are listed in Table 5.2-8.

Table 5.2-8 Compounds detected and their internal standard (IS) or surrogate IS

Validated for quantification	Internal Standard/surrogate IS
2-amino-butyric acid	-
Betaine	-
Choline	Choline-d ₄
Cystathionine	Cystathionine-d ₄
Cysteic acid	-
Cysteine	Glycine-d ₅
Cysteinyl-glycine	DL-Methionine- ¹³ C ¹⁵ N
Cystine	Cystine-d ₆
γ -glutamyl-cysteine	-
Glutamic acid	DL-Glutamic acid-d ₃
Glutamine	L-Glutamine-d ₅
Glutathione, reduced (GSH)	-
Glutathione, oxidized (GSSG)	Glycine-d ₅
Glycine	GSSG- ¹³ C ₄ ¹⁵ N ₂
Homocystine	Cystathionine-d ₄
Homoserine	Glycine-d ₅
Hypotaurine	Hypotaurine-d ₄
Methionine	DL-Methionine- ¹³ C ¹⁵ N
Ophthalmic acid	-
Pyroglutamic acid	Pyroglutamic acid-d ₅
S-adenosyl-homocysteine	-
s-adenosyl-methionine	-
Serine	DL-Serine-d ₃
Taurine	Taurine-d ₄

“-“ indicates that no internal standard was used

5.2.7 Data Processing

Data were processed in Targetlynx (Waters), where automated peak integration followed by manual checking and correction were performed. Data were then exported to Excel (Microsoft) for validation calculations.

5.2.8 Validation Calculations and Criteria

Evaluation of this method was based on FDA Guidance for Bioanalytical Method Validation (2001, 2013), and included an assessment of assay selectivity, accuracy, precision, carryover, and stability. The FDA recommended acceptance criteria have been cited, but are used here only as a guide to highlight assay limitations, and not for pass/fail categorisations.

5.2.8.1 Selectivity

Matrix (solvent) to Analyte Interference

The assessment of matrix interference was limited to aqueous - organic solvent. Six double blanks (water, diluted with 1/4 v/v with 0.2% FA CH₃CN, containing no IS and no analytes) were processed. Any response at the retention time of each analyte was compared to the mean of the analyte response in the LLOQ calibration standards. A minimum of five of the six double blanks should meet the following acceptance criteria:

$$\frac{\text{Double Blank Response}}{\text{LLOQ STD Response}} \times 100 \leq 20 \%$$

Matrix (solvent) to Internal Standard Interference

Six double blanks (water, processed as samples and stock calibration solvents i.e. diluted with 1/4 v/v with 0.2% FA CH₃CN, containing no IS and no analytes) were processed. Any response at the retention time of the IS was compared to the average response of the IS of all standards accepted in the calibration curve (including the single blanks). A minimum of five of the six double blanks should meet the following acceptance criteria:

$$\frac{\text{Double Blank Response}}{\text{IS Average Response}} \times 100 \leq 5 \%$$

Internal Standard to Analyte Interference

Three aliquots of the same lot of blank calibration solvent spiked with IS (single blank) were processed to assess interference due to the IS at the retention time of the analytes. Any response at the retention times of the analytes was compared to mean of the analyte response in the LLOQ calibration standards. Single blanks must meet the following acceptance criteria:

$$\frac{IS\ Interference\ Sample}{LLOQ\ STD\ Response} \times 100 \leq 20\%$$

Analyte to Internal Standard Interference

Three aliquots of each individual analyte were prepared in CH₃CN + 0.2% formic acid without any IS, at a concentration of ULOQ. They were processed to assess any interference at the retention time of internal standards, any response was compared to the average IS detected in the curve. Analytes must meet the following criteria:

$$\frac{Analyte\ interference\ sample\ response}{IS\ Average\ Response} \times 100 \leq 5\%$$

Analyte to Analyte Interference

The three aliquots of the individual analyte were also processed to assess any interference at the retention time of other analytes in the assay. Where any interference was detected calculations were performed to establish if these were significant/ met the following acceptance criteria:

$$\frac{Analyte\ interference\ sample\ response}{LLOQ\ STD\ Response} \times 100 \leq 20\%$$

5.2.8.2 Accuracy and Precision

Within-run and between-run accuracy

Six replicates of LLOQQC, LQC, MQC, HQC and ULOQQC were freshly prepared and analysed on three separate days. The within-run accuracy was calculated by comparing the mean QC concentration detected, to the nominal concentration. The acceptance criteria for accuracy was defined as:

$$\frac{\text{Mean calculated QC concentration}}{\text{Nominal QC concentration}} \times 100 \leq 15 \% \text{ (20\% for LLQC)}$$

Between-run accuracy was calculated from the mean QC concentration determined using the QC samples analysed over the three batches (n=18 per concentration).

Within-run and between-run precision

The within-run precision was calculated on three separate days, by calculating the coefficient of variation of six replicates of LLOQQC, LQC, MQC, HQC and ULOQQC. Acceptable precision was defined as:

$$\text{QC coefficient of variation} \leq 15 \% \text{ (20\% for LLQC)}$$

The between-run precision was determined by calculating the coefficient of variation of all 18 QC samples (per concentration), over the three batches.

5.2.8.3 Sensitivity

LLOQ

The accuracy and precision of the LLOQQCs, should be < 20% to be accepted. The LLOQ was also only accepted if it was 5x the intensity of carryover.

Carryover

Carryover was assessed by running a double blank (calibration solution, 0.2% FA CH₃OH (v/v), containing no IS and no analytes) immediately after a ULOQ calibration standard. The level of carryover for the analyte was considered acceptable if the response in the double blank sample was ≤20% of the average response from the acceptable LLOQ standards in the batch. The level

of carryover for the IS was considered acceptable if the response in the double blank sample is ≤5% of the average response from the acceptable calibration standards (including single blank) in the batch.

5.2.8.4 Stability

Processed sample stability/autosampler stability

The stability of samples in an autosampler were assessed by reanalysing the QCs from one batch 36h later, with a freshly prepared standard curve. The accuracy acceptance criteria remained the same:

$$\frac{\text{Mean calculated QC concentration}}{\text{Nominal QC concentration}} \times 100 \leq 15 \% \text{ (20\% for LLQC)}$$

5.2.9 Statistical analyses

Statistical significant between plasma samples from the TA/TAI study were calculated using two-tailed Mann Whitney (Prism 6.0, GraphPad, La Jolla, California, USA).

5.3 RESULTS

5.3.1 LC development and characteristics

To determine which LC column was most suitable for the quantification of the metabolites involved in GSH metabolism, several columns were tested on a subset of the compounds. The most polar retaining reversed-phase column was tested (Waters HSS T3), but did not provide sufficient retention for many of the metabolites with the majority eluting with the solvent front (Table 5.3-1). Waters BEH Amide and BEH HILIC columns were subsequently tested with various mobile phases. The BEH Amide column with formic acid in water (A) and formic acid in acetonitrile (B) found to be the best for both chromatography; peak shape and retention (refer to appendix for HILIC retention times).

Table 5.3-1 Retention time comparison between a HSS T3 and BEH Amide columns

Compound	HSS T3 15cm 99% H ₂ O+ FA hold	BEH Amide 15cm 16% H ₂ O+FA hold
2-amino-butyrate	N.A	1.44
Betaine	0.61	1.47
Choline	0.55	0.77
Cystathionine	0.55	6.59
Cysteic acid	0.58	5.95
Cysteine	0.62	2.11
Cysteinyl-glycine	0.53	1.41
Cystine	0.55	6.73
γ-glutamyl-cysteine	0.79	2.44
Glutamic acid	0.59	2.55
Glutamine	0.57	3.26
GSH	1.29	3.18
GSSG	2.55-2.72 (split peak)	6.78
Glycine	0.68	2.23
Homocystine	N.A	6.05
Homoserine	N.A	2.48
Hypotaurine	0.57	3.75
Methionine	0.96-1.15 (split peak)	1.31
Ophthalmic acid	1.63	2.98
Pyroglutamic acid	1.57	0.89
SAH	0.79	5.35
SAM	0.58	6.35
Serine	0.55	3.38
Taurine	0.57	2.75

N.A Not included in analysis, shading indicated elution less than 1min.

Further optimisation found a critical factor for symmetric and narrow peak shape was increasing the percentage of formic acid in acetonitrile and water from 0.1% to 0.2%. This was most dramatic for GSSG, as shown in Figure 5.3-1.

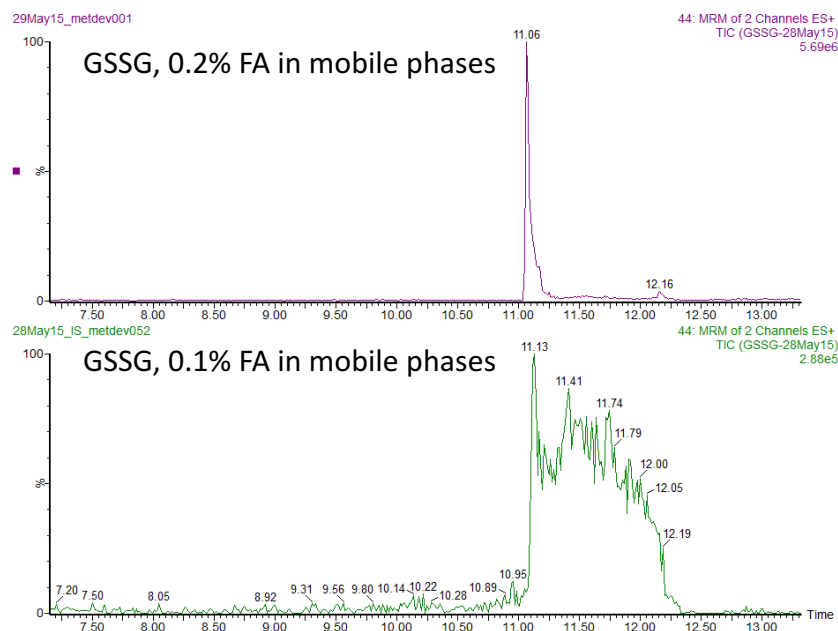


Figure 5.3-1 The impact of 0.2% FA compared to 0.1% FA on the peak shape of GSSG.

The final gradient developed for the assay is shown in Figure 5.3-2. Whilst improved chromatography (retention) could be obtained using a lower percentage of H₂O in the starting conditions, this led to a significant carryover which was prevented by not dropping below 16% H₂O. This may be due to the very polar compounds coming out of solution in the tubing when concentrations of very high acetonitrile were used.

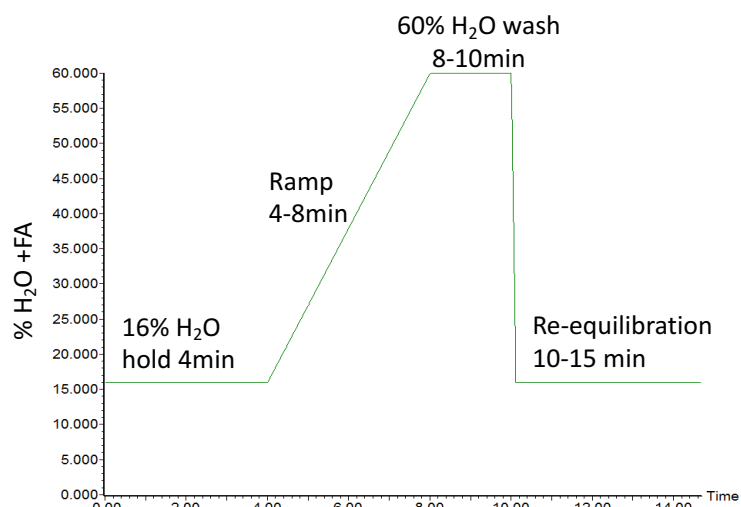
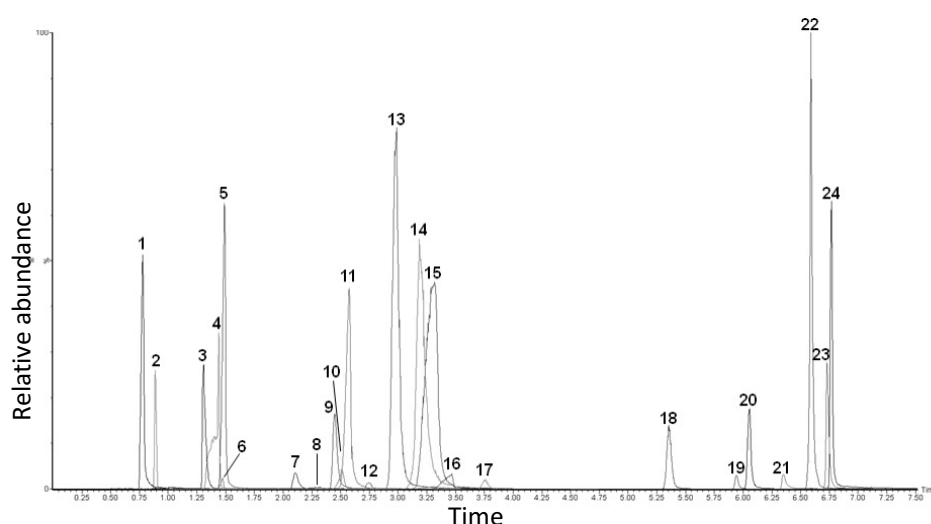


Figure 5.3-2 The gradient developed for the optimal analysis of polar GSH related metabolites, line represents %

The ramp between 4-8 min was optimised for peak shape and separation. The 4 min hold at 60% H₂O+ 0.2%FA was designed to flush the column, by giving time for non-polar compounds to elute. A higher percentage of H₂O may have been optimal to remove the most non-polar analytes, however, this increased the back pressure to a value that was too high (15 000 PSI). The hold at the end provided sufficient re-equilibration for reproducible retention times.

A typical chromatogram for the UPLC-MS/MS analysis of the standards in solvent at the ULOQ is shown in Figure 5.3-3. The co-eluting compounds did not affect the method, as these compounds could be separated by mass differences in the mass spectrometer. The separation was shown to be reproducible, and stable retention times were observed over the course of the 3-day validation (Table 5.3-2). However, the peak shape of glycine was particularly poor (asymmetrical, jagged and tailing), and GSSG deteriorated at lower concentrations over the course of the run.



1	Choline	9	γ -Glutamyl-cysteine	17	Hypotaurine
2	Pyroglutamic acid	10	Homoserine	18	S-adenosyl-homocysteine
3	Methionine	11	Glutamic acid	19	Cysteic acid
4	Cysteinyl-glycine	12	Taurine	20	Homocystine
5	Betaine	13	Ophthalmic acid	21	S-adenosyl-methionine
6	2-Amino butyrate	14	Glutathione, reduced	22	Cystathionine
7	Cysteine	15	Glutamine	23	Cystine
8	Glycine	16	Serine	24	Glutathione, oxidised

Figure 5.3-3 Chromatographic separation of the standard mix at ULOQ concentrations- showing 0-7.5min of 15min run.

Table 5.3-2 Mean retention time of each metabolite of 3 batch/day validation experiment

Compound	Mean retention	Standard deviation
Choline	0.77	0.00
Pyroglutamic acid	0.89	0.00
Methionine	1.31	0.00
Cysteinyl-glycine	1.41	0.01
2-amino-butyrates	1.44	0.01
Betaine	1.47	0.01
Cysteine	2.11	0.02
Glycine	2.23	0.03
γ-glutamyl-cysteine	2.44	0.01
Homoserine	2.48	0.01
Glutamic acid	2.55	0.01
Taurine	2.75	0.01
Ophthalmic acid	2.98	0.01
GSH	3.18	0.03
Glutamine	3.26	0.02
Serine	3.38	0.04
Hypotaurine	3.75	0.01
SAH	5.35	0.01
Cysteic acid	5.95	0.01
Homocystine	6.05	0.00
SAM	6.35	0.01
Cystathionine	6.59	0.00
Cystine	6.73	0.00
GSSG	6.78	0.01

5.3.2 MS Development and Assay Selectivity

The MS transitions were determined by direct infusion of the compounds in the MS, and using Intellistart to automatically determine the product ions, and optimal cone voltages and collision energies. To assess the selectivity of the assay for each metabolite, interferences between the matrix, other analytes, and the internal standards were calculated.

5.3.2.1 Matrix (solvent) to Analyte or Internal Standard Interference

Analyte free plasma was not available, therefore the matrix used for the QC and calibration curve standards was aqueous-organic solvent. All of the analytes examined for this solvent matrix to analyte interference met the acceptance criteria as there were no peaks above the LOD (data not shown). In the absence of internal standard in surrogate matrix (blank solvent), no peaks were detected in any of the internal standard transitions (data not shown), indicating

no matrix to internal standard interference.

5.3.2.2 Internal Standard to Analyte, and Analyte to Internal Standard Interference

All compounds passed the acceptance criteria for internal standard to analyte interference (representative chromatograms are shown in the Appendix). For analyte to internal standard interference, cystathionine and GSSG were the only analytes where peaks were detected in the internal standard transition, and therefore underwent further calculations to determine the percentage of cross-talk from analyte to internal standard (Table 5.3-3). Acceptable interference levels were defined at below 5% of the average internal standard response in the standard curve. Cystathionine interference was below 3% and was therefore deemed acceptable. However, GSSG was found to very significantly interfere with GSSG-¹³C₄¹⁵N₂, showing GSSG interference was 34-36% of the mean concentration of GSSG-¹³C₄¹⁵N₂ detected in the curve.

Table 5.3-3 Analyte to internal Standard interference

Analyte	Average Response	IS	Internal standard	Response at IS	Interference (%)
Cystathionine 1	448830		Cystathionine-d ₄	10495	2.3
Cystathionine 2				16692	3.7
Cystathionine 3				13642	3
GSSG 1	64329		GSSG- ¹³ C ₄ ¹⁵ N ₂	23151	36
GSSG 2				21730	33.8
GSSG 3				21655	33.7

Shaded=failed to meet validation criteria

5.3.2.3 Analyte to Analyte Interference

Analyte to analyte interference was studied between all analytes included in this assay. Where any interference was detected, calculations were performed to establish if these were significant; defined as breaching the acceptance criteria of below 20% of the LLOQC (Appendix). Analyte- analyte interference was detected between seven compounds tested in this assay. However, of these, the only compounds that closely eluted, and where therefore likely to display true interference and not the result of degradation or contamination of the standards, was GSSG interfering with cystathionine signal. However, this is unlikely to impact any results in the plasma samples reported later in this thesis, due to the low levels of GSSG present. However, this should be further investigated and potentially alternative, more specific transitions determined for cystathionine.

5.3.2.4 Concentration- response

Analyte linearity over the specified concentration range was calculated, and typically ranged between 0.999 – 0.945 (Table 5.3-4). Those below 0.99 were 2-amino-butyrates, betaine, glycine and SAM.

Table 5.3-4 Typical linearity of the compounds tested

Compound	Range tested (ng/mL)	R ²
2-amino-butyrates	10-1000	0.979
Betaine	10-1000	0.987
Choline*	10-1000	0.996
Cystathionine*	10-1000	0.999
Cysteic acid	10-1000	0.998
Cysteine	20-2000	0.995
Cysteinyl-glycine	20-2000	0.999
Cystine*	20-2000	0.999
γ-glutamyl-cysteine	10-1000	0.996
Glutamic acid	20-2000	0.992
Glutamine*	200-20000	0.994
GSH	10-1000	0.998
GSSG*	200-20000	0.994
Glycine*	200-20000	0.974
Homocystine	10-1000	0.999
Homoserine	20-2000	0.995
Hypotaurine*	20-2000	0.999
Methionine*	20-2000	0.999
Ophthalmic acid	10-1000	0.996
Pyroglutamic acid *	20-2000	0.996
SAH	10-1000	0.998
SAM	10-1000	0.945
Serine*	200-20000	0.998
Taurine*	200-20000	0.999

Figures have been reported to 3 significant figures. * Indicates an internal standard was used. Shaded=failed to meet validation criteria

5.3.3 Accuracy and Precision

5.3.3.1 Within-Batch Accuracy and Precision

Within-batch (intra-day) accuracy and precision was assessed over three days, results from Batch 1, Batch 2 and Batch 3, are summarised in Table 5.3-5, and Table 5.3-7, respectively. Where the relative error (accuracy), or coefficient of variation (CV, precision) exceeded 15% (20% for LLOQ), these have been shaded in grey.

2-aminobutyrate, glycine, SAM and GSSG consistently failed to come close to the accuracy (bias) acceptance criteria. In addition, the bias ranged from -57 to -77% for betaine at the LLOQ. The majority of the remaining compounds met the accuracy acceptance criteria at each QC concentration (where a QC has failed this has been highlighted in grey). The CV acceptance criteria (20/15%) was consistently breached by SAM across the concentration range. The CV acceptance criteria was also exceeded at LLOQ by 2-amino butyrate, betaine and glycine. The remaining compounds were within the acceptance criteria across the concentration ranges for precision.

5.3.3.2 Between-batch Accuracy and Precision

The between-batch (inter-day) precision and accuracy was determined for each compound over three batches/days. The between-batch validation results for the QCs are summarised in Table 5.3-8. 2-amino butyrate and SAM consistently failed the accuracy and precision acceptance criteria. In addition, betaine and glycine had poor bias and CV at the LLOQ. For the remaining 20 compounds the LLOQ bias did not exceed 23%, and the CV was below 20%. At the higher QC concentrations these 20 compounds had a bias below 15% and CV not exceeding 18%.

Table 5.3-5 Within Batch Accuracy and Precision Batch 1

Compound	Accuracy- Bias (%)					Precision- Coefficient of Variance (%)				
	LLOQ 10ng /mL	LQC 30ng /mL	MQC 100n g/mL	HQC 400n g/mL	ULOQ 1000 ng/mL	LLOQ 10ng/ mL	LQC 30ng/ mL	MQC 100ng /mL	HQC 400ng /mL	ULOQ 1000 ng/mL
2-amino- butyrate	-43.5	0.70	16.1	14.4	-10.4	21.6	10.5	1.19	6.97	2.71
Betaine	-57.0	6.90	16.6	9.60	-9.10	13.1	5.35	2.26	3.51	0.67
Choline*	-12.5	0.70	4.10	6.00	-2.40	2.37	4.23	3.15	3.83	4.78
Cystathionine*	-0.30	1.60	0.90	3.80	23.3	2.17	2.77	4.13	3.13	2.83
Cysteic acid	2.70	-6.40	-8.70	-3.60	-6.10	9.15	6.10	3.86	4.22	3.80
GSH	-2.20	-4.80	-6.40	-3.00	-5.10	3.38	3.29	1.85	1.90	2.32
γ-glutamyl- cysteine	-3.30	-2.70	-2.10	3.00	-4.60	4.77	4.83	2.41	2.80	3.25
Homocystine	-4.50	-3.10	-2.90	1.30	-0.10	1.96	3.05	2.54	3.78	1.71
Ophthalmic acid	0.80	1.20	1.60	4.20	-7.00	3.16	4.24	1.87	2.16	2.92
SAH	-15.7	-11.8	-9.70	-5.70	-3.40	17.2	6.39	3.42	2.02	1.37
SAM	40.0	-35.7	-52.1	-47.3	-11.8	6.35	13.3	28.2	15.3	27.3
Compound	LLOQ 20ng /mL	LQC 60ng /mL	MQC 200n g/mL	HQC 800n g/mL	ULOQ 2000 ng/mL	LLOQ 20ng/ mL	LQC 60ng/ mL	MQC 200ng /mL	HQC 800ng /mL	ULOQ 2000 ng/mL
Cysteine	8.70	-11.7	-14.0	-5.20	3.20	6.85	4.73	5.13	3.99	4.94
Cysteinyl- glycine	-0.30	-6.80	-1.50	-4.10	-1.10	6.07	6.57	3.92	3.61	5.29
Cystine*	13.6	11.7	4.10	4.50	17.7	9.62	1.47	0.55	0.12	0.04
Glutamic acid	-1.60	5.10	5.20	6.50	-0.80	4.38	6.42	4.09	3.52	2.37
Homoserine	22.9	-7.00	-14.4	-3.40	9.90	11.8	5.67	4.78	5.16	2.90
Hypotaurine*	13.38	2.16	-6.87	1.75	6.78	12.13	6.04	3.42	1.65	2.46
Methionine*	0.30	-1.90	-0.70	-0.70	1.10	5.63	3.54	4.82	1.33	3.99
Pyroglutamic acid *	8.70	-11.7	-14.0	-5.20	3.20	7.20	5.89	6.00	3.00	5.38
Compound	LLOQ 200n g/mL	LQC 600n g/mL	MQC 2000 ng/m L	HQC 8000 ng/m L	ULOQ 20μg/ mL	LLOQ 200ng /mL	LQC 600ng /mL	MQC 2000n g/mL	HQC 8000n g/mL	ULOQ 20μg/ mL
Glutamine*	-1.80	-5.90	-7.50	4.80	3.60	13.1	5.77	2.78	11.1	6.30
Glycine*	5.90	-7.30	-10.1	0.10	6.40	25.7	13.0	9.22	8.00	10.4
GSSG*	-17.7	-17.2	-14.2	-2.50	-6.00	13.6	9.06	8.56	9.16	8.07
Serine*	-2.20	-6.30	-4.60	0.40	2.90	12.1	6.04	3.42	1.65	2.46
Taurine*	-16.5	0.10	-0.20	3.40	0.90	8.52	5.29	7.60	2.75	2.57

Shaded=failed to meet validation criteria

Table 5.3-6 Within Batch Accuracy and Precision Batch 2

Compound	Accuracy- Bias (%)					Precision- Coefficient of Variance (%)				
	LLOQ 10ng /mL	LQC 30ng /mL	MQC 100n g/mL	HQC 400n g/mL	ULOQ 1000 ng/mL	LLOQ 10ng/ mL	LQC 30ng/ mL	MQC 100ng /mL	HQC 400ng /mL	ULOQ 1000 ng/mL
2-amino-butyrates	-19.17	29.94	50.20	45.66	16.91	22.6	6.78	4.92	4.56	3.05
Betaine	-70.50	7.22	19.83	9.03	-4.44	23.1	5.02	5.26	2.93	2.64
Choline*	-22.67	6.72	15.93	7.48	0.94	9.63	2.77	4.06	3.45	4.22
Cystathionine*	1.17	12.39	13.72	14.39	13.83	6.97	2.68	1.33	2.95	2.93
Cysteic acid	-3.33	10.22	10.55	15.43	15.40	8.06	9.57	4.48	2.45	2.50
GSH	8.67	16.50	14.30	16.22	17.00	7.82	3.68	4.36	3.40	3.10
γ-glutamyl-cysteine	-9.00	9.56	13.90	15.10	12.83	4.66	2.59	4.30	3.60	4.29
Homocystine	6.50	16.28	18.88	21.44	23.91	6.63	2.76	3.08	2.90	3.07
Ophthalmic acid	-0.83	17.94	19.37	20.44	11.28	6.10	2.87	3.27	2.08	2.40
SAH	19.33	16.94	14.63	19.25	19.84	7.77	2.82	1.38	1.99	2.53
SAM	35.00	-36.28	-47.08	-8.40	41.55	2.10	23.5	43.1	25.8	24.9
Compound	LLOQ 20ng/ mL	LQC 60ng/ mL	MQC 200ng /mL	HQC 800ng /mL	ULOQ 2000 ng/mL	LLOQ 20ng/m L	LQC 60ng/ mL	MQC 200ng/ mL	HQC 800ng/ mL	ULOQ 2000 ng/mL
Cysteine	17.25	2.44	5.93	10.47	13.78	17.3	2.44	5.93	10.5	13.8
Cysteinyl-glycine	-7.25	-3.83	1.38	7.23	5.87	7.17	4.38	4.74	2.09	3.26
Cystine*	8.17	19.22	18.03	13.62	11.88	5.61	5.21	3.60	4.46	3.25
Glutamic acid	-5.58	23.75	29.08	26.48	19.82	10.2	5.56	5.76	5.32	3.77
Homoserine	10.42	-0.42	-3.22	0.05	11.69	9.51	6.12	4.21	5.46	4.67
Hypotaurine*	-6.43	0.61	2.57	0.45	2.57	7.95	7.39	3.51	4.69	1.19
Methionine*	-5.92	4.17	1.53	3.02	0.52	8.43	3.84	3.75	3.27	3.19
Pyroglutamic acid *	-18.50	-5.92	-3.89	-3.74	-1.43	9.76	1.09	3.40	2.15	1.26
Compound	LLOQ 200ng /mL	LQC 600ng /mL	MQC 2000n g/mL	HQC 8000n g/mL	ULOQ 20μg/ mL	LLOQ 200ng/ mL	LQC 600ng/ mL	MQC 2000ng /mL	HQC 8000ng /mL	ULOQ 20μg/mL
Glutamine*	-5.29	-0.09	4.27	-0.76	-1.11	4.86	3.54	8.80	10.67	8.57
Glycine*	92.27	23.24	-9.02	-9.40	-6.58	9.85	10.6	6.85	9.42	3.86
GSSG*	17.34	0.88	-7.86	6.09	6.92	8.84	3.47	1.71	9.96	1.68
Serine*	-16.78	-5.84	-2.01	-0.10	1.53	9.61	6.05	1.89	1.70	1.26
Taurine*	41.25	5.22	-3.50	-6.20	-0.99	10.4	4.55	7.50	9.43	11.7

Shaded=failed to meet validation criteria

Table 5.3-7 Within Batch Accuracy and Precision Batch 3

Compound	Accuracy- Bias (%)					Precision- Coefficient of Variance (%)				
	LLOQ 10ng/ mL	LQC 30ng/ mL	MQC 100ng /mL	HQC 400ng /mL	ULOQ 1000 ng/mL	LLOQ 10ng/m L	LQC 30ng/ mL	MQC 100ng/ mL	HQC 400ng/ mL	ULOQ 1000 ng/mL
2-amino- butyrate	-13.83	-7.50	-4.17	-4.73	-23.22	26.05	16.56	13.55	7.58	9.05
Betaine	-77.00	-0.83	11.35	4.03	-11.78	31.35	7.56	6.07	2.46	4.67
Choline*	-10.67	1.56	1.80	-2.20	-14.08	9.62	4.06	5.67	3.22	5.96
Cystathionine*	-2.83	-3.33	-5.77	-5.35	-7.47	1.77	5.35	5.55	4.29	4.85
Cysteic acid	-11.83	-6.28	-11.10	-8.76	-11.29	8.83	7.35	7.60	2.62	9.21
GSH	3.83	-1.67	-5.65	-6.81	-9.70	8.60	2.35	6.77	2.83	6.83
γ-glutamyl- cysteine	-0.83	-2.39	-4.63	-4.81	-8.62	7.42	3.48	5.60	3.48	7.03
Homocystine	-6.50	-12.06	-15.60	-14.42	-18.05	12.70	10.92	10.92	9.17	13.73
Ophthalmic acid	-1.17	-2.28	-4.03	-4.37	-11.87	4.41	4.30	5.31	2.37	4.09
SAH	5.00	-5.56	-11.72	-10.70	-13.10	3.24	4.01	4.34	2.98	6.16
SAM	219.33	32.83	-26.15	-20.80	-34.20	3.81	13.11	11.25	26.70	43.33
Compound	LLOQ 20ng/ mL	LQC 60ng/ mL	MQC 200ng /mL	HQC 800ng /mL	ULOQ 2000 ng/mL	LLOQ 20ng/m L	LQC 60ng/ mL	MQC 200ng/ mL	HQC 800ng/ mL	ULOQ 2000 ng/mL
Cysteine	41.25	5.22	-3.50	-6.20	-0.99	10.41	4.55	7.50	9.43	11.71
Cysteinyglycine	1.75	-3.11	-4.24	-3.02	-10.02	7.67	4.14	4.12	4.32	6.03
Cystine*	14.83	8.39	-0.89	0.44	-1.87	1.50	3.85	3.38	3.43	6.06
Glutamic acid	-12.42	-2.22	-4.73	-8.25	-14.77	11.73	9.26	8.44	4.88	6.85
Homoserine	15.42	-1.92	-3.63	-4.58	6.18	12.51	8.69	7.02	11.73	12.74
Hypotaurine*	6.13	-9.52	-3.72	-4.32	-6.48	10.91	10.88	7.63	4.92	5.54
Methionine*	-16.83	-1.44	-1.14	-0.94	-7.19	5.69	5.26	6.30	3.78	6.03
Pyroglutamic acid *	2.75	-3.69	-7.74	-6.23	-8.05	7.20	5.92	6.38	3.20	5.31
Compound	LLOQ 200ng /mL	LQC 600ng /mL	MQC 2000n g/mL	HQC 8000n g/mL	ULOQ 20μg/ mL	LLOQ 200ng/ mL	LQC 600ng/ mL	MQC 2000ng /mL	HQC 8000ng /mL	ULOQ 20μg/ mL
Glutamine*	0.29	-6.00	-3.40	-5.96	-6.97	7.97	7.77	8.29	12.94	18.85
Glycine*	47.86	-9.40	-24.88	-10.57	-3.35	32.72	12.30	15.48	12.26	8.04
GSSG*	-	-	-18.72	7.49	2.35	-	-	11.26	10.72	6.46
Serine*	12.17	-5.25	-10.08	-6.92	-7.97	11.87	7.63	7.57	3.73	5.60
Taurine*	-16.20	-3.46	0.03	0.24	-8.06	12.83	6.35	7.42	5.91	6.40

Shaded=failed to meet validation criteria

Table 5.3-8 Between-batch Accuracy and Precision

Compound	Accuracy- Bias (%)					Precision- Coefficient of Variance (%)				
	LLOQ 10ng/ mL	LQC 30ng/ mL	MQC 100ng /mL	HQC 400ng /mL	ULOQ 1000 ng/mL	LLOQ 10ng/m L	LQC 30ng/m L	MQC 100ng/ mL	HQC 400ng/ mL	ULOQ 1000 ng/mL
2-amino- butyrate	-25.50	7.72	20.72	18.44	-5.57	28.98	18.52	20.27	18.95	18.83
Betaine	-67.07	4.44	15.94	7.55	-8.44	30.77	6.73	5.46	3.71	4.47
Choline*	-15.28	3.00	7.29	3.75	-5.18	9.79	4.38	7.22	5.37	8.39
Cystathionine*	-0.67	3.54	2.96	4.29	9.90	4.48	7.39	8.85	8.59	12.49
Cysteic acid	-4.17	-0.82	-3.09	1.03	-0.66	10.39	11.08	11.48	11.00	13.01
GSH	3.44	3.33	0.75	2.12	0.75	8.20	10.00	10.79	10.57	12.61
γ-glutamyl- cysteine	-4.39	1.50	2.38	4.44	-0.13	6.34	6.47	9.15	8.59	10.61
Homocystine	-1.50	0.39	0.13	2.77	1.94	9.73	13.39	15.65	15.51	18.49
Ophthalmic acid	-0.39	5.63	5.63	6.76	-2.54	4.51	9.30	10.31	10.12	10.92
SAH	2.89	-0.15	-2.26	0.96	1.11	17.09	13.38	12.93	13.52	14.47
SAM	136.91	-11.71	-41.76	-25.51	-1.49	40.18	41.35	32.59	32.73	43.58
Compound	LLOQ 20ng/ mL	LQC 60ng/ mL	MQC 200ng /mL	HQC 800ng /mL	ULOQ 2000 ng/mL	LLOQ 20ng/m L	LQC 60ng/m L	MQC 200ng/ mL	HQC 800ng/ mL	ULOQ 2000 ng/mL
Cysteine	22.39	-1.34	-3.84	-0.32	5.32	13.98	8.87	10.49	10.16	9.09
Cysteinyglycine	-2.75	-3.47	-1.43	2.11	-2.08	8.59	4.08	5.19	6.11	9.56
Cystine*	12.19	13.09	7.08	6.19	9.23	6.65	5.97	8.29	6.27	8.74
Glutamic acid	-6.53	8.87	9.85	8.25	1.42	9.86	12.26	14.50	14.24	15.00
Homoserine	16.25	-3.11	-7.09	-2.63	9.26	11.67	7.22	7.74	7.76	7.68
Hypotaurine*	3.76	-2.34	4.45	6.65	6.93	12.05	9.13	4.99	3.06	1.89
Methionine*	-7.50	0.27	-0.12	0.47	-1.87	10.10	4.91	4.89	3.36	5.77
Pyroglutamic acid *	-4.56	-4.07	-4.65	-3.21	-6.27	12.78	3.95	5.44	3.65	4.89
Compound	LLOQ 200ng/ mL	LQC 600ng /mL	MQC 2000n g/mL	HQC 8000n g/mL	ULOQ 20,000 ng/mL	LLOQ 200ng/ mL	LQC 600ng/ mL	MQC 2000ng /mL	HQC 8000ng /mL	ULOQ 20g/m L
Glutamine*	-2.26	-4.00	-2.22	-0.64	-1.51	9.12	6.26	8.61	11.78	12.17
Glycine*	48.79	2.18	-14.65	-6.64	-1.18	32.41	18.70	13.21	10.69	9.60
GSSG*	-0.17	-8.18	-13.58	3.68	1.10	21.02	11.96	9.13	10.39	7.72
Serine*	-2.28	-5.80	-5.57	-2.21	-1.17	15.83	6.04	6.87	4.38	6.00
Taurine*	-20.46	0.07	3.38	3.21	-1.11	13.83	6.00	8.15	4.51	6.42

Shaded=failed to meet validation criteria

5.3.4 Carryover

The calculated percentage carryover for each analyte is shown in Table 5.3-9. Carryover response was below 20% of the LLOQ for each compound excluding GSSG, and glycine (26%) and homocystine (22%). For the internal standards carryover was below 1 % for all compounds.

Table 5.3-9: Carryover Detected in a Double Blank Following a ULOQ Standard

Compound	Carryover Response	Average LLOQ Response	Carryover (%)
2-amino-butyrate	-	1977	-
Betaine	-	41946	-
Choline*	144	25466	1
Cystathionine*	3949	20380	19
Cysteic acid	-	1680	-
γ-glutamyl-cysteine	208	N.D	N.D
GSH	1892	37998	5
Homocystine	1496	6894	22
Ophthalmic acid	2651	51226	5
SAH	1048	9053	12
SAM	1831	N.D	N.D
Compound	Carryover Response	Average LLOQ Response	Carryover (%)
Cysteine	-	1636	-
Cysteinyl-glycine	-	13744	-
Cystine*	1291	7307	18
Glutamic acid	-	44620	-
Homoserine	-	1844	-
Hypotaurine*	10	807	1
Methionine*	-	7526	-
Pyroglutamic acid *	-	7967	-
Compound	Carryover Response	Average LLOQ Response	Carryover (%)
Glutamine*	3061	83385	4
Glycine*	37	141	26
GSSG*	3931	4308	91
Serine*	57	2197	3
Taurine*	-	1465	-

Shaded=failed to meet validation criteria, “-” indicates the response was below the limit of detection. N.D indicates value was not determined.

5.3.5 Stability

5.3.5.1 Autosampler Stability

The stability of the compounds in the autosampler were tested over a 36h time period, by reanalysing Batch 2 QCs with a freshly made curve. The results are shown in Table 5.3-10. There were no compounds that had worse accuracy or stability after 36h in the autosampler.

Table 5.3-10 Accuracy and Precision of QCs re-analysed after 36h in autosampler

Compound	Accuracy- Bias (%)					Precision- Coefficient of Variance (%)				
	LLOQ 10ng/ mL	LQC 30ng/ mL	MQC 100ng /mL	HQC 400ng /mL	ULOQ 1000 ng/mL	LLOQ 10ng/m L	LQC 30ng/ mL	MQC 100ng/ mL	HQC 400ng/ mL	ULOQ 1000 ng/mL
2-amino- butyrate	8.50	9.33	6.64	4.48	-7.20	9.23	6.00	6.64	4.48	2.67
Betaine	-49.83	17.06	24.75	11.88	-2.41	14.83	2.52	1.07	1.89	3.94
Choline*	-11.67	6.56	-2.34	9.13	-6.41	3.63	2.52	-2.34	9.13	4.22
Cysteic acid	3.00	6.78	4.48	8.76	9.01	6.35	4.52	2.66	3.07	3.84
GSH	-5.50	5.89	6.67	4.41	5.85	3.40	1.25	1.05	1.58	3.20
γ-glutamyl- cysteine	13.17	5.94	8.00	6.77	9.43	9.38	3.70	2.56	2.00	3.26
Homocystine	-1.50	1.83	1.60	4.27	7.13	4.62	4.90	3.39	2.39	4.77
Ophthalmic acid	0.50	7.22	7.37	6.91	2.77	3.60	1.92	1.96	1.75	2.18
SAH	4.50	1.33	-0.07	2.32	5.83	4.79	3.03	1.84	2.19	3.74
SAM	24.75	-35.44	-48.17	-5.03	13.45	8.26	18.34	47.75	18.22	20.46
Compound	Accuracy- Bias (%)					Precision- Coefficient of Variance (%)				
	LLOQ 20ng/ mL	LQC 60ng/ mL	MQC 200ng /mL	HQC 800ng /mL	ULOQ 2000 ng/mL	LLOQ 20ng/m L	LQC 60ng/ mL	MQC 200ng/ mL	HQC 800ng/ mL	ULOQ 2000 ng/mL
Cysteine	4.25	-13.72	-2.72	1.94	10.95	11.79	11.11	9.20	6.53	4.61
Cysteinyl- glycine	-5.33	-7.19	-3.87	1.12	0.13	4.05	7.10	2.40	1.28	1.47
Cystine*	17.50	19.03	8.01	5.14	7.93	6.40	6.19	4.26	2.86	3.89
Glutamic acid	-20.17	6.19	11.12	7.19	2.48	8.85	3.19	2.85	3.97	3.12
Homoserine	7.58	-5.64	-5.10	-4.08	10.94	8.33	9.14	6.30	7.39	4.54
Hypotaurine*	3.76	-2.34	4.45	6.65	6.93	12.05	9.13	4.99	3.06	1.89
Methionine*	-14.75	6.64	7.23	5.26	2.50	11.51	4.48	2.44	2.99	2.07
Pyroglutamic acid *	-15.58	1.61	-1.04	-0.26	2.06	3.25	8.09	1.61	2.33	1.65
Compound	Accuracy- Bias (%)					Precision- Coefficient of Variance (%)				
	LLOQ 200ng /mL	LQC 600ng /mL	MQC 2000n g/mL	HQC 8000n g/mL	ULOQ 20μg/ mL	LLOQ 200ng/ mL	LQC 600ng/ mL	MQC 2000ng /mL	HQC 8000ng /mL	ULOQ 20μg/m L
Glutamine*	-11.63	6.09	9.20	3.21	18.63	16.26	14.90	4.36	10.24	11.53
Glycine*	20.96	-9.18	-1.08	2.59	13.98	14.9	21.9	15.25	9.51	8.70
GSSG*	-12.04	-9.77	-2.22	5.47	4.37	17.84	5.65	13.35	4.92	2.61
Serine*	24.68	-1.90	0.44	2.99	4.59	6.06	5.69	3.84	1.99	0.72
Taurine*	2.96	-3.75	8.72	5.17	-2.58	17.61	7.27	6.06	3.23	2.13

5.3.6 Validation overview

Overall the same four compounds performed poorly across validation criteria, 2-amino butyrate, glycine, SAM and GSSG. In addition, betaine performed badly at the LLOQ. The remaining 19 compounds showed good linearity, precision and accuracy, selectivity and stability.

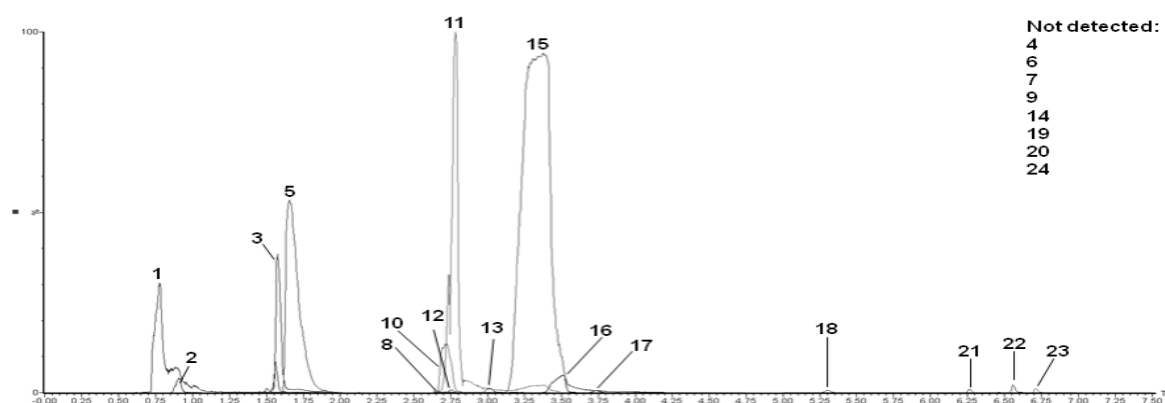
Table 5.3-11: A visual overview of pass or failure of the different criteria for each

Compound	Validation criteria assessed				
	Linearity	Precision and accuracy	Carry-over	Selectivity	36h stability
2-amino-butyrate					
Betaine					
Choline*					
Cystathionine*					
Cysteic acid					
γ-glutamyl-cysteine					
GSH					
Homocystine					
Ophthalmic acid					
SAH					
SAM					
Cysteine					
Cysteinyl-glycine					
Cystine*					
Glutamic acid*					
Homoserine					
Hypotaurine*					
Methionine*					
Pyroglutamic acid*					
Glutamine*					
Glycine*					
GSSG*					
Serine*					
Taurine*					

Red indicates failed acceptance criteria as defined in the methods failed, green is passed, orange is marginal failure

5.3.7 Application of the assay to rat plasma samples

Rats (n=46 total) were treated with equimolar doses of TA, TAI or vehicle, plasma collected at 2h, 6h and 24h as detailed in Chapter 3. A representative chromatogram is shown in Figure 5.3-4 of what a rat plasma sample at 2h from control. Metabolites that were below the limit of detection across the groups included: cysteinyl-glycine, cysteine, γ -glutamyl-cysteine, cysteic acid, homocysteine, reduced glutathione and oxidised glutathione. The corrected concentrations (x5 for dilution factor) for the remaining metabolites are shown in Figure 5.3-7, where the upper and lower limits of quantification (LLOQ and ULOQ) are shown where samples have exceeded these boundaries. For all samples 2-amino butyrate fell below the LLOQ, the LLOQ was also too low for the quantification of ophthalmic acid and SAH in several samples. Compounds that had many samples exceeding the ULOQ included betaine, homoserine, glutamine, and methionine. A few samples also exceeded the ULOQ for hypotaurine, glutamate and pyroglutamic acid.



1	Choline	9	γ -Glutamyl-cysteine	17	Hypotaurine
2	Pyroglutamic acid	10	Homoserine	18	S-adenosyl-homocysteine
3	Methionine	11	Glutamic acid	19	Cysteic acid
4	Cysteinyl-glycine	12	Taurine	20	Homocystine
5	Betaine	13	Ophthalmic acid	21	S-adenosyl-methionine
6	2-Amino butyrate	14	Glutathione, reduced	22	Cystathionine
7	Cysteine	15	Glutamine	23	Cystine
8	Glycine	16	Serine	24	Glutathione, oxidised

Figure 5.3-4 Representative chromatogram of compounds in rat plasma- showing 0-7.5min of 15min run.

5.3.7.1 The comparative impact of TA and TAI on plasma GSH metabolites

Ophthalmic acid was below the LOQ at all time points, so concentrations obtained can only be considered as approximate values. Ophthalmic acid was elevated in TAI treated animals at 2h (median control= 32ng/mL, TA=34.5ng/mL and TAI=90ng/mL), which was found to be statistically significant ($p<0.05$) by Mann Whitney test. At 6h OA in both TA and TAI treated animals was significantly elevated (median control= 17.5ng/mL, TA=175.5ng/mL and TAI=112.5ng/mL). At 24h OA had decreased below the LOQ in all groups.

Pyroglutamic acid was significantly elevated ($p<0.05$) at 2h in the TAI treated animals, (median control= 3859ng/mL, TA=3521ng/mL and TAI=6376ng/mL). At 6h there was a greater elevation in TAI treated animals, and a slight but significant elevation in TA group (median control= 3559ng/mL, TA=5564ng/mL and TAI=10908ng/mL). At 24h pyroglutamic acid from TAI treated animals remained significantly elevated (median control= 3640ng/mL, TA=3810ng/mL and TAI=8284ng/mL).

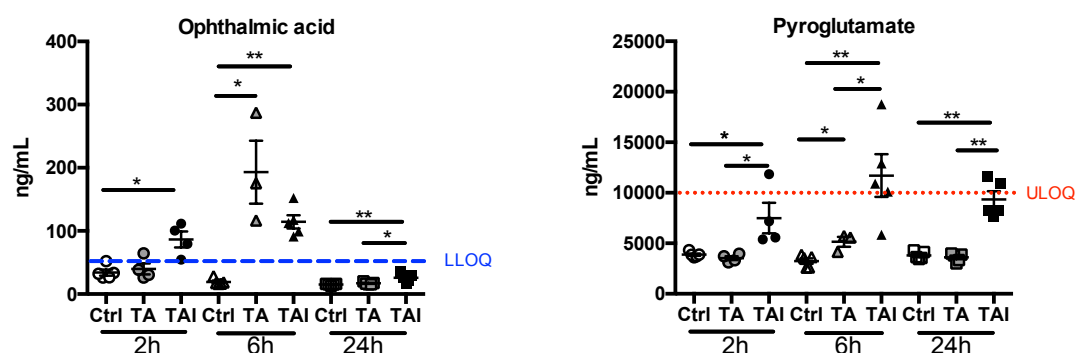


Figure 5.3-5 Plasma concentrations of ophthalmic acid and pyroglutamic acid following treatment with TA or TAI

At 2h there were three statistically significant and unique changes in the TA v Ctrl treated animals compared to TAI v Ctrl treated group, these included small depletions in glutamate and glutamine and an increase in hypotaurine (Figure 5.3-6 and Figure 5.3-7). The median hypotaurine concentration was detected as 11.6 μ g/mL in the TA-treated animals, almost double the concentration detected in vehicle (5.7 μ g/mL) and TAI (4.6 μ g/mL) treated animals. In addition to the unique elevations in pyroglutamic acid and ophthalmic acid, there were three statistically significant changes that were only found in the TAI samples at this time-point. These included a depletion in serine and elevations in SAM and cystine (Figure 5.3-6 and Figure 5.3-7). At 2h there were no statistically significant alterations in both TA v Ctrl and TAI v Ctrl.

At 6h the only significant changes observed in the TA treated group were the small elevation in pyroglutamic acid and larger elevation in ophthalmic acid that were also seen in response to TAI. However, there were four unique and statistically significant alterations in the TAI treated animals. These included elevations in glycine, glutamine, cystine and taurine (Figure 5.3-6 and Figure 5.3-7).

At 24h there were four statistically significant alterations that were unique to TA treated animals. These include decreases in choline, methionine, cystathionine and taurine (Figure 5.3-6 and Figure 5.3-7). Unique alterations in response to TAI included elevations in cystine, cystathionine, methionine, pyroglutamic acid, SAM and SAH. Three shared alterations were also observed, including depletion in betaine, glycine and hypotaurine (Figure 5.3-6 and Figure 5.3-7).

Venn diagrams highlighting the common and unique statistically significant alterations in these metabolites is shown in Figure 5.3-8.

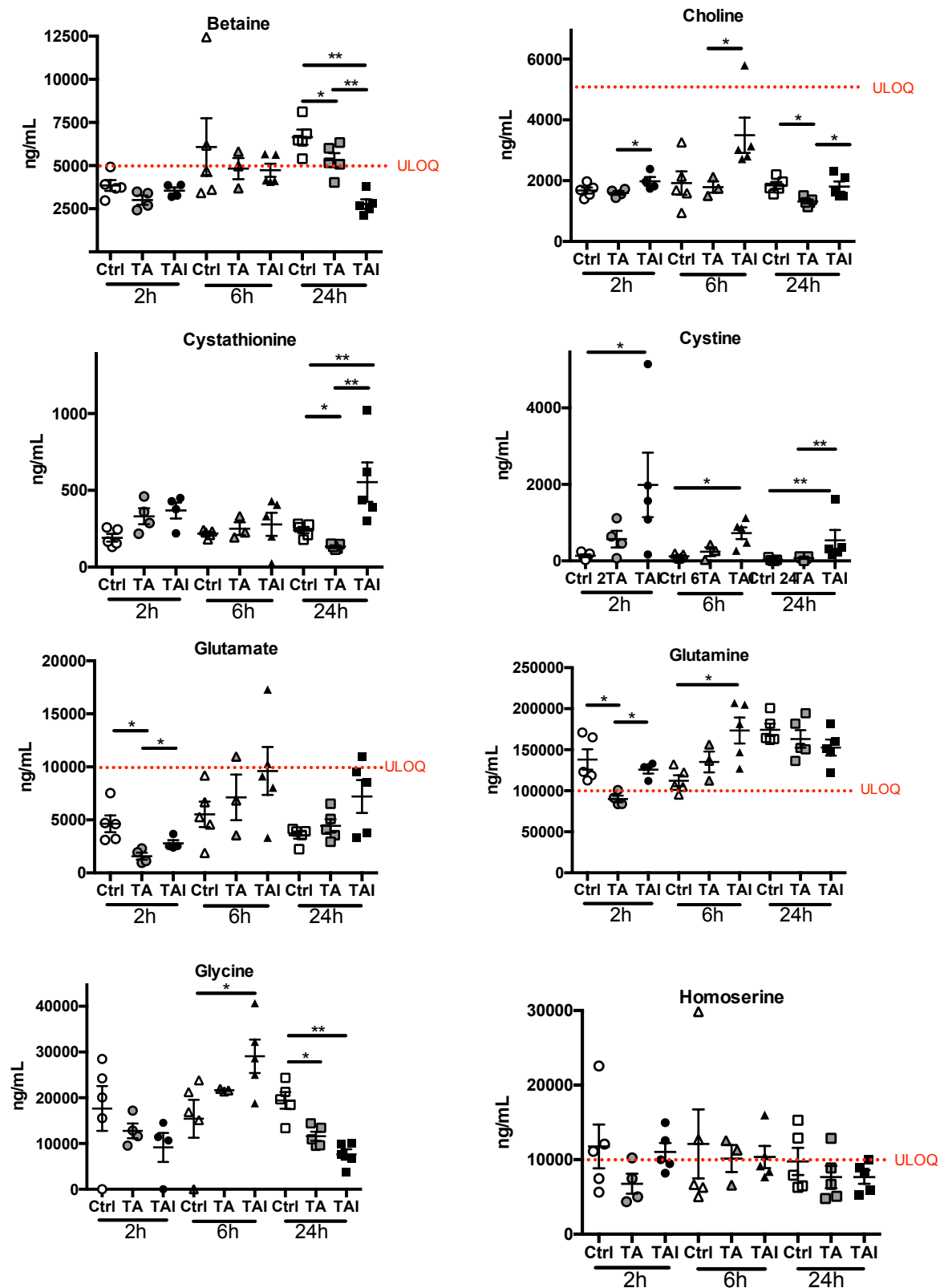


Figure 5.3-6 The corrected (for 5x dilution during sample preparation) plasma concentrations of glutathione related metabolites in plasma, shown in ng/mL. LLOQ marks the corrected lower limit of quantification. ULOQ indicates the corrected upper limit of quantification. Lines indicate the mean, error bars indicate S.E.M..

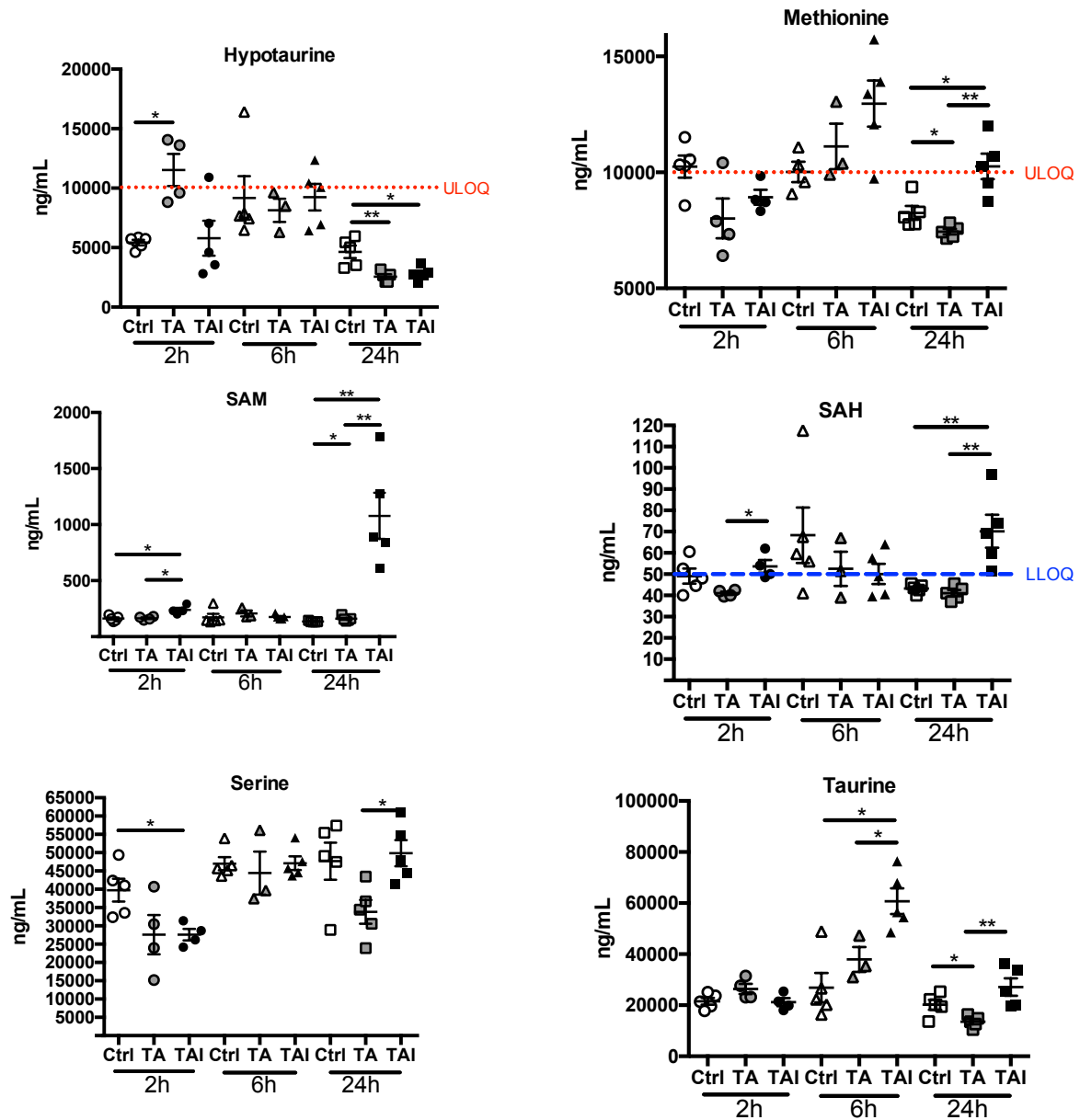


Figure 5.3-7 The corrected (for 5x dilution during sample preparation) plasma concentrations of glutathione related metabolites in plasma, shown in ng/mL. LLOQ marks the corrected lower limit of quantification. ULOQ indicates the corrected upper limit of quantification. Lines indicate the mean, error bars indicate S.E.M..

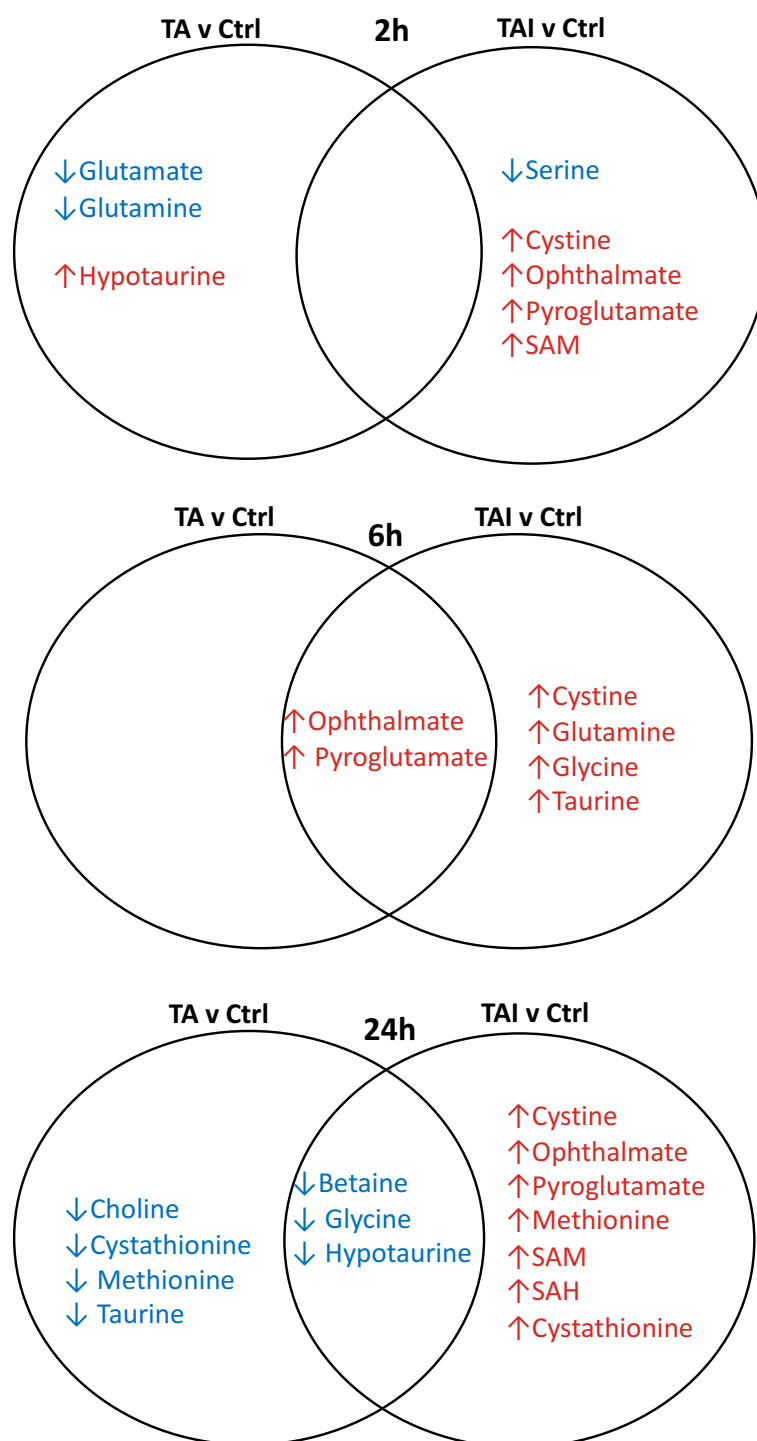


Figure 5.3-8 Venn diagrams showing unique and shared significantly elevated or depleted metabolites in TA or TAI treated animals compared to vehicle treated animals. Significance was determined by a Two-tailed Mann-Whitney test.

5.4 DISCUSSION

To further understand the comparative impact of TA and TAI on glutathione metabolism, and look how this was reflected in plasma, the aim of this work was to develop and validate a targeted LC-MS/MS assay to simultaneously quantify metabolites linked to GSH metabolism in rat plasma. Whilst other quantitative methods exist to quantify many of the selected metabolites, a single, high-throughput method to cover the full range of metabolites has not previously been reported. It was necessary to develop a single method to cover as many metabolites as possible, due to restricted sample volumes, as well as the time and resource investment required for implementing multiple methods for each analyte.

5.4.1 Chromatography

Ion-pair chromatographic methods have previously been shown to be useful in profiling polar metabolites, such as the method utilised in Chapter 4 (Michopoulos et al., 2014). However, as ion-pairing reagents are known to permanently contaminate the mass spectrometer, other options were investigated. Reversed phase columns (e.g. HSS T3 or C18) are more commonly used in metabolomic studies, but are unsuitable here due to the large number of very polar metabolites. Although derivatisation of the compounds may have been an option, a single derivatising agent may not have been suitable, as the chemical structure of these compounds varied widely and would have greatly increased the sample preparation time. A HILIC approach was therefore preferred, and BEH HILIC and BEH Amide columns were compared. As previously reported for similar compounds, the BEH Amide column provided superior chromatography in terms of peak shape and retention (Gika et al., 2012).

Although solvent buffers are often used in HILIC (such as by Gika et al., 2012), others have reported good chromatography using 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile as mobile phases (Paglia G, 2013). An adapted version of this method developed by Paglia et al was found to be optimal for the current compounds. Alterations included increasing the concentration of formic acid from 0.1% to 0.2 %, and altering the gradient to reduce carryover and improve peak shape. Although the final analytical conditions selected were optimal for the majority of the compounds, glycine and 2-amino butyrate had very asymmetric peak shapes, and GSSG often resulted in a broad and tailing peak, and likely contributed to their poor performance during the validation experiments.

Overall, the chromatographic method developed requires simple sample and solvent preparation, provides sufficient retention, resolution and peak shape, and the 15min run time ensures it is appropriate for high-throughput analysis.

5.4.2 Validation

Evaluation of the method was based on FDA Guidance for Bioanalytical Method Validation (FDA, 2001, FDA, 2015, FDA, 2013), and included an assessment of assay selectivity, accuracy, precision, carryover, and short-term stability. The FDA guidelines were developed as recommendations for bioavailability, bioequivalence and pharmacokinetic studies reported in new drug applications, which are predominantly quantitative assays for a single exogenous metabolite. Although the same parameters are relevant for the purposes of validating the present assay, as these data are not being used in a clinical or regulatory context, there is a lower requirement for absolute accuracy and precision. Therefore, the acceptance criteria were only used as a benchmark to highlight the limitations of the assay, and not for absolute pass and fail categorisations.

Selectivity was evaluated here by assessing interference between the matrix, other analytes, and the internal standards. Overall, the selectivity of this assay appears to be acceptable, with the exception of GSSG influencing its internal standard. However, the impact of the matrix was only studied using solvent as the matrix. This was because the metabolites of interest are endogenous, so making the curve and QCs in plasma would have been impacted by any endogenous analyte present. Other approaches have previously been used by others, such as using diluted plasma as the QC/calibration curve matrix and making adjustments for the endogenous analyte present (Klepakic et al, 2013), and a similar approach may be useful to further validate this assay. In the current validation, the use of two fragments per compound in the mass spectrometer; one for identification and one for quantification, should reduce the chance of co-eluting endogenous metabolites of a similar mass impacting quantification. In addition, the use of internal standards, should compensate for ion suppression by other compounds in the plasma, however, to further validate the assay, this should be assessed by studying matrix effects. This could be achieved by spiking an equal concentration of internal standard into a prepped sample and solvent blank, and comparing the concentrations detected.

The inter and intra-day precision and accuracy of 19 of 24 compounds was acceptable with bias and coefficient of variation rarely exceeding 20%. Compounds with poor accuracy and precision included 2-aminobutyrate, betaine, glycine, GSSG and SAM. As previously mentioned, 2-aminobutyrate, glycine and GSSG had poor peak shape, making accurate quantification less likely. It may be more appropriate to monitor these compounds, and not attempt to quantify them using this assay. For betaine, the LLOQ precision and accuracy was poor, but acceptable at the higher concentrations, therefore a higher LLOQ would be optimal. The other metabolite that failed the validation criteria was SAM, and this may be improved with the introduction of an internal standard. Carryover was within (or close to) acceptable limits for all metabolites, except for GSSG, where a ULOQ far higher than biologically likely, and should therefore be lowered.

The short term (36h) stability of processed samples in a 4°C autosampler was also studied. None of the analytes showed less accurate or precise quantification after 36h. Other areas of stability that remain to be assessed include: freeze and thaw, bench-top, long-term and stock solution stability.

One of the biggest areas for improvement for the assay would be optimising the range of concentrations covered, as these were not low enough for some compounds, and not high enough for others. For the compounds that were detected in plasma above the validated ULOQ (betaine, homoserine, glutamine, methionine, hypotaurine, glutamate and pyroglutamic acid) this could be re-validated with a higher ULOQ. Where only a few samples are likely to exceed the ULOQ, or where increasing the ULOQ would exceed the linear range, evaluation of the dilution integrity of compounds would enable the dilution and reanalysis of samples that fall above the ULOQ.

For compounds that were detected but fell below the LLOQ, the standard curves could be lowered (e.g. OA and SAH). However, where compounds were below the limit of detection (cysteine, cysteinyl-glycine, γ -glutamyl-cysteine, reduced glutathione, cysteic acid, homocystine, oxidised glutathione), adjustments in sample preparation would be required. For example, the recovery during sample preparation should be assessed further (comparison of equal concentrations of IS in solvent and IS spiked in plasma prior to extraction, minus IS spiked into an extracted plasma sample, to account for loss due to ion suppression) and optimized (e.g. different extraction solvent, or drying down sample following protein precipitation).

Whichever approach is taken, this may result in some of the other metabolites falling above their linear concentration range. Therefore, it may not be possible to quantify all of these compounds in plasma using a single sample preparation and this assay.

5.4.3 Application: The impact of TA and TAI on plasma GSH related metabolites

The elevations observed in Chapter 4 with respect to pyroglutamic acid and ophthalmic acid were replicated. Additionally, the concentrations observed in this study were similar with previous reports in male Sprague Dawley rats using two alternative assays (Geenen et al., 2011b, Geenen et al., 2011a). In that work the authors studied the impact of chronic dosing of methapyrilene on plasma pyroglutamic acid and ophthalmic acid. They found control ranges of pyroglutamic acid of 2500- 4500ng/mL and 30-60ng/mL of ophthalmic acid with little elevation in either metabolite in response to drug treatment. Control ranges of pyroglutamic acid in the present study were between ~3000-4000ng/mL, rising to over 10 000ng/mL in response to TAI. Control ranges of ophthalmic acid in this study were extrapolated as between 15 and 50ng/mL which were below the LLOQ of 50ng/mL, this rose to over 100-200ng/mL in response to both TA and TAI. The advantage of the present assay is it quantifies both metabolites, as well as others, in a single run, which is particularly important for the application of this method in pre-clinical models of toxicity where 'in-life' plasma samples are taken that are low volume.

There were a number of other perturbations in the related metabolites studied, showing overall despite both depleting hepatic glutathione the impact on related metabolites in plasma was very different.

The only unique elevation observed in response to TA was in plasma hypotaurine concentrations. Interestingly, the elevation in hypotaurine in TA samples reflects a similar pattern as the hepatic hypotaurine content detected by NMR (Coen et al., 2012), including a unique elevation at 2h in TA treated animals. Elevated hypotaurine has been reported in liver and urine following partial hepatectomy (Sturman, 1980, Brand et al., 1998), indicating their potential markers of liver regeneration, suggested that it indicates a reduced capacity for oxidation of hypotaurine to taurine (Sturman et al., 1982).

The further break down product of hypotaurine, taurine, was elevated at 6h in response to TAI, but uniquely depleted in TA samples at 24h. Both taurine and hypotaurine perturbations have

been reported in response to other hepatotoxins, and are suggested to reflect altered glutathione homeostasis (Yamazaki et al., 2013). In that study of 13 hepatotoxins in the rat at 2 day and 5 day time points, the authors reported a pattern of depleted taurine associated with elevated pyroglutamic acid and ophthalmic acid, suggesting methionine/cysteine was preferentially shunted to glutathione not taurine pathway. If that hypothesis is correct, the depleted taurine in TA may be indicative of TA inducing a more adaptive response by 24h.

Cystine and methionine are other sulphur containing amino acids that are essential in GSH biosynthesis, and methionine has been shown to impact pyroglutamic acid and ophthalmic acid concentrations (Geenen et al., 2013a). Using this assay cystine was found to be elevated at all time points in response to TAI, and methionine was also elevated in TAI at 24h, whereas it was depleted in TA samples. Methionine and cystine also measured using the IPC-MS/MS approach (Chapter 4) where both were found to be depleted in plasma. This discrepancy could be related to ion suppression effects as both methionine and cystine elute at the start of the run 0.6 and 0.9 min, therefore they are more likely to be impacted by ion suppression caused by any metabolites not retained on the column. They were both well retained using the HILIC assay and internal standards were used to correct for any ion suppression, however as the assay has not yet been fully validated for extraction/recovery reproducibility, variability in this could provide another possible explanation.

Other sulphur containing metabolites linked to methionine included SAH, SAM and cystathionine, which were all found to be uniquely elevated in TAI treated animals at 24h. Although these elevations should be interpreted with caution as SAM failed validation, and SAH was below or close to the LLOQ, perturbations in cystathionine and SAH were also reported in the study of 13 other hepatotoxins (Yamazaki et al., 2013).

The concentration of betaine was depleted in both TA and TAI at 24h, although it was significantly more depleted in the TAI group. This was also observed by NMR in the liver, where betaine was shown to correlate with histopathological liver glycogen scores (as were glycogen and glucose detected by NMR). Betaine supplementation has previously been shown to alleviate ethanol induced liver injury and metabolomics changes in S-containing metabolites (Kim et al., 2008), and to influence hepatic glutathione content. In the plasma of TAI-treated group, there was a unique but non-significant alteration in the related metabolite, choline, this mirrors an elevation at 6h in the TAI group liver by NMR, and provides an example of the utility

of a more sensitive analytical approach. The precursor to betaine is choline, and in this study it was found to be uniquely depleted in response to TA at 24h. Supplementation with choline has been shown to improve GSH/GSSG balance (Innis et al., 2007).

Overall, despite both depleting hepatic GSH, and elevating OA, this work has demonstrated the very different impact TA and TAI have on metabolites linked to GSH. Consistent with the hypothesis, TAI had a greater impact on metabolites linked to GSH metabolism. As TA does not lead to toxicity in this model, metabolic changes observed in response to TA could be reflective of recovery/adaption, whereas those specific to TAI could be reflective of failure to adapt and resultant toxicity. Unravelling the mechanism behind these fluctuations, will be important in determining which are truly indicative of adaption/recovery, and which are indicative of poor adaption/ progression to toxicity.

5.4.4 Limitations and future work

Further development and validation experiments are required to ensure the reproducibility and accuracy of the assay developed, including experiments to test matrix effects, dilution integrity, recovery and an appropriate concentration range should be undertaken. The assay could also be improved by the addition of internal standards for all compounds, to ensure matrix effects are countered. The assay could be extended to include further polar metabolites, however, this could be limited by the poor peak shape that is widely reported with HILIC (Naidong, 2003), and thought to be caused by the multiple retention mechanisms interacting.

This study has demonstrated that monitoring a single biomarker, such as ophthalmic acid, may not accurately predict toxicity. However, as related metabolites were also impacted, and this appears to differ between animals that go on to suffer DILI and those that do not, determining the concentration of multiple metabolites may be useful in predicting DILI. Future studies will be required to determine if perturbations in multiple metabolites can be used to determine hepatic oxidative stress or risk of DILI. Further work into the stability, time course, and relationship between these different metabolites in response to a toxin insult will be important in establishing their utility to accurately/robustly monitor hepatic glutathione status or risk of hepatotoxicity. To further explore these metabolites, work in the next chapter involved the quantification of these metabolites in rats treated with the model hepatotoxin paracetamol (APAP).

RESULTS

CIRCULATING PLASMA CONCENTRATIONS OF OPTHALMIC
ACID, PYROGLUTAMATIC ACID AND RELATED METABOLITES
FOLLOWING PARACETAMOL DOSING IN THE RAT

6.1 INTRODUCTION

6.1.1 Rationale and aims

Paracetamol (acetaminophen, APAP) is a very widely used antipyretic and analgesic. APAP overdose is the leading cause of acute liver failure in the United States and United Kingdom (Lee, 2003), and a significant proportion are due to chronic overuse which are particularly challenging to identify. Therefore, there is an ongoing need for improved biomarkers for APAP induced DILI. APAP toxicity has been extensively studied, and due to its translatable mechanism of toxicity between mouse and man, is considered a model hepatotoxin, making it a useful drug for biomarker discovery and development (Kaplowitz, 2004a).

APAP is an intrinsic hepatotoxin; during APAP metabolism a reactive metabolite N-acetyl-p-benzoquinone (NAPQI) is produced, leading to the rapid depletion of the hepatic antioxidant glutathione (GSH). Depleted GSH can lead to NAPQI binding mitochondrial proteins resulting in intracellular stress and subsequent cellular dysfunction (Kaplowitz, 2004b). Previous metabonomic studies of APAP have revealed perturbations in circulating metabolites linked to GSH metabolism, including pyroglutamic acid and ophthalmic acid. Elevated ophthalmic acid has been reported in liver and plasma from APAP dosed mice (Soga et al., 2006) and rats (Yamazaki et al., 2013). Clinically, a more recent study found human serum from APAP overdose patients revealed ophthalmic acid elevated more often in non-survivors of APAP induced acute liver failure compared to survivors (Kaur et al., 2015). Similarly, pyroglutamic acid has also been linked to APAP induced hepatic glutathione depletion, with pyroglutamic acid accumulation in urine and plasma following both chronic and acute APAP dosing in the rat (Sun et al., 2009, Ghauri et al., 1993). Clinically pyroglutamic acid has also been linked to several cases of APAP induced toxicity (Fenves et al, 2006). This has led to interest in whether ophthalmic acid and pyroglutamic acid can act as systemic biomarkers of hepatic oxidative stress and/or APAP toxicity (Geenen et al., 2013a).

Interestingly, these perturbations in ophthalmic acid and pyroglutamic acid that have previously been observed in response to APAP, parallel work presented earlier in this thesis for the idiosyncratic toxin Tienilic acid (TA) and the intrinsic hepatotoxin, Tienilic acid Isomer (TAI). In Chapters 4, depleted hepatic glutathione (GSH) and elevated ophthalmic acid were found in response to TA and TAI. Conversely, pyroglutamic acid was only elevated in response to TAI.

To further our understanding of the knock-on impact of drug induced glutathione depletion a quantitative UPLC-MS/MS method for ophthalmic acid, pyroglutamic acid and related endogenous compounds in plasma was developed in Chapter 5. In the present chapter this quantitative method is used to study the impact of APAP dosing on plasma ophthalmic acid, pyroglutamic acid and related metabolites in the rat.

Although both ophthalmic acid and pyroglutamic acid have already been reported in response to APAP, further understanding about the timing and stability of these biomarkers in response to APAP is required. For example, *in vitro* work by Geenen et al., suggests that multiple biomarkers are required to accurately predict hepatic glutathione content, as both ophthalmic acid and pyroglutamic are affected by methionine availability (Geenen et al., 2013b). Quantitative data from *in vivo* studies such as this could be used to validate and improve the models such as those developed by Geenen et al., as this is yet to be tested and validated on *in vivo* data.

Although not the focus of this study, but to serve as an interesting comparator to the response observed in TA and TAI, a fuller range of endogenous compounds involved in energy and amino acid metabolism were also measured using the ion-pair approach previously applied in Chapter 4 (Michopoulos et al., 2014). As one of the major pathways effected in response to TA and TAI dosing was tryptophan metabolism, a secondary aim of this work was to study the impact of APAP dosing on tryptophan metabolism. Although the animal study designs were different, it provides a useful comparison for the TA/TAI study to look at a well-studied model hepatotoxin which also perturbs glutathione homeostasis.

6.1.2 Hypothesis

The hypothesis of this Chapter is that APAP induced glutathione depletion would perturb the concentration of circulating metabolites linked to glutathione, including increases in the circulating concentrations of ophthalmic acid and pyroglutamic acid.

6.2 MATERIALS AND METHODS

6.2.1 Contributions of others

The animal study, sample collection, histopathology and clinical chemistry analysis were undertaken by collaborators at Drug Safety, AstraZeneca, UK. The application of an IPC-MS assay was conducted by the author whilst at Oncology iMed, AstraZeneca. All remaining analytical sample preparation, UPLC-MS/MS analysis and the related data processing were performed by the author at Imperial College, London.

6.2.2 Animal handling and sample collection

6.2.2.1 Animals

36 male Wistar Han (CrI:WI(Han)) rats weighing 300-350g were purchased from Harlan Laboratories (Bicester, UK). Upon arrival animals were randomly assigned groups, and then acclimated to the facility for 14-16 days prior to the study commencing. Animals were housed under standard temperature (294–300 K) and humidity conditions (30-70%), with a 12 hour light dark cycle. Animals were provided with food and drinking water ad libitum.

6.2.2.2 APAP administration

Animals were orally dosed with APAP (Sigma, batch number SLBC6391V, purity determined as 100.3%) dissolved in 0.5% methylcellulose at doses of 500mg/kg or 1500mg/kg, or vehicle, according to the table of groups below.

Table 6.2-1 Animal groups and dosing

Group	Animal reference numbers	Treatment	Dose (parent) mg/kg	Necropsy Hours
Vehicle 4h	301 - 306	Vehicle	0	4
Low 4h	307 - 312	Paracetamol	500	4
High 4h	313 - 318	Paracetamol	1500	4
Vehicle 24h	319 - 324	Vehicle	0	24
Low 24h	325 - 330	Paracetamol	500	24
High 24h	331 - 336	Paracetamol	1500	24

6.2.2.3 Sample Collection

In life blood sampling from tail vein, took place at -1,1,2h post-dose for 4h necropsy groups, and at -1,4,8h post-dose for 24h necropsy groups. At each time-point approximately 0.8mL was placed into lithium heparin vacutainer tubes, and centrifuged to separate the plasma. 200µL of the plasma was aliquoted for APAP and biomarker analysis and stored at -70°C or below until analysis. The remainder was aliquoted for clinical pathology analysis, and stored at -20°C or below.

At termination, at either 4h or 24h, 2mL blood samples were taken from the vena cava into lithium heparin vacutainer tubes, and centrifuged to separate the plasma. 300µL of the plasma was aliquoted for APAP and biomarker analysis and stored at -70°C or below until analysis. The remainder was aliquoted for clinical pathology analysis, and stored at -20°C or below.

Sections of liver and kidney tissues were taken for histological examination. The remainder of the left lateral lobe of the liver and remaining liver lobes were fresh frozen in liquid nitrogen as two separate samples, and stored at -70°C until biomarker analysis.

6.2.2.4 Overview of study design

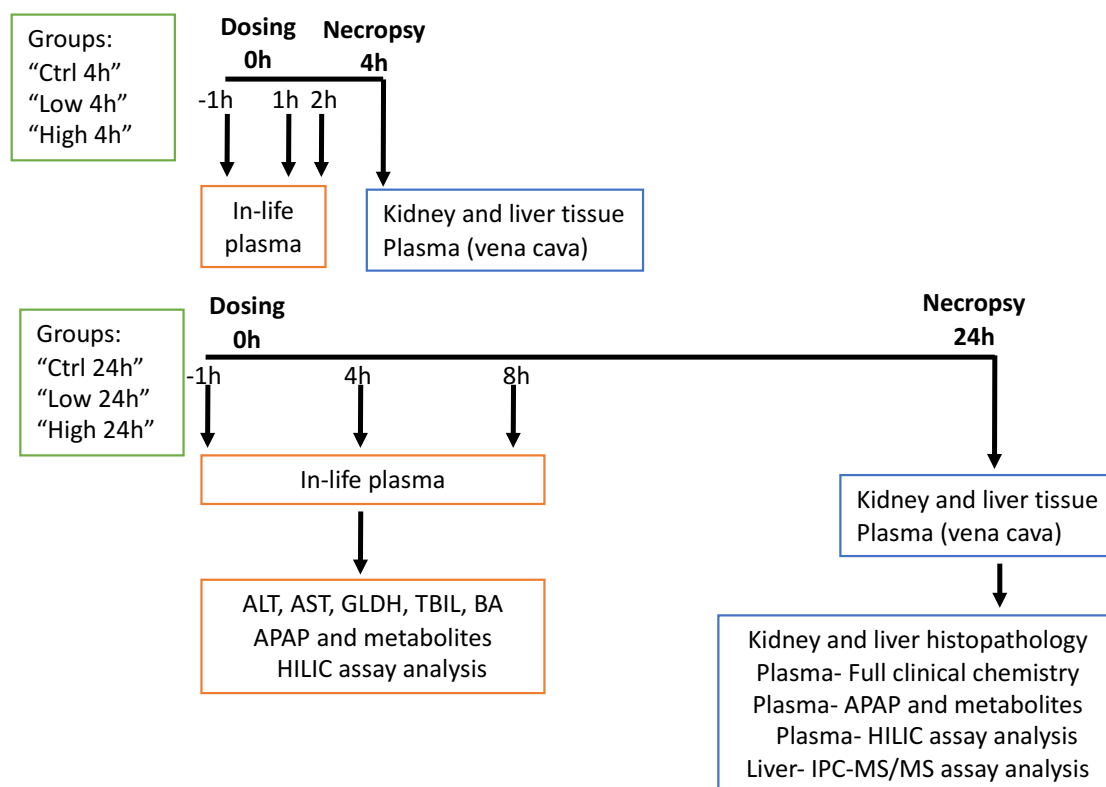


Figure 6.2-1. A figure depicting the study design, and sample collection points.

6.2.3 Histopathology and clinical chemistry

6.2.3.1 Kidney and liver histopathology

Sections of the left lateral lobe of the liver were cut approximately 3-4mm thick and immersed in 10% neutral buffered formalin, before being fixed for 48h prior to tissue processing into paraffin wax. Slices (4-5µm) thick were stained with haematoxylin and eosin (H&E) and examined by light microscopy for congestion, inflammation, vacuolation, and degeneration/necrosis. Slides were assessed on a scale from 0 (no abnormality detected), 1 (minimal), 2 (mild), 3 (moderate), 4 (marked) and 5 (severe). Kidney sections were examined for abnormalities and graded for hydronephrosis, dilation and unilateral basophilia, and marked using the same scaling system as the liver.

6.2.3.2 Clinical Chemistry

In-life samples were analysed on a Roche P Modulae analyser (Roche Diagnostics, Burgess Hill, West Surrey, UK) using an alanine transferase (ALAT/GPT) assay (Roche Diagnostics), total bilirubin (Roche Diagnostics), total bile acids (Alere, Stockport, UK), AST and GLDH, in line with manufacturers' instructions. Terminal plasma samples were analysed, in accordance with AstraZeneca protocols, for glucose, urea, creatinine, total protein, albumin, globulin, total bile acids, total bilirubin, conjugated bilirubin, cholesterol, triglycerides, ALP, ALT, AST, GLDH, total calcium, potassium and sodium levels.

6.2.4 UPLC-MS/MS of APAP and metabolites

6.2.4.1 Chemical standards and reagents

Paracetamol (APAP) was purchased from Sigma Aldrich, Gillingham, UK, whilst APAP-D3 was purchased from Toronto Research chemicals, Toronto, Canada. Optima LC-MS grade 0.1% formic acid in water was obtained from Fisher Scientific, Loughborough, UK. LC-MS Chromasolv grade methanol, LC-MS Chromasolv grade acetonitrile, and formic acid, approximately 98% for MS, were obtained from Sigma Aldrich, Gillingham, UK.

6.2.4.2 Calibration standard and quality control preparation

From a solution of 1mg/mL APAP in water, stock solutions for the standard curve were diluted in water to create concentrations of 1, 5, 10, 50, 100, 500, 1000, 5000 and 10 000ng/mL. Stock solutions for low, mid and high quality control samples (QCs) were made in water, at concentrations of 7.5, 200 and 8000ng/mL respectively. A stock solution for the internal

standard APAP-D3 was prepared at 100µg/mL in 1:10 (v:v) water/methanol.

For each point of the calibration curve and QCs, 20µL of control plasma, comprised of a composite of vehicle treated animals, was diluted in 170µL methanol and 10µL internal standard stock solution. Samples were kept at -20°C for 20 min to precipitate the proteins, before being centrifuged for 10min at 10 000g. 20µL of the supernatant was added to 880µL water and 100µL standard stock solution to create a standard curve with final concentrations of 0.1, 0.5, 1, 5, 10, 50, 500 and 1000 ng/ml, LQC 0.75ng/mL, MQC 20ng/mL and HQC of 800ng/mL. The upper limit of quantification QC (ULOQQC) was defined as the highest value on curve, and the lowest limit of quantification (ULOQQC) was defined as the lowest point of the curve. Solvents blanks, single blanks (plasma with internal standard, but no standard) and double blanks (plasma not spiked with standard or internal standard) were also prepared. The standard curve was run at the start, middle and end of the run, and the QCs were run at the start and at evenly spaced intervals throughout the run.

6.2.4.3 Sample preparation

Plasma samples were defrosted at room temperature and randomised prior to sample preparation. 10µL of plasma was added to 170µL of methanol and 100µL of internal stock solution. The samples were then kept at -20°C before being centrifuged at 10 000g for 10min. 20µL of the resultant supernatant was diluted in 980µL water, to create a final plasma dilution of 1:1000 and an internal standard concentration of 200ng/mL. Samples were re-randomised before 600µL was transferred into 96 well plates, prior to UPLC-MS analysis.

6.2.4.4 Chromatography

For APAP quantification Ultra Performance- Liquid Chromatography (UPLC) was performed on an Acquity Chromatography system, made up of a binary solvent manager, heated column manager, and an automated, temperature controlled, sample manager (Waters Corporation, MA, USA). For the duration of the experiment, samples were kept at 5°C. In each analysis, a 2 µL injection of plasma was injected onto a 2.1 X100mm 1.8um 130 A C18 ACQUITY HSS T3 column (Waters Corporation), maintained at 40°C.

A reversed phase gradient consisting of water and 0.1% (v/v) FA (solvent A) and methanol and 0.1% (v/v) FA (solvent B) was applied to elute samples over a 5min run. A flow rate of 0.6mL/min was used over a linear gradient, as shown in Table 6.2-2.

Table 6.2-2 Chromatography conditions for APAP quantification

Time	%A	%B
0	95	5
0.5	95	5
3.5	90	10
3.6	5	95
4.0	5	95
4.1	95	5
5.0	95	5

6.2.4.5 Mass spectrometry

For both experiments, data were acquired using a Waters Xevo tandem quadrupole (TQ)-S mass spectrometer (Waters corporation, Manchester, UK) operating in positive ion electrospray mode for MS/MS detection. The capillary voltage was set to 3kV, the source temperature was 150°C, the desolvation temperature was 500°C, and the gas flow was 1000L/h. The compound specific MS parameters, detailed in Table 6.2-3 include: parent/product ions, cone voltage and collision energy. These were determined by the direct infusion, at 10-20 µL/min, of 100-500 ng/mL solutions of each analyte, combined with an LC flow of 50:50 H₂O/CH₃OH at 0.2 mL/min.

Table 6.2-3 MS conditions and MRM transitions for APAP quantification

Compound	Parent ion (m/z)	Product ions (m/z)	Cone voltage (V)	Collision energy (V)	Interscan delay & Dwell time (s)
APAP	152.10	65.00 92.88 110.06	30	26 22 16	0.029
APAP-D4	155.07	111.00	30	20	0.029
APAP-cysteine	271.03	96.09 139.99 182.06	34	36 24 16	0.033
APAP-sulphate	276.22	142.81 186.84	34	26 12	0.033
APAP-glucuronide	328.13	110.01 152.09	30	34 14	0.079

6.2.4.6 Data processing

The UPLC-MS instrument was operated with MassLynx V4.1 software, and data were analysed using TargetLynx (Waters Corporation).

6.2.5 Measurement of endogenous hepatic metabolites

An Ion-pair UPLC-MS/MS, detailed in Chapter 4, was used to measure hepatic GSH, GSSG, ophthalmic acid, pyroglutamic acid, cystine and methionine.

6.2.6 Quantification of endogenous plasma metabolites

The HILIC- MS/MS method detailed in Chapter 5, was used to quantify plasma metabolites. The assay was originally optimised on plasma from this study, so no alterations to the ranges were made prior to analysis.

6.2.7 Statistical Analysis

A two-tailed Mann-Whitney test was applied where statistical significance was calculated. Spearman's correlation coefficient was calculated to analyse the relationship between plasma ophthalmic acid concentrations and hepatic glutathione.

6.3 RESULTS

6.3.1 Clinical Chemistry

Plasma samples were collected at -1, 1, 2, 4, 8 and 24h post-dose. There were no alterations seen between the low dose and vehicle treated groups in ALT, AST and GLDH. In contrast, statistically significant (by two-tailed Mann Whitney test) elevations in ALT, AST and GLDH were observed at 24h in the high dose APAP group compared to controls. There was wide variability observed in this high dose group, with four out of the six high dose animals having increased ALT and AST levels. The highest ALT activity detected was rat number 332 with 1376IU/L, the lowest was rat 331 with 37IU/L. There were no significant alterations observed in total bilirubin or total bile acids over the 24h period in either high or low dose compared to the vehicle group.

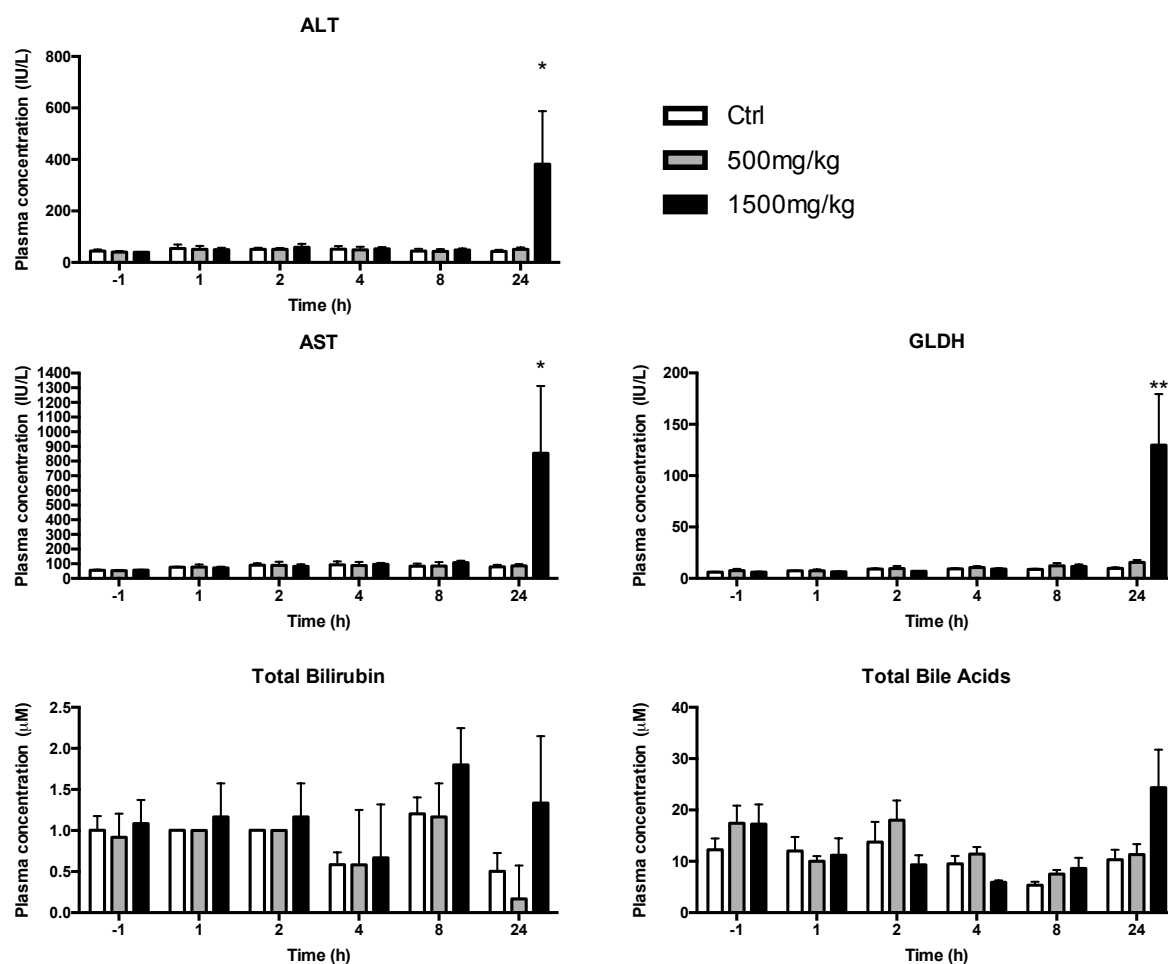


Figure 6.3-1 In-life clinical chemistry, significance Mann-Whitney test, between high/low APAP group and vehicle at each time-point. * $p < 0.05$. Bars indicate the mean, error bars indicate S.E.M. ($n=12$ at -1 and 4h, remaining time-points $n=6$). Corresponding graphs showing individual animals/data points are in the appendix.

At necropsy at 4h or 24h, a wider panel of clinical chemistry parameters were analysed. A significant but small decrease in urea was found in the low dose group at 4h and a slight increase in potassium at 24h. In the high dose group, there were small but statistically significant decreases in urea, creatinine, sodium and calcium, as well as elevations in glucose and albumin. At 24h in the high dose group, there was a statistically significant decrease in sodium, triglycerides, cholesterol, and an elevation in potassium. The animal with the greatest perturbations was rat 332; this was the animal that showed the highest elevation of ALT/AST.

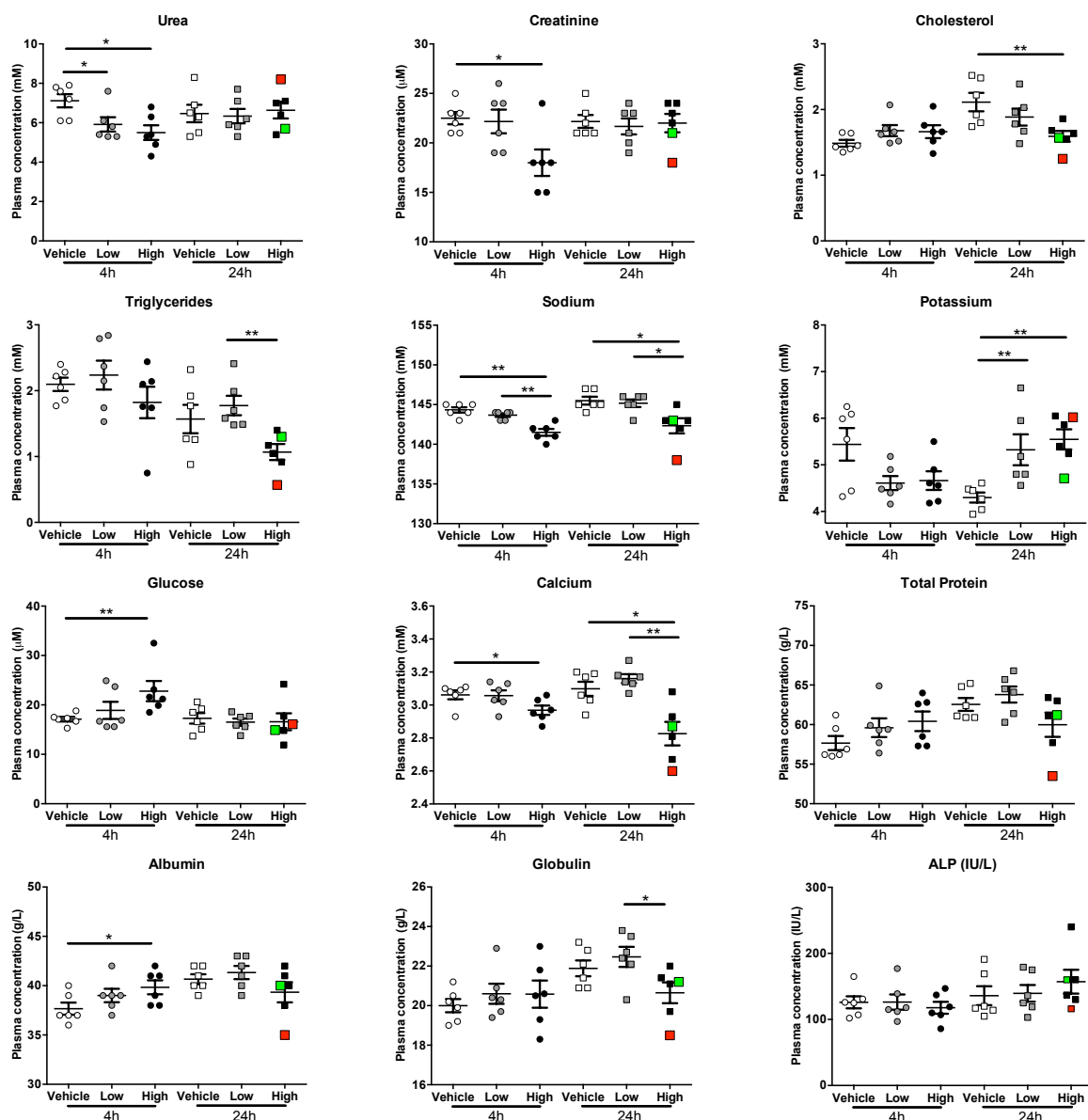


Figure 6.3-2 Necropsy plasma Clinical chemistry. Mann-Whitney test. Red- rat 332, highest ALT, green- rat 331, lowest ALT. Lines indicate the mean, error bars indicate $\pm$ S.E.M. (n=6 per group).

6.3.2 Histopathology

Histopathological analyses of the liver and kidney were performed at necropsy, looking for incidences of gross and microscopic abnormalities. There were no treatment-related gross findings noted in this study. Microscopic analyses revealed treatment-related changes in the kidneys and liver. In the liver at 4h, treatment with APAP resulted in minimal centrilobular inflammation in one animal treated at 500mg/kg and two animals from the 1500mg/kg group. In the liver at 24h, treatment with APAP resulted in minimal to severe centrilobular degeneration/necrosis, minimal to mild centrilobular inflammation and minimal vacuolation in animals treated at 500 and 1500mg/kg. This was accompanied by mild to moderate congestion in animals treated at 1500mg/kg only.

In the kidney at the 4-hour time-point, treatment with APAP resulted in minimal to mild renal cortical tubular dilation. This change was characterised by loss of apical cytoplasm with a consequent dilation of the tubular lumen. At the 24h time-point, treatment with APAP resulted in a similar minimal to mild renal cortical tubular dilation but at decreased frequency compared to the 4-hour time-point possibly indicating recovery in the intervening time period.

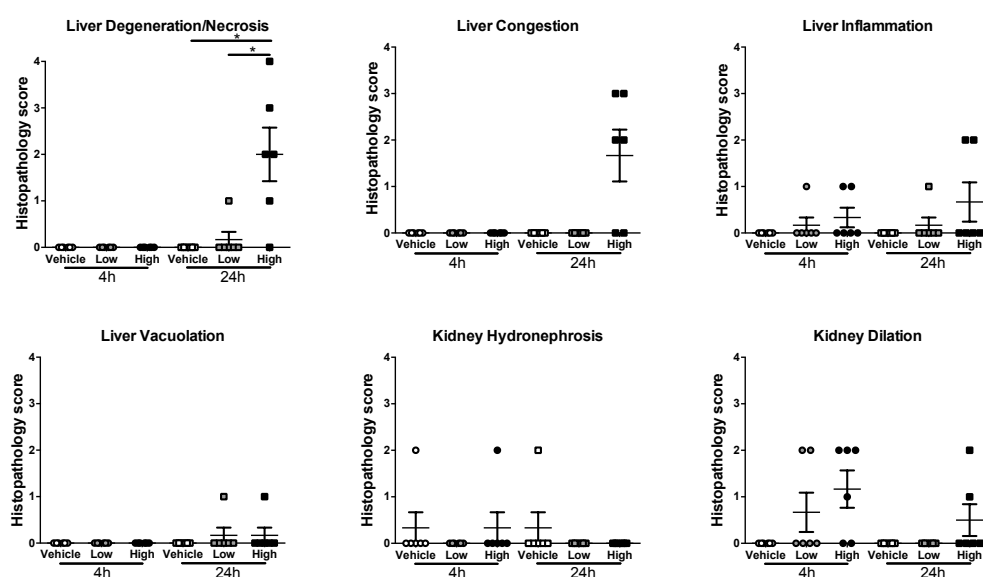


Figure 6.3-3 Histopathology scores 0- no abnormality detected, 1- minimal, 2- mild, 3- moderate, 4- marked. Lines indicate the mean, error bars indicate $\pm$ S.E.M. (n=6 per group).

6.3.3 Plasma concentration of APAP and APAP metabolites

The concentration of plasma APAP and the relative concentrations of the APAP sulphate, glucuronide and cysteinyl metabolite (Figure 6.3-4) were calculated at 1h, 2h, 4h, 8h and 24h post-dose using UPLC-MS/MS. The median plasma concentration of APAP at 1h post-dose was

95.95µg/mL for animals dosed with 500mg/kg, and 238.9µg/ml in animals dosed with 1500mg/kg. The concentration of APAP decreased over time, and at 24h the low dose group had a median concentration of 23.65µg/mL and the median high group concentration was 69.00µg/mL.

The concentration of sulphate metabolite detected was similar between the groups treated with low and high doses of APAP, whereas far higher concentrations of the glucuronide metabolite were detected in the higher dose group. There was an increase in the concentration of APAP-sulphate, and APAP-glucuronide detected at 24h time-point compared to 8h in both the low and high dose groups.

The cysteinyl conjugate was detected at a low level in both low and high dose groups. At 24h there was a variable but marked increase in APAP-cys only in the high dose group. The animal with the highest cysteinyl-APAP, was rat 332, the same animal with greatest disturbance in clinical chemistry.

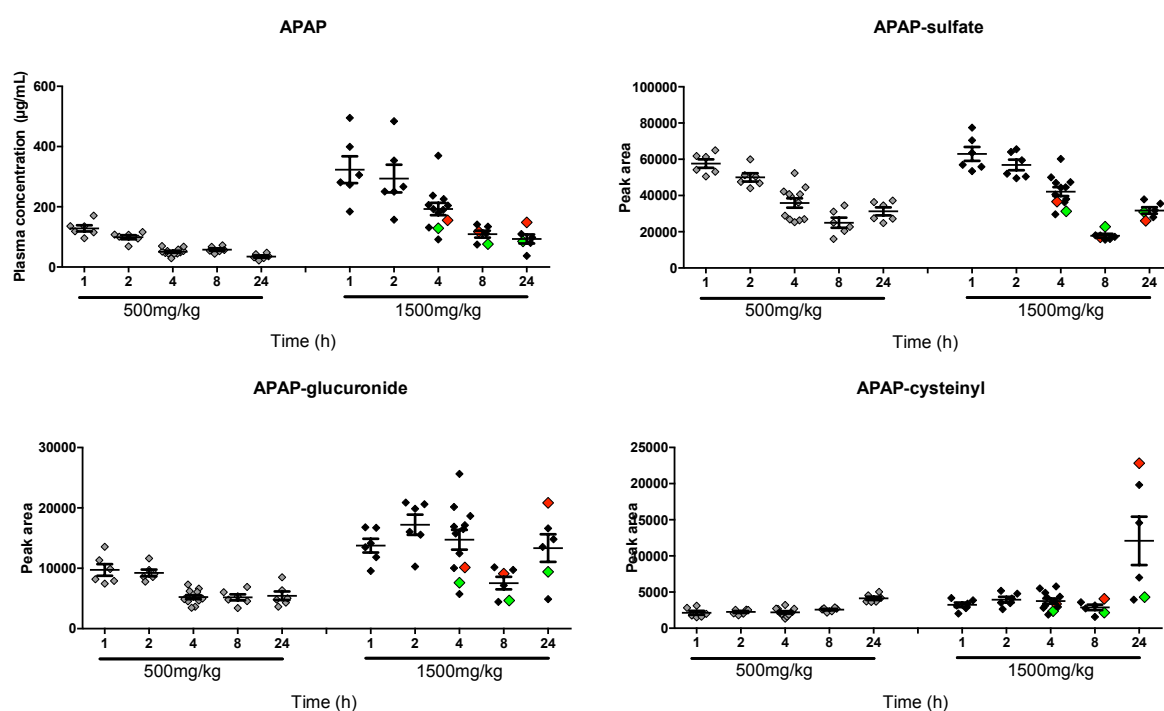


Figure 6.3-4 Quantification of plasma paracetamol (APAP) quantified using UPLC-MS. Red- rat 332, highest ALT, green- rat 331, lowest ALT. Lines indicate the mean, error bars indicate ± S.E.M. (n=12 at -1 and 4h, remaining time-points n=6).

6.3.4 Hepatic glutathione, pyroglutamic acid and ophthalmic acid

Hepatic glutathione and ophthalmic acid and pyroglutamic acid were measured using an IPC-MS/MS approach. A significant depletion in reduced glutathione (GSH) and oxidized

glutathione (GSSG) were detected, with the high dose group having lower concentrations of both GSH and GSSG. There were no alterations in pyroglutamic acid detected at either dose at these time-points, however there were significant elevations in ophthalmic acid at 24h in both high and low doses. The same animal with the highest ALT has one of the lowest glutathione levels, and highest ophthalmic acid. There was also a statistically significant decrease in hepatic methionine detected in the high-dose group, and a slight and variable increase in cysteine only seen in the low dose group.

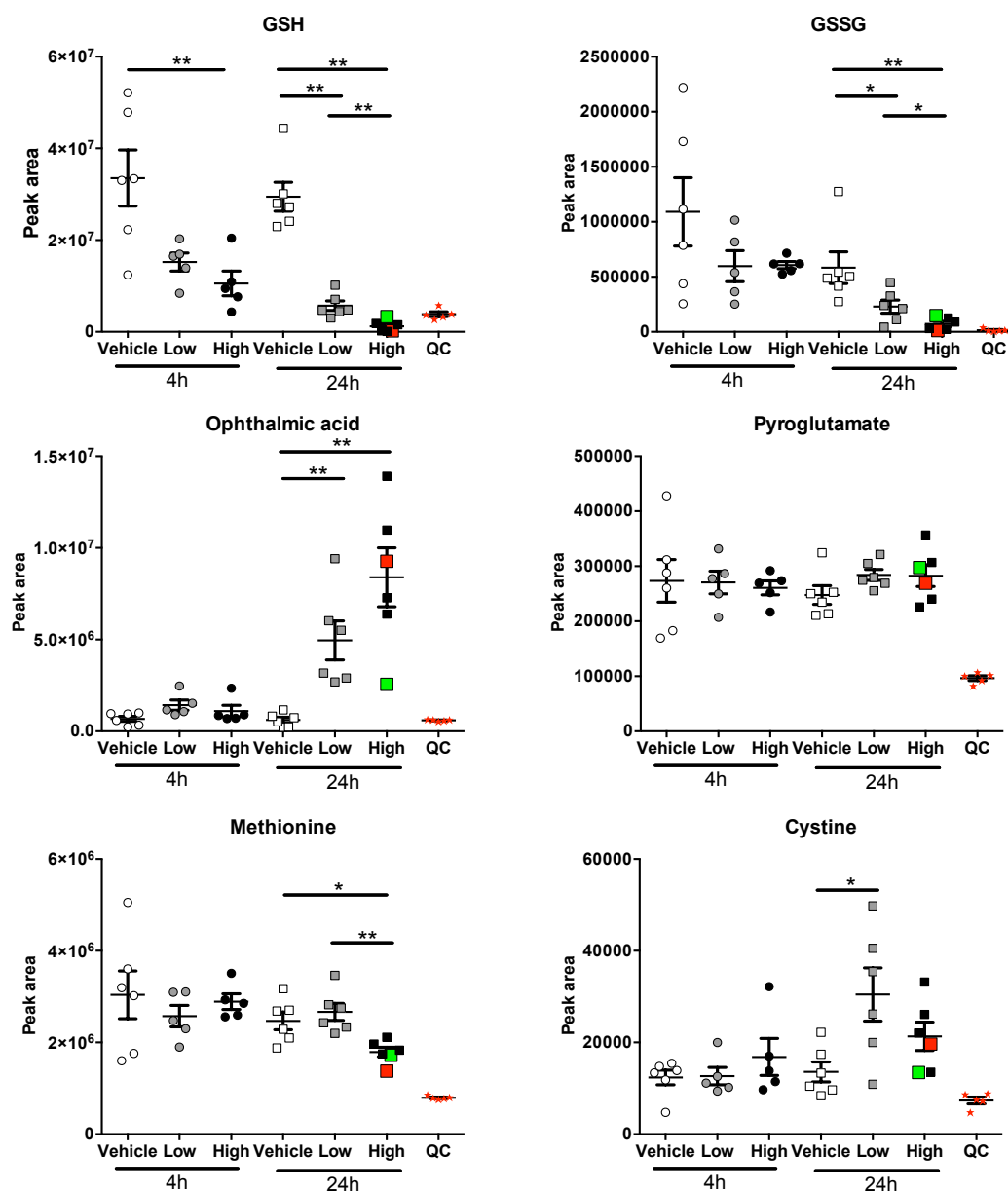


Figure 6.3-5 Impact of 500mg/kg and 1500mg/kg doses of APAP on hepatic glutathione, ophthalmic acid, pyroglutamic acid and sulphur containing amino acids methionine and cysteine. 332=red, 331=green. Lines indicate the mean, error bars indicate $\pm$ S.E.M. (n=6 per group).

6.3.5 In-life quantification of endogenous metabolites linked to glutathione

Ophthalmic acid, pyroglutamic acid and 12 other related metabolites were quantified in plasma. The most marked perturbation detected was in ophthalmic acid which was significantly elevated at 8h and 24h in both the high and low dose groups (median high group 24h= 535ng/mL, low group= 228ng/mL, vehicle= 25ng/mL), with the majority of the earlier samples below the LLOQ (50ng/mL) for all treatment groups. The highest ophthalmic acid concentration at 24h was rat number 332 (1151ng/mL), the lowest was rat 331 (59ng/mL).

The concentrations of pyroglutamic acid in the vehicle treated groups varied between 977.5ng/mL- 7298ng/mL over the time-course. There were no elevations in pyroglutamic acid detected at any time-point. There was a small but statistically significant ($p<0.05$) decrease at 1h and 2h in the high-dose group (median of 1015ng/mL and 1139ng/mL respectively), and at 8h in the low dose group (median 1105ng/mL).

Methionine was detected at a relatively stable concentration in the vehicle treated samples across the time-course (~8-10 μ g/mL). There were consistent and statistically significant decreases in methionine at 1, 4 and 8h post-dose observed in the high-dose group compared to vehicle-treated animals, that was recovered to vehicle concentrations by 24h. The low dose group also has significantly depleted methionine at 2h and 4h post-dose, recovered to control concentrations by 8h. At 4h the median control concentration detected was 9.5 μ g/mL, compared to the low dose group with 7.7 μ g/mL, and the high dose group with 6.6 μ g/mL.

There were no significant alterations in taurine at any time-point. There was a significant depletion of hypotaurine detected at 24h in both dose groups (median vehicle-treated =8.0 μ g/mL, low dose= 2.5 μ g/mL, high dose= 1.5 μ g/mL).

Median betaine concentrations were consistently lower in the APAP dosed animals, significant depletion in betaine detected at 1, 4 and 24h in the high dose group and at 2h in the low dose group. In contrast choline was significantly elevated in both APAP treated groups at 24h, as well as the low dose at 2h. At 24h the median vehicle treated choline concentration was 2.3 μ g/mL, and 2.9 μ g/mL and 4.0 μ g/mL in the low and high APAP dosed groups respectively.

Details for the remaining compounds analysed are in the appendices.

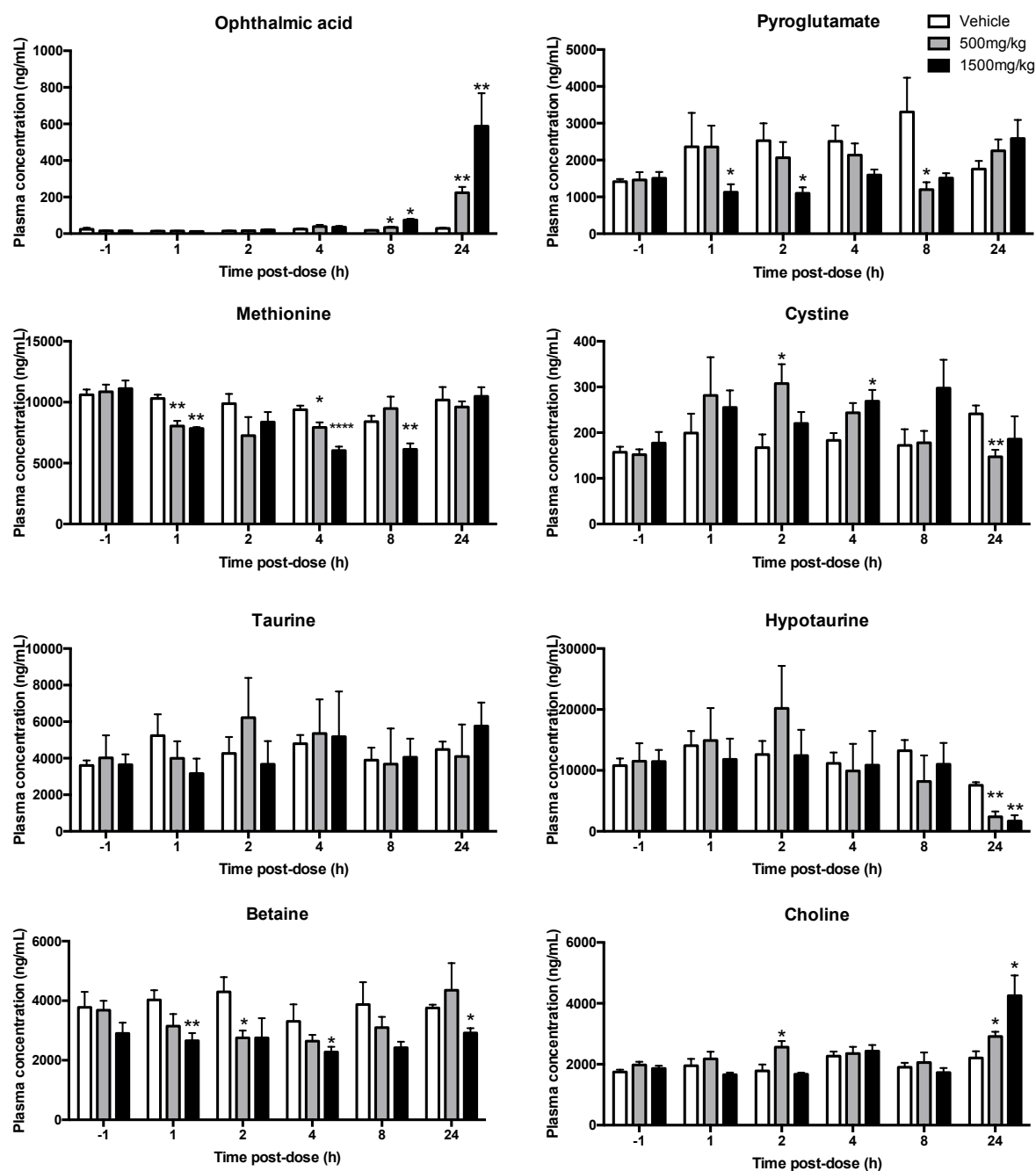


Figure 6.3-6 Selected metabolites from the quantification of 14 metabolites in plasma following APAP dosing, significance Two tailed Mann-Whitney test compared to vehicle treated at each time-point. Bars represent the mean, error bars indicate $\pm$ S.E.M. ($n=12$ at -1 and 4h, remaining time-points $n=6$). Corresponding graphs showing individual animals/data points are in the appendix.

6.3.6 Plasma ophthalmic acid correlation with hepatic GSH

The correlation between plasma ophthalmic acid and hepatic GSH was assessed by calculating the Spearman's correlation coefficient (Figure 4.3-29). There was a strong and significant ($r = -0.7653$, $p < 0.0001$, $n = 34$) correlation between plasma ophthalmic acid and hepatic glutathione detected in this study.

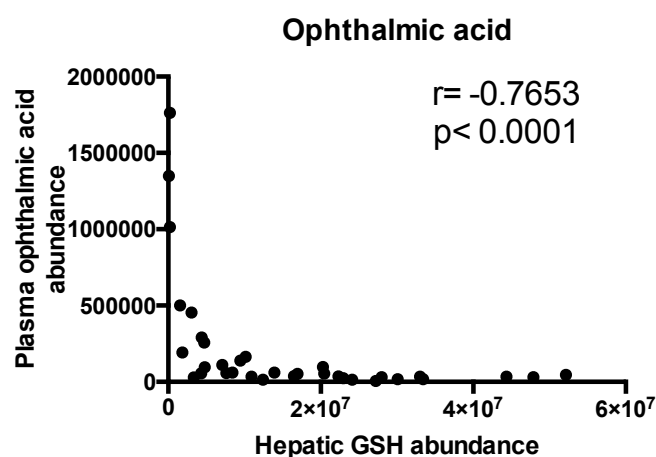


Figure 6.3-7 The correlation of plasma ophthalmic acid with hepatic glutathione. Spearman's rank correlation coefficient is " r ", and " p " indicates statistical significance ($n = 36$)

6.3.7 The impact of APAP on tryptophan metabolism

Tryptophan and kynurenine were measured in aqueous liver extracts using an IPC-UPLC-MS/MS approach. There were no alterations detected in tryptophan at either time point, while at 24h there was a significant decrease in kynurenine in both treatment groups.

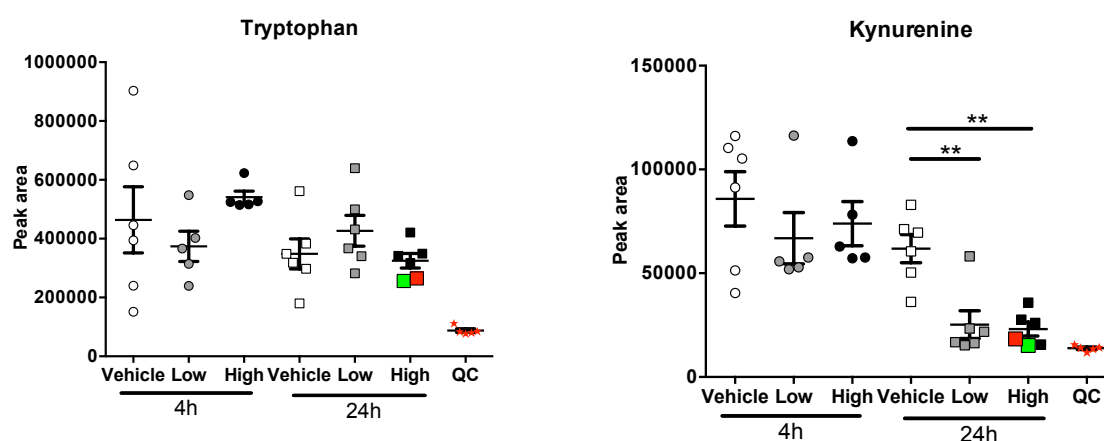


Figure 6.3-8 Hepatic tryptophan and metabolites. Statistical significance was calculated using Mann Whitney test, * $p < 0.05$, ** $p < 0.01$. Symbols are the individual animals, and the line represents the mean, error bars indicate S.E.M ($n = 6$ per group).

In plasma there was no change in tryptophan concentration detected in either dose group, however there was a significant elevation in kynurenine and kynurenic acid in the high dose group ($p<0.01$) at 4h.

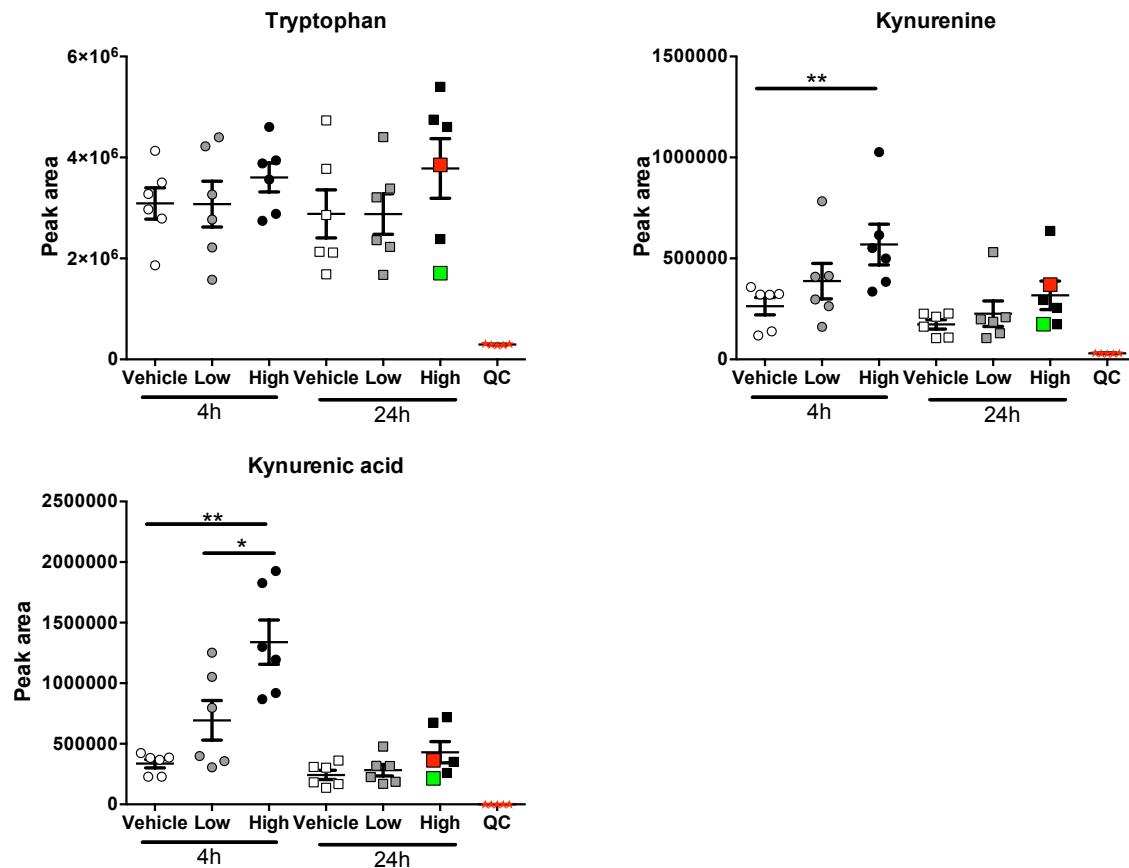


Figure 6.3-9 Plasma tryptophan and metabolites, statistical significance was calculated using Mann Whitney test, * $p<0.05$, ** $p<0.01$. Symbols are the individual animals, the line represents the mean, error bars indicate S.E.M (n=6 per group).

The ratio of kynurenine to tryptophan in serum has been previously reported to correlate to indoleamine 2,3-dioxygenase (IDO) activity: the enzyme that is involved in the metabolism of tryptophan. To assess this relationship in the present study the peak area ratio of kynurenine and tryptophan was calculated from the IPC-MS/MS liver and plasma analyses (Figure 6.3-10). There was a statistically significant depletion in kynurenine to tryptophan ratio at the high and low dose group at 24h in the liver. There was a statistically significant elevation in the ratio in plasma at the high dose at 4h.

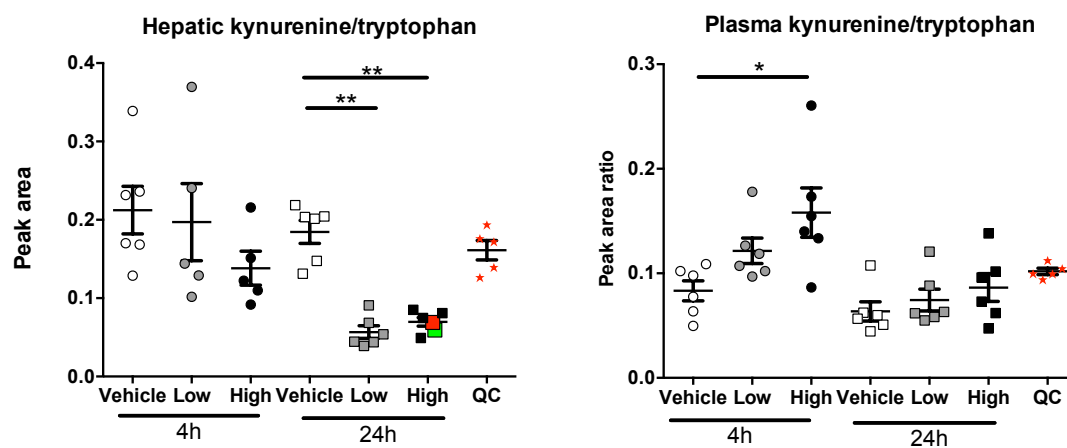


Figure 6.3-10 The ratio of liver and plasma kynurenine and tryptophan abundance (peak area from IPC-MS/MS analyses). Statistical significance was calculated using Mann Whitney test, * $p < 0.05$, ** $p < 0.01$. Symbols are the individual animals, and the line represents the mean, error bars indicate S.E.M ($n=6$ per group).

6.4 DISCUSSION

The aim of this study was to determine the plasma concentration of pyroglutamic acid, ophthalmic acid and other metabolites related to glutathione following single 500mg/kg and 1500mg/kg APAP dosing in the rat.

Histopathology examination of the liver and clinical chemistry analyses revealed that despite the high dose of 1500mg/kg there was marked necrosis and very high ALT activity detected in only one animal. This is consistent with previous studies showing APAP is well tolerated in rats at this dose. In another single dose study APAP was dosed at 1600 to 2000 mg/kg (McGill et al., 2012b), with only minor or no change in plasma ALT values 24 hours post dose. There were smaller elevations in ALT, AST and perturbations in several other clinical chemistry parameters, but these were only minor; hence APAP was tolerated at this dose.

The quantification of APAP and measurement of the glucuronide, sulphate and cysteinyl conjugates revealed an unusual elevation at 24h. At the 'in-life' time points blood was taken from the tail, in contrast when they were euthanised at either 4h and 24h blood was taken from the vena cava. This sampling difference may have led to this unusual elevation, supported by observation that the 4h samples taken from the animals euthanised at 24h were higher than the 4h samples taken from group euthanised at 4h (data not shown). Alternatively, a biological explanation could be enterohepatic recirculation; the re-absorption of APAP from intestines after excretion into bile, or alternatively a coprophagic effect.

IPC-MS/MS analyses of the liver revealed a dose dependent depletion in GSH and GSSG at both time point, showing that this was a sufficient dose to deplete GSH/GSSG, likely caused by the generation of the reactive metabolite of APAP, NAPQI. A dose dependent elevation in hepatic ophthalmic acid was also observed at 24h, indicative of the upregulation of glutathione cysteine ligase (Soga et al., 2006). In contrast, hepatic pyroglutamic acid was not altered at either dose. The 24h high dose group had a depletion in hepatic methionine, a sulphur containing amino acid, which may be indicative of its conversion to support GSH synthesis.

Quantitative analyses of the plasma metabolites using the HILIC assay, revealed a variable elevation in ophthalmic acid at both 8h and 24h. Interestingly, the animals with high ALT, and most perturbed clinical chemistry, also have the greatest ophthalmic acid elevation. There was a much stronger correlation between hepatic glutathione and plasma ophthalmic acid found

in response to APAP than in the TA/TAI study. Additionally, the median ophthalmic acid concentration detected was far higher in response to APAP than detected in response to TA or TAI and in the APAP treated animals' ophthalmic acid was highest at 24h, whereas in both TA and TAI ophthalmic acid was higher at 6h. This highlights the continued need for better characterisation of this potential biomarker. Interestingly, the plasma ophthalmic acid was elevated at 8h, which was prior to the ALT elevation, suggesting it could be an earlier marker before ALT elevation.

In contrast and in opposition to the hypothesis, pyroglutamic acid was not elevated in plasma at either dose or at any time point. However, pyroglutamic acid can be readily excreted into urine and this was not collected or analysed in this study, and so it is unknown whether elevation of pyroglutamic acid was rapidly excreted. Therefore, in subsequent studies of these biomarkers it will be important to collect urine. The other most significant and consistent changes in circulating metabolites were depletions of methionine and betaine, possibly due to increased requirement of methionine to be converted to cystine, or cystathionine, for glutathione synthesis.

In future, it will be interesting to attempt to test the model developed by Geenen et al., 2013. The glutathione model was more recently developed to make it more suitable for *in vivo* studies by incorporating it into a physiologically based pharmacokinetic (PBPK) model of paracetamol, as rate of elimination of APAP can also impact hepatic GSH. This work generated predicted changes to hepatic GSH and circulating ophthalmic acid, pyroglutamic acid and related metabolites following APAP dosing in both humans and rats. To date, the model has only been tested using limited *in vivo* concentration data available in the literature. However, as there are only very limited reports of pyroglutamic acid and ophthalmic acid in the literature this model remains largely untested. Therefore, the data generated here may be useful in the development and validation of this model.

A secondary aim of this work was to study the impact of APAP on tryptophan metabolites in liver and plasma. There was no alteration in hepatic tryptophan detected and a reduction in kynurenine, this is in contrast to TA and TAI treated animals which both had dramatic elevations in kynurenine. Serotonin is the other metabolite of tryptophan, and was not measured in this study, but has previously been found to increase after APAP dosing (Yamazaki et al., 2013). A recent study has shown serotonin deficiency is associated with increased APAP

toxicity, as serotonin acts to decrease CYP2E1 expression, thereby reducing NAPQI production, as well as inhibiting ROS and RNS formation and promoting hepatocyte proliferation (Zhang et al., 2015). The administration of tryptophan has also been found to alleviate APAP induced toxicity (Kimura and Watanabe, 2016).

Plasma tryptophan was also not elevated in response to APAP in this study, in contrast plasma tryptophan was depleted in response to both TA and TAI at 2h. There were dose dependent increases in both kynurenine and kynurenic acid in response to APAP, but these were far lower than the elevations observed in response to TA and TAI. As kynurenine is elevated in response to pro-inflammatory cytokines, this could indicate that TA and TAI induce a greater degree of inflammation than APAP.

6.4.1 Limitations

The sampling differences between time point may have affected the PK of APAP, and in future study design considerations sampling would be conducted from the same location. The IPC-MS/MS assay had the same limitations with the possibility of ion-suppression, and the quantitative HILIC method still requires optimisation as discussed in Chapter 5. Multi-dose studies would be more representative of chronic, unintentional overdose and may therefore be more useful for future studies.

The quantitative data for ophthalmic acid, pyroglutamic acid and related metabolites could be useful in the validation and optimisation of a predictive model of hepatic glutathione depletion (Geenen et al., 2013b). In addition, the differing impacts of APAP and TA and TAI on tryptophan metabolism would be interesting to further investigate.

DISCUSSION

A UPLC-MS BASED EXPLORATION OF THE XENOBIOTIC AND
ENDOGENOUS METABOLIC PHENOTYPES OF PRE-CLINICAL
MODELS OF HEPATOTOXICITY

7.1 THESIS CONTEXT

DILI is a significant cause of costly late stage attrition during drug development and post release withdrawal (Lee, 2003). Furthermore, DILI is the largest cause of acute liver failure in the U.K. and U.S. (Bernal and Wendon, 2013). Consequently, there remains a pressing need for improved mechanistic understanding and biomarkers to predict and monitor DILI. The broad aim of the work presented in this thesis was to contribute to an improved understanding of DILI and aid in the search for improved predictive biomarkers of DILI. To tackle this, a UPLC-MS based metabonomics approach was utilised to study the drug metabolism and metabolic impact of known hepatotoxins, TA, TAI and APAP in the rat. The drug metabolism and endogenous metabolic impact were studied as both may have a role in DILI, as highlighted in Figure 7.1-1.

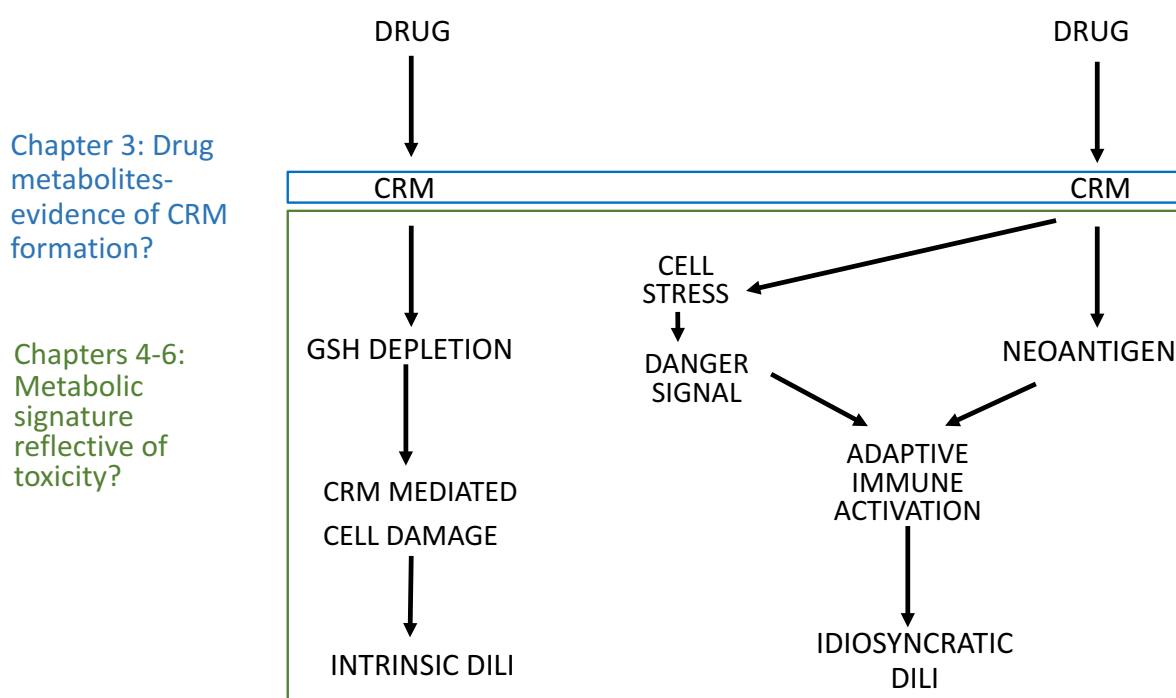


Figure 7.1-1 Context of project to mechanism of intrinsic and idiosyncratic DILI. CRM=chemically reactive metabolites.

Herein, I shall discuss the overall success of the thesis by assessing if the aims were met, summarising what biological and methodological contributions have been made, as well as considering some of the limitations of the work.

7.2 THESIS SUMMARY

The aim, methods and outcome of each of the four results chapters have been detailed in Table 7.2-1.

Table 7.2-1 A summary table detailing the aims, methods and outcome of this thesis.

Chapter	Aim	Methods	Outcomes
3	Characterise the <i>in vivo</i> metabolites of TA and TAI	Untargeted RP UPLC-MS RP UPLC-MS/MS	Successfully revealed numerous TA and TAI metabolites Limited success identifying/determining the structure of the metabolites
4	Study the impact of TA and TAI on endogenous metabolism	Untargeted RP UPLC-MS Targeted ion-pair LC-MS/MS	Successfully revealed numerous TA and TAI metabolic changes, including metabolites linked oxidative stress including ophthalmic acid and pyroglutamate Limited success identifying/determining metabolites from untargeted analysis
5	Develop an assay to simultaneously quantify ophthalmic acid, pyroglutamate and related metabolites in plasma from TA/TAI treated animals	Quantitative HILIC UPLC-MS/MS	HILIC UPLC-MS/MS assay developed and partially validated HILIC assay was applied to TA/TAI samples Further validation and optimisation is still required
6	To apply the HILIC assay to plasma samples from an <i>in vivo</i> APAP study to further investigate the relationship between hepatic GSH depletion and plasma metabolites linked to GSH metabolism	Quantitative HILIC UPLC-MS/MS Targeted ion-pair LC-MS/MS	Quantitative data acquired for 14 metabolites linked to GSH metabolism Not yet incorporated into a mathematical model, which limited the interpretation of the quantitative data acquired

7.3 BIOLOGICAL CONTRIBUTION

TA and TAI metabolism

The initial aim of this thesis was to compare the *in vivo* metabolism of TA and TAI. This was addressed using a UPLC-MS based metabolomic approach, which was effective in revealing both previously characterised and novel metabolites. The *in vivo* metabolism of TA and TAI were found to be strikingly different. The major metabolites of TA that were detected here corresponded to the known major metabolite, 5-hydroxy-TA, as well as several glucuronide conjugates, whereas for TAI a large number of glutathione conjugates were detected.

TAI-GSH conjugates provide evidence for a large CRM burden generated during TAI metabolism, and are consistent with TAI being an intrinsic toxin. The ability to further our mechanistic understanding of TA toxicity through this study is limited, partly due to the incomplete identification of some of the metabolites, but more profoundly because there are no animal models that mimic TA toxicity. One potential way to explore the role of different metabolites in the absence of toxicity, could be to study the correlation of drug metabolites with markers of oxidative stress such as elevated ophthalmic acid or GSH depletion. Additionally, these data may be used alongside other studies to improve our understanding of how structure impacts toxicity, for example, when interpreted alongside data on the metabolism of other toxic and non-toxic thiophenes.

As further work would be required to confirm the structures of the compounds, the aim of this study was only partially achieved.

TA and TAI metabolic impact

Another aim of the thesis was to compare the endogenous metabolic impact of TA and TAI, adding to information reported in the NMR study of these samples (Coen et al., 2012). To do so both untargeted UPLC-MS and targeted IPC-MS/MS assays were applied. Using both approaches, TA and TAI were found to perturb metabolism at earlier time-points, however, samples from TA treated animals were more like controls by 24h, indicative of their adaption/recovery, whereas TAI samples remained distinct, indicating their progression to toxicity.

Far more metabolic differences were revealed using these LC-MS approaches compared to an

NMR spectroscopic based approach (Coen et al., 2012), supporting the rationale that the superior sensitivity of LC-MS compared to NMR would reveal greater metabolic information. As no other metabonomic studies have been reported with TA or TAI, much of the metabolic information gathered was novel. Numerous metabolic changes common to TA and TAI, and unique to either TA or TAI were observed. Although originally hypothesised that the common metabolic alterations may relate to their shared pharmacological action and the different changes to their mechanisms of toxicity, it seems more likely that some of the common alterations are stress related, and following administration of TA but not TAI the rat can adapt/recover. This can make the interpretation of metabolic changes unique to TA difficult to link to toxicity, as unlike TAI there are no other established markers to correlate to e.g. ALT, in the absence of models of idiosyncratic toxicity this will be difficult to resolve.

One of the most prominent observations was the elevation of tryptophan metabolites; kynurenine and kynurenic acid. This is interesting as it could be indicative of an inflammatory stimulus, and was particularly prominent in response to TA, despite no toxicity being observed. Additionally, perturbations in oxidative stress related metabolites were detected including pyroglutamic acid, specific to TAI, and ophthalmic acid, elevated in response to both TA and TAI. These metabolites were selected to study further due to their previous association with DILI, including some reports of translatability to humans (Fenves et al., 2006, Kaur et al., 2015).

Ophthalmic acid and pyroglutamate as biomarkers of DILI

The second half of the thesis was focused on developing and applying a method to quantify ophthalmic acid, pyroglutamate and other glutathione related metabolites in plasma.

The development of a quantitative assay confirmed the ophthalmic acid and pyroglutamate observations from untargeted and ion-pair LC-MS/MS, and enabled the further characterisation of the impact of TA and TAI on glutathione related metabolites. Interestingly, despite TA and TAI both perturbing hepatic glutathione, their very different impacts on related metabolites, may be indicative of an adaptive response (TA) compared to a response leading to toxicity (TAI). Further understanding of how these metabolites correspond to hepatic GSH depletion could be useful in predicting mild from toxic oxidative stress, based on circulating metabolite concentrations.

The targeted HILIC assay was subsequently utilised to further study these glutathione related

metabolites in response to the hepatotoxin, APAP; known to deplete hepatic GSH. Data from the APAP study could be very useful for the testing and optimisation of a previously developed mathematical model for predicting hepatic glutathione content from circulating biomarkers such as ophthalmic acid and pyroglutamic acid (Geenen et al., 2013b). However, these data have not yet been used to determine if they can be used to predict hepatic GSH. Further work into the stability, time course, and relationship between these different metabolites in response to a toxin insult will be important in establishing their utility to accurately/robustly monitor hepatic glutathione status or risk of hepatotoxicity.

As pyroglutamate and ophthalmic acid have both been proposed as potential biomarkers of DILI, quantitative data such as these may be useful for comparing between other drugs, animals or time-points in independent studies.

Mechanism of IDRs

One of the most unresolved area of IDR research is how the immune system is activated to allow an autoimmune response to occur (Kaplowitz, 2005). Theories have suggested both external ‘danger signals’ such as co-infection, others have suggested that the drug itself could cause sufficient cellular stress to induce a danger signal. This UPLC-MS based metabonomic study supports the hypothesis that drug-intrinsic factors are sufficient to induce oxidative stress, and some evidence that an immune response is initiated, but controlled. This is further supported by similar observations from transcriptomic studies showing the alteration of immune and oxidative stress genes. Further mechanistic experiments would be required to confirm if the oxidative stress caused by TA is sufficient to produce a danger signal, and what it is, but the discovery of a disturbance in tryptophan metabolism provides interesting indirect evidence that it may be sufficient to cause inflammation. This provides an example of how metabonomics can be applied in hypothesis generation.

7.4 METHODOLOGICAL CONTRIBUTION

The application of untargeted UPLC-MS to liver extracts, plasma and urine samples collected from TA and TAI dosed rats, was found to be a useful approach to reveal both known and unknown drug metabolites of TA and TAI. One benefit of this approach is that it was not biased to a pre-determined set of commonly reported metabolites e.g. drug metabolite identification

software, or neutral loss experiments for glutathione conjugates. Direct comparisons using different approaches (neutral loss experiments, isotope pattern recognition or other software) could have been informative in assessing this approach. It was used here principally due to the simultaneous acquisition of endogenous metabolic phenotype data.

The use of a global/untargeted LC-MS based metabonomic approach was only of limited success in this project. Whilst there was a very large volume of data produced, this was of limited use, due to the difficulties involved in metabolite identification (time and resources). In this instance, preliminary identifications from untargeted analyses were predominantly used in informing targeted assay choice, and further confirmational studies would be required to obtain a fuller metabolic picture.

Instead of investing a huge amount of time/resources in metabolite identification, the project was progressed using a more targeted approach; applying a targeted IPC-MS/MS assay which covered over 100 polar metabolites. This was found to be particularly time and resource efficient, and appears to be a good compromise between the difficult to interpret untargeted approaches, and costlier quantitative assays using internal standards.

However, due to the risks of ion suppression, quantitative methods including internal standards will remain important for verification. This was highlighted by the discrepancy between IPC-MS/MS and the quantitative HILIC methods for methionine and cystine. Additionally, quantitative assays, such as the assay developed here, are key for determining clinically relevant perturbations, as well as the potential to help develop and validate robust predictive models, that utilise several metabolite concentrations to predict risk or clinical outcome.

7.5 KEY LIMITATIONS

The untargeted metabonomic approach successfully revealed numerous metabolites, however, as only limited structural information can be obtained from MS/MS, the structure of these remained unconfirmed. To enable the identification and quantification of these drug metabolites, as they are not commercially available, the compounds would need to be synthesised for use as standards to enable the comparison of the retention time and fragmentation of each suspected metabolite. One of the key conclusions of the drug metabolism work was that TAI creates a far greater CRM burden, compared to TA, indicated

by the large proportion of GSH conjugates. Although this is likely to be true, as TA-GSH have previously been reported, but only in the bile in the rat (Nishiya et al., 2006), which was not collected as part of this study, it would be useful to investigate bile in the future.

Similarly, the untargeted metabolite data presented were greatly limited by the absence of confirmational experiments. However, unlike the drug metabolites, as many of the suggested compound identifications are commercially available, these could be used in confirmational studies. Additionally, in future studies it would be helpful to run analyses applying MSe mode, where high and low collision energies are applied in the collision cell, enabling fragmentation data at the time of acquisition. Newer UPLC-MS analysis software (e.g. within Progenesis) can use this fragmentation data in the initial analysis, and should enable more accurate preliminary identifications to be obtained.

Although numerous metabolic changes have been demonstrated in this work, the biological significance or their role in TA/TAI DILI is unproven, and other factors such as food intake should be considered, as several metabolic changes may be affected by a difference in food consumption of the animals. In addition, due to the low animal number and low sample volumes, there was insufficient sample volume to perform all analyses with sufficient animals in a group, this restricted data interpretation at certain groups.

Time pressure meant that it was not possible to develop all aspects of the method as much as I would have liked, and it would have been preferable if the matrix effects, recovery and an appropriate concentration range to be determined and optimised, prior to study sample application. This may have allowed the quantification of a greater number of metabolites.

Although the hypothesis that hepatic glutathione metabolism could be ascertained/extrapolated from the concentration of multiple circulating metabolites, using a mathematical modelling approach, was discussed, this has not been tested or demonstrated in this thesis. The testing and further development of such models would have greatly strengthen the usefulness of the data obtained in the second part of this thesis.

7.6 CONCLUSION

This thesis has described an LC-MS based exploration of the xenobiotic and endogenous metabolic phenotypes of pre-clinical models of hepatotoxicity, with the aim of contributing to an improved understanding of DILI and aid in the search for improved predictive biomarkers. Despite the limitations discussed, novel drug metabolites of TA and TAI have been revealed, and differences in the endogenous metabolic impact of these drugs have been further characterised following on from work by Coen et al. (2012).

Elevations in metabolites previously linked to oxidative stress, including pyroglutamate and ophthalmic acid were further studied by developing a targeted UPLC-MS/MS assay for these are related metabolites. Despite TA and TAI both depleting hepatic GSH and elevating ophthalmic acid, they were found to impact other circulating metabolites in different ways. To further explore the dynamics of these metabolites, the assay was applied to plasma from paracetamol (APAP) dosed rats; a model GSH depleting hepatotoxin. Quantitative data such as these may contribute to the further development and validation of mathematical models to predict hepatic glutathione status from multiple circulating plasma biomarkers.

Throughout this thesis a spectrum of LC-MS approaches to study xenobiotic and endogenous metabolism have been successfully applied; demonstrating the utility of a UPLC-MS based approach for hypothesis generation and biomarker development.

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Appendix

8.1 CHAPTER 1- SUPPORTING DATA/DOCUMENTS

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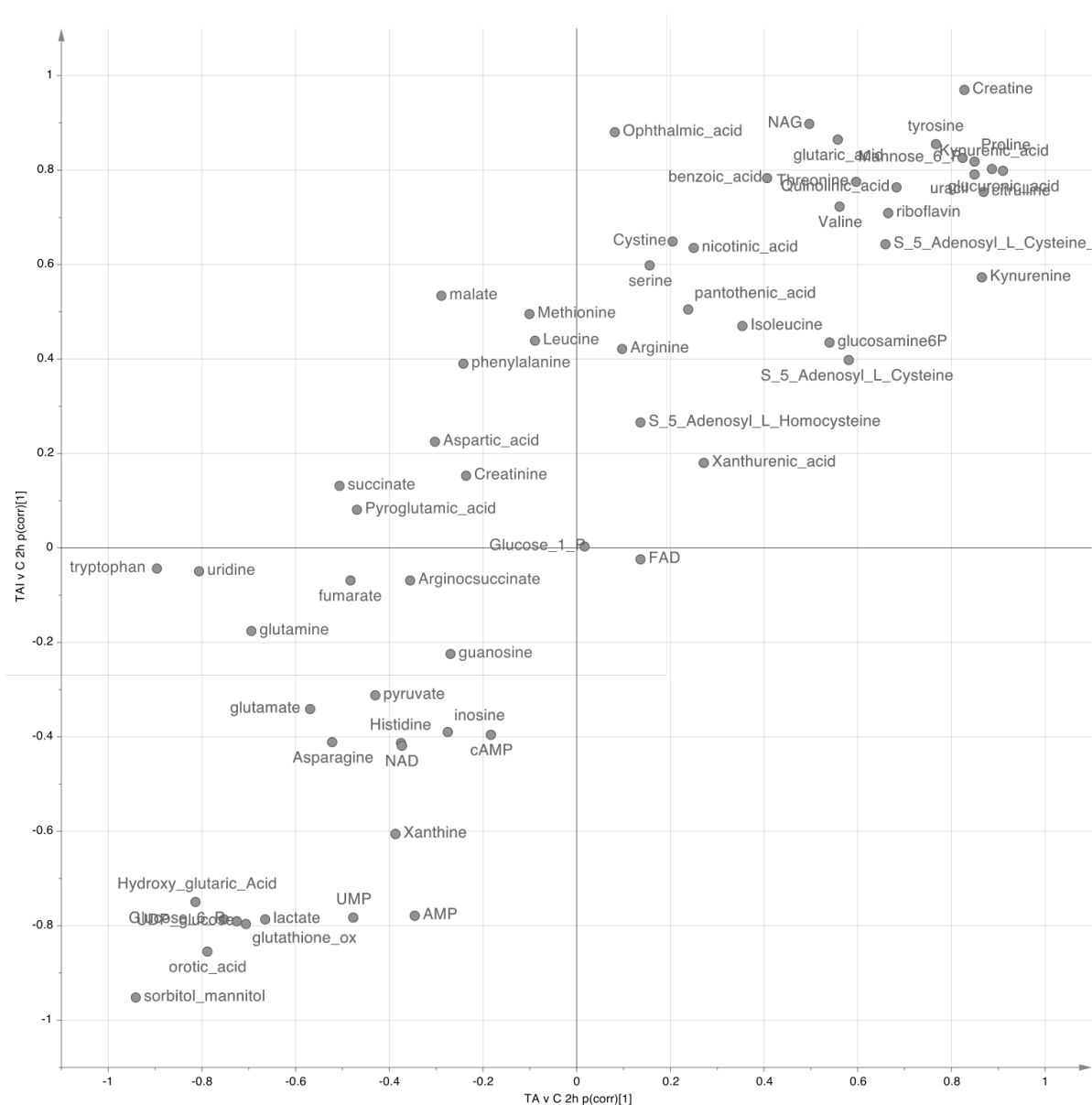


Figure 8.4-1 An SUS plot generated from 2h TA v Ctrl and TAI v Ctrl OPLA-DA models of ion-pair LC-MS analyses of liver extracts.

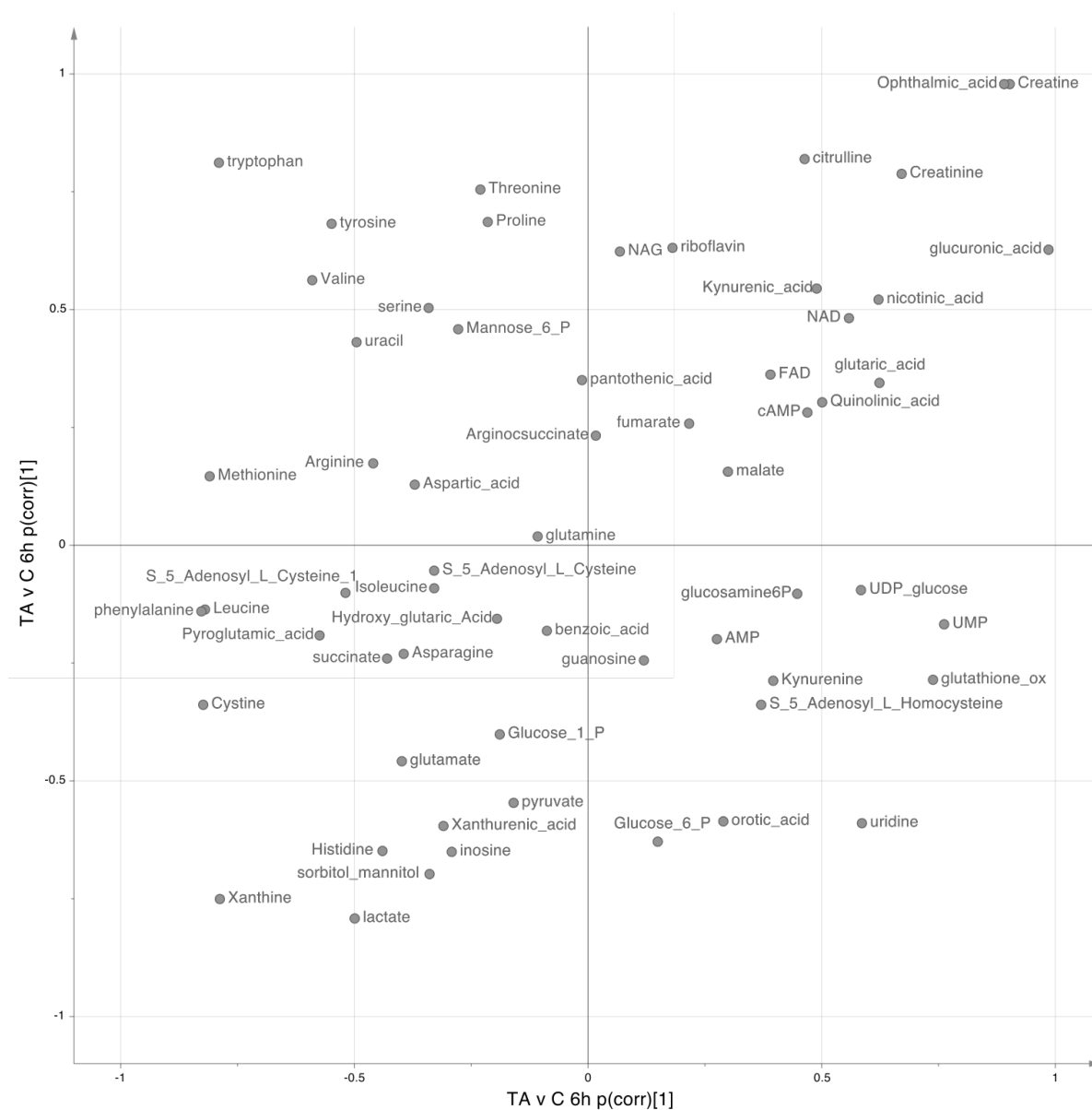


Figure 8.4-2 An SUS plot generated from 6h TA v Ctrl and TAI v Ctrl OPLS-DA models of ion-pair LC-MS analyses of liver extracts.

8.5 CHAPTER 5- SUPPORTING DATA/DOCUMENTS

Table 8.5-1 A comparison of retention times of compounds analysed using 15cmx 2.1mm HSS T3, HILIC and amide columns.

Compound	HSS T3	BEH HILIC	BEH Amide 15cm
Solvents	ACN + 0.1%FA H₂O+ 0.1%FA	H₂O+10mM Ammonium acetate ACN 0.1%FA	H₂O+ 0.1%FA ACN + 0.1%FA
Gradient	99% H₂O hold	10% H₂O hold	16% H₂O hold
2-amino-butyrate	N.A	0.55	1.47
Betaine	0.61	3.44	1.49
Choline	0.55	2.69	0.78
Cystathionine	0.55	0.52/5.33	6.59
Cysteic acid	0.58	N.A	5.95
Cysteine	0.62	0.81/2.78	2.12
Cysteinyl-glycine	0.53	N.A	1.42
Cystine	0.55	5.23	6.73
γ-glutamyl-cysteine	0.79	N.A	2.46
Glutamic acid	0.59	3.30	2.58
Glutamine	0.57	3.58	3.33
GSH	1.29	3.88	3.19
GSSG	2.55-2.72 (split peak)	0.51/5.92	6.77
Glycine	0.68	N.A	2.31
Homocystine	N.A	N.A	6.05
Homoserine	N.A	N.A	2.52
Hypotaurine	0.57	N.A	3.74
Methionine	0.96-1.15 (split peak)	2.67	1.32
Ophthalmic acid	1.63	4.00	2.97
Pyroglutamic acid	1.57	1.07	0.89
SAH	0.79	4.07	5.35
SAM	0.58	N.A	6.34
Serine	0.55	3.27	3.48
Taurine	0.57	1.37	2.75

N.A Not included in analysis

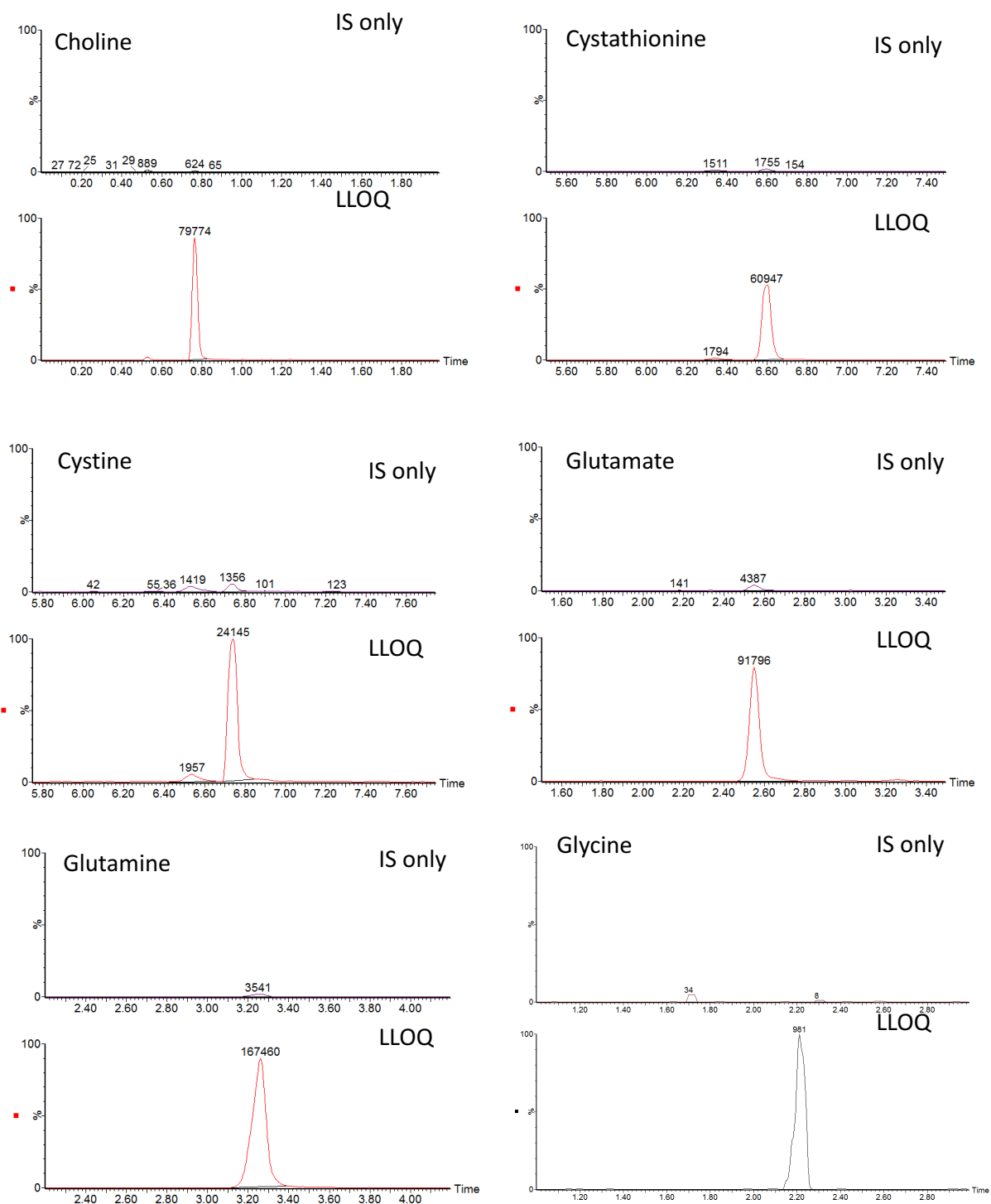


Figure 8.5-1 Part 1/2. Representative chromatograms of internal standard interference to analyte experiments. Upper panels labelled "IS only" are chromatograms from analysis of samples containing only internal standard. Lower panels are the LLOQ of the standard.

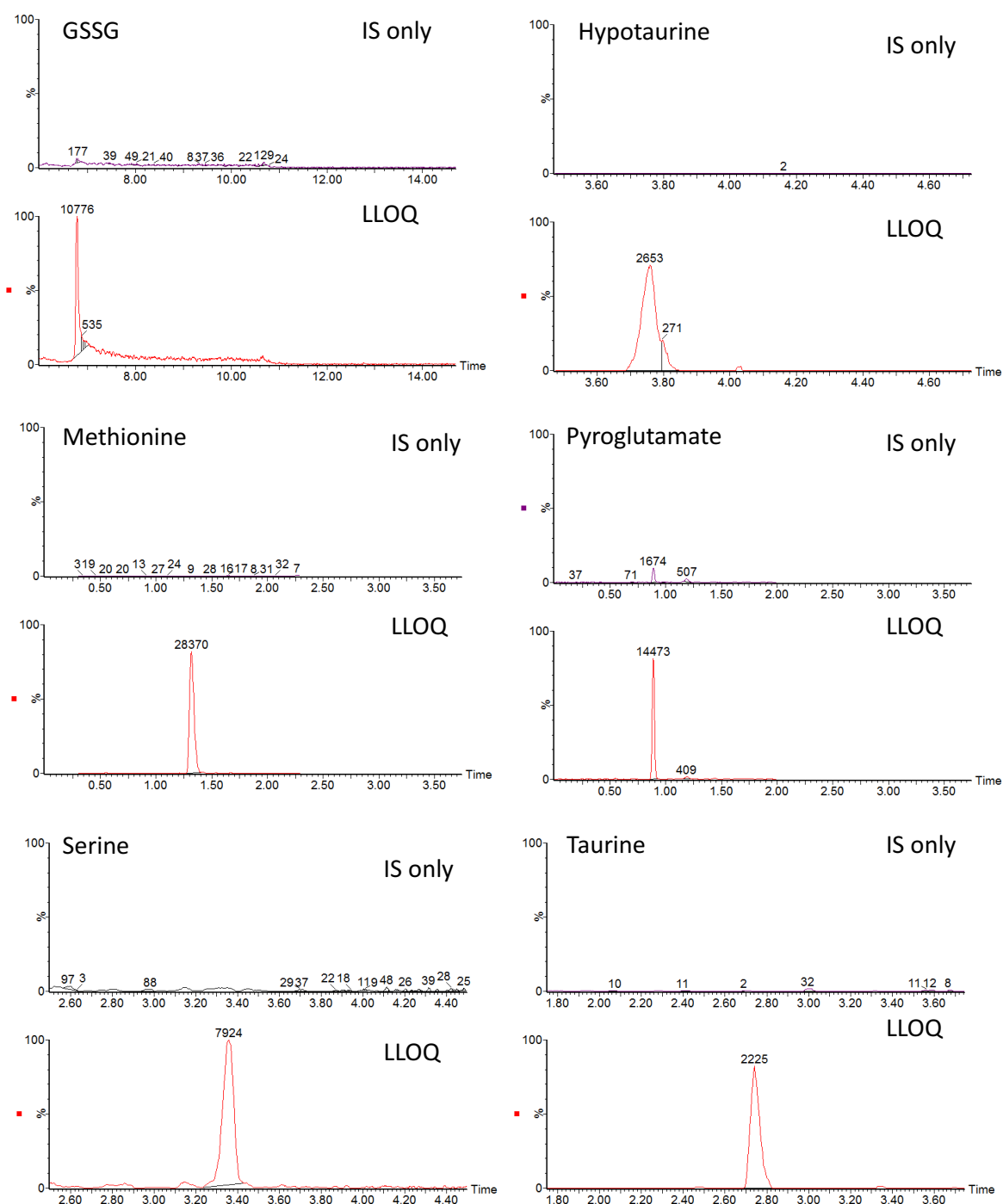


Figure 8.5-2 Part 2/2. Representative chromatograms of internal standard interference to analyte experiments. Upper panels labelled "IS only" are chromatograms from analysis of samples containing only internal standard. Lower panels are the LLOQ of the standard.

Table 8.5-2 Analyte to analyte interference

Analyte tested	Average response LLOQ Analyte	Analyte	Response at Analyte	Interference (%)
Cysteine 1	1036	Taurine	5614	5.4
Cysteine 2			5785	5.6
Cysteine 3			5988	5.8
Cysteine 1		γ-glutamyl-cysteine	3734	3.6
Cysteine 2			4610	4.4
Cysteine 3			3087	3.0
Cystine 1	6952	Cysteine	266409	38.3
Cystine 2			234848	33.8
Cystine 3			210217	30.2
Cystine 1		Cystathionine	10320	1.5
Cystine 2			9942	1.4
Cystine 3			9759	1.4
Cystine 1		γ-glutamyl-cysteine	575001	82.7
Cystine 2			632288	91.0
Cystine 3			554347	79.7
Cystine 1		GSSG	2999	0.4
Cystine 2			4576	0.7
Cystine 3			3765	0.5
Cystine 1		Taurine	29928	4.3
Cystine 2			24706	3.6
Cystine 3			21816	3.1
Glutamate 1	5454	γ-glutamyl-cysteine	1428	0.3
Glutamate 2			-	0.0
Glutamate 3			-	0.0
Glutamate 1		Glutamine	1493569	273.8
Glutamate 2			1484616	272.2
Glutamate 3			1557351	285.5
Glutamate 1		GSSG	874	0.2
Glutamate 2			954	0.2
Glutamate 3			659	0.1
Glutamate 1		Ophthalmic acid	787	0.1

Glutamate 2			721	0.1
Glutamate 3			35578	6.5
Glutamate 1			-	0.0
Glutamate 2		SAH	2635	0.5
Glutamate 3			-	0.0
Glutamaine 1	59322	Cystathionine	1441.842	2.4
Glutamaine 2			1416.46	2.4
Glutamaine 3			1293.275	2.2
Glutamaine 1		Cysteine	2690.812	4.5
Glutamaine 2			2329.731	3.9
Glutamaine 3			2046.548	3.4
Glutamaine 1		GSSG	5874.89	9.9
Glutamaine 2			5147.397	8.7
Glutamaine 3			4786.746	8.1
Glutamaine 1		GSH	1232.114	2.1
Glutamaine 2			1086.162	1.8
Glutamaine 3			1006.622	1.7
Glutamaine 1		Homocystine	4845.921	8.2
Glutamaine 2			4422.922	7.5
Glutamaine 3			3469.488	5.8
Glutamaine 1		Taurine	7271.154	12.3
Glutamaine 2			6756.338	11.4
Glutamaine 3			6178.858	10.4
GSSG 1	4692	Cystathionine	947.577	20.2
GSSG 2			2307.261	49.2
GSSG 3			1585.697	33.8
GSSG 1		Glutamine	2846.086	60.7
GSSG 2			361.835	7.7
GSSG 3			139.339	3.0
GSSG 1		GSH	5300.88	113.0
GSSG 2			5292.134	112.8
GSSG 3			4046.992	86.3
Homocystine 1	3203	γ-glutamyl-cysteine	241.564	7.5
Homocystine 2			324.58	10.1
Homocystine 3			300.292	9.4
Homocystine 1		GSH	1687.393	52.7
Homocystine 2			886.009	27.7

Homocystine 3			680.437	21.2
Homocystine 1		Ophthalmic acid	714.435	22.3
Homocystine 2			648.327	20.2
Homocystine 3			532.295	16.6
Homocystine 1		SAH	691.696	21.6
Homocystine 2			397.972	12.4
Homocystine 3			-	0.0

“-“ represents no peak detected.

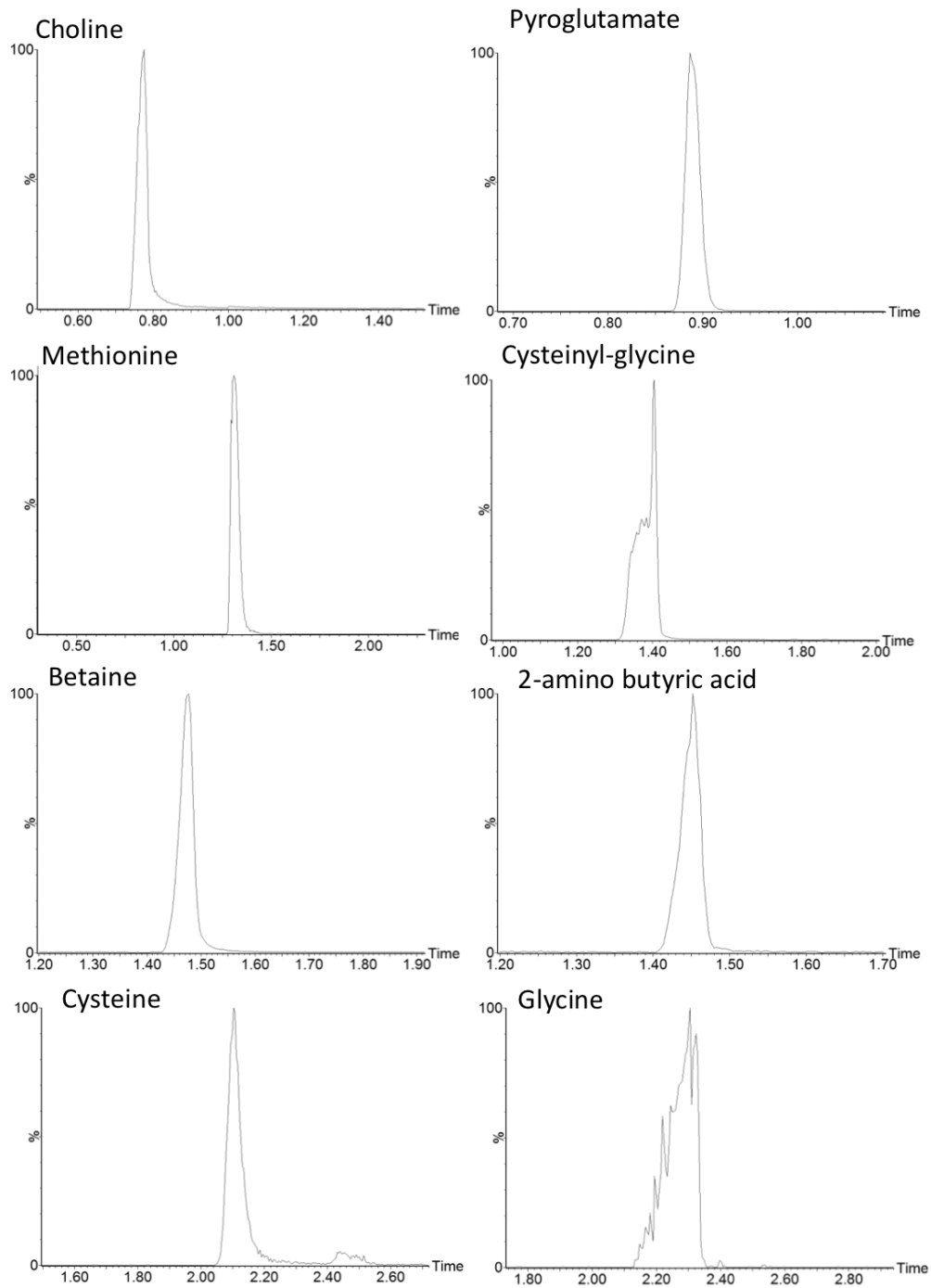


Figure 8.5-3 Peak shapes from HILIC-MS/MS assay, in order of elution (1/3).

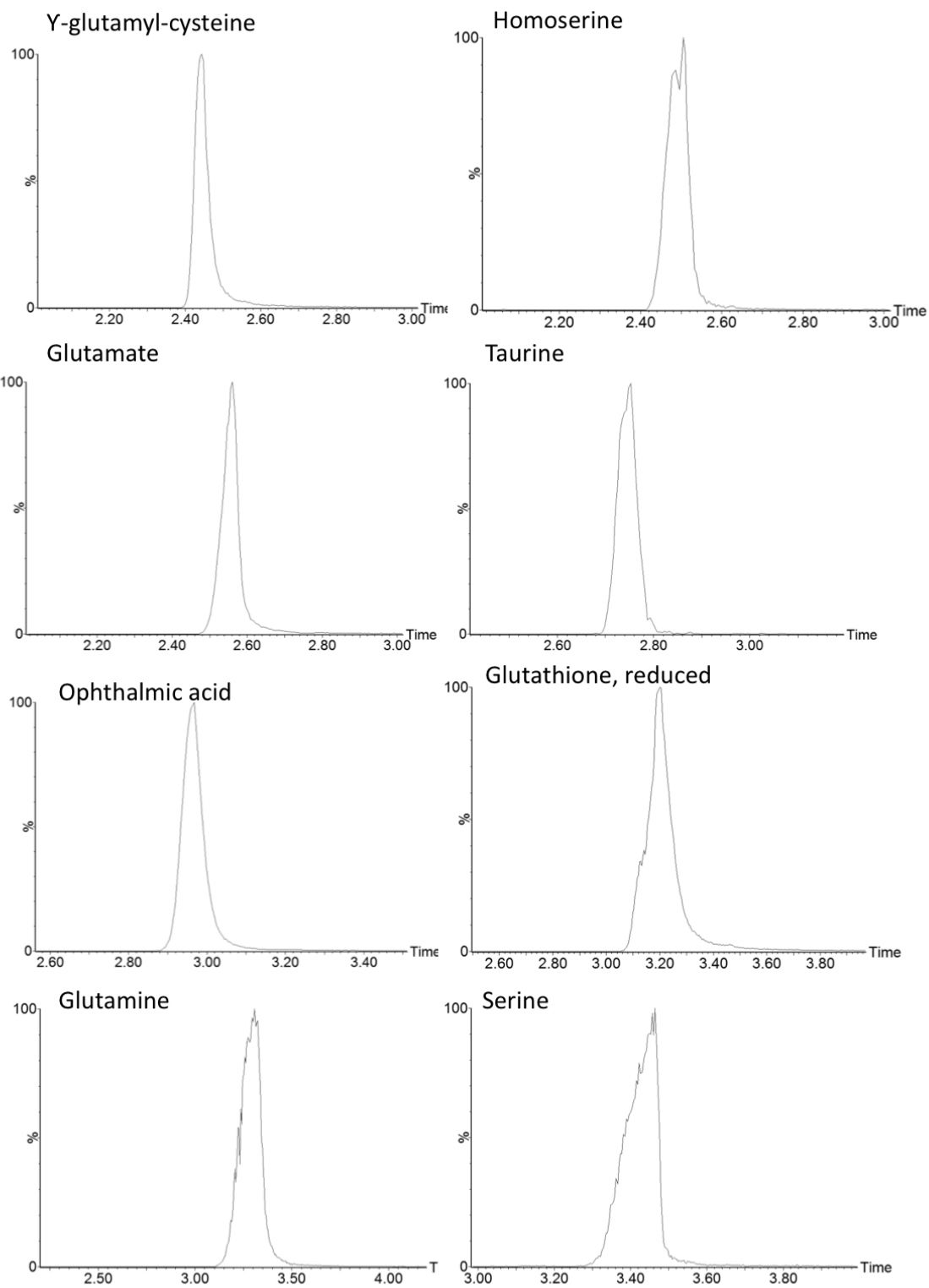


Figure 8.5-4 Peak shapes from HILIC-MS/MS assay, in order of elution (2/3).

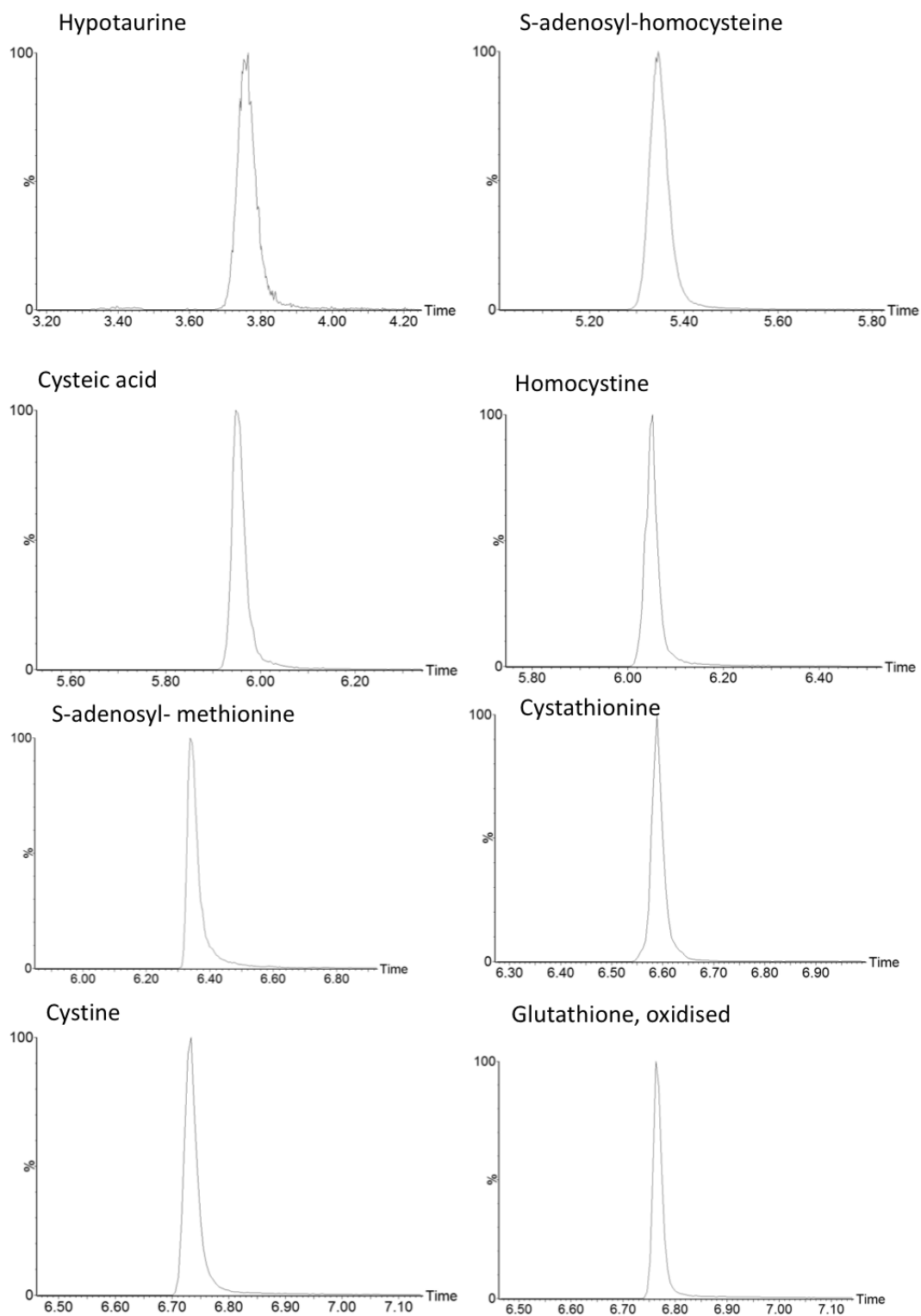


Figure 8.5-5 Peak shapes from HILIC-MS/MS assay, in order of elution (3/3).

Table 8.5-3 Median, min, max concentrations (ng/mL) of metabolites in plasma from TA/TAI study quantified using HILIC-MS/MS

Compound	2h			6h			24h		
	Ctrl	TA	TAI	Ctrl	TA	TAI	Ctrl	TA	TAI
Betaine	Median	3736	3051	3563	4708	4979	4152	6471	5164
	Min	2977	2418	3198	3419	3698	4070	5400	4029
	Max	4918	3489	3883	12458	5816	5676	8124	6334
Choline		1697	1612	1894	1690	1742	3022	1756	1285
		1398	1441	1741	940.0	1504	2718	1555	1134
		1976	1723	2386	3263	2127	5798	2203	1515
Cystathionine		166.5	323.8	403.0	227.0	226.5	332.0	259.0	132.5
		132.0	217.0	219.5	181.0	194.0	25.00	179.0	114.0
		258.5	460.5	450.0	242.5	331.5	430.0	282.0	152.5
Cystine		111.0	546.0	1569	95.00	265.5	858.5	21.50	71.00
		24.00	69.50	166.0	62.00	30.00	266.0	N.D	N.D
		246.0	1117	5143	194.0	422.5	11	98.00	119.0
Glutamate		4614	1531	2547	5272	6839	9112	3912	4087
		3113	955.5	2396	1889	3552	3331	2247	2940
		7533	2301	3681	9199	10980	17297	4140	6519
Glutamine		123120	87663	128811	106236	136459	181380	164240	152248
		112507	83663	112013	95338	112470	126908	162063	136411
		170945	100970	132972	132208	156331	207043	200660	194524
Glycine		20065	12214	11045	16897	21632	28629	19668	10936
		N.D	N.D	N.D	N.D	21395	18810	13362	9513
		28470	17176	14513	23825	21939	40667	24367	14437
Homoserine		11110	6237	9993	6670	11291	9161	7904	6682
		5629	4350	8180	5046	6584	7696	6279	4790
		22565	10246	14957	29856	12556	15988	15279	12867
Hypotaurine		5726	11607	4601	7674	8500	10077	5030	2670
		4629	8825	2815	6483	6295	6432	3287	2124
		5863	14064	10914	16408	9622	12364	5964	3183
Methionine		10330	7615	8766	9960	10386	13383	8064	7422
		8566	6409	8332	9079	9912	9735	7759	7149
		11511	10417	9844	11087	13056	15731	9369	7829
Pyroglutamate		3859	3521	6376	3559	5564	10908	3640	3810
		3608	3112	5379	2584	4159	5838	3442	3006
		4322	3966	11850	3861	5716	18769	4295	3981
Ophthalmic acid		32.00	34.50	90.00	17.50	175.5	112.5	15.50	16.50
		26.00	26.00	54.50	16.00	116.5	91.00	13.00	15.50
		52.00	64.50	111.5	28.00	287.0	152.0	16.00	20.00
SAM		159.0	165.8	231.0	145.5	185.0	174.5	134.5	156.0
		134.0	147.0	200.0	132.5	178.0	157.5	132.0	139.0
		196.5	180.5	292.5	297.0	259.5	210.0	146.5	197.0
SAH		48.00	41.00	52.00	59.50	51.50	49.00	43.50	41.00
		40.00	39.50	48.50	41.00	39.00	39.50	40.00	37.00
		60.50	42.50	62.00	117.5	67.00	64.00	45.50	45.50
Serine		41029	27230	27420	45704	39694	45698	49110	34429
		32385	15206	24201	43621	37445	43659	28924	23915
		49349	40680	31404	53918	56132	54111	57374	43445
Taurine		21373	25419	20650	22333	35395	56454	20226	13538
		17791	23147	18076	16346	31071	48522	13587	10460
		25108	31469	25398	48844	47291	76398	25330	16334

Orange= Unique statistically significant change in TAI group v C

Blue= Unique statistically significant change in TA group v C

Yellow= Statistically significant elevation in both TA v C and TAI v C.

Green= Statistically significant elevation in TAI, depletion in TA.

8.6 CHAPTER 6 - SUPPORTING DATA/DOCUMENTS

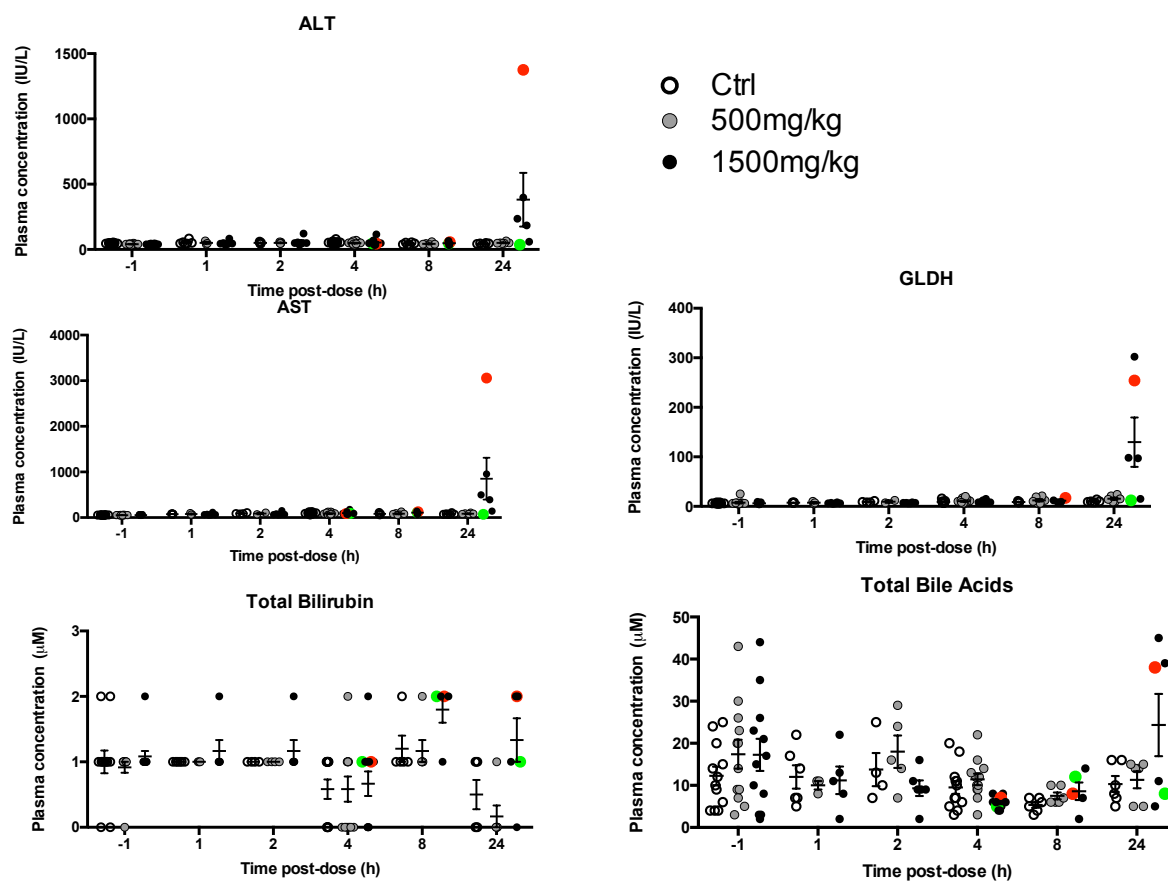


Figure 8.6-1 In-life clinical chemistry. Bars indicate the mean, error bars indicate S.E.M. (n=12 at -1 and 4h, remaining time-points n=6).

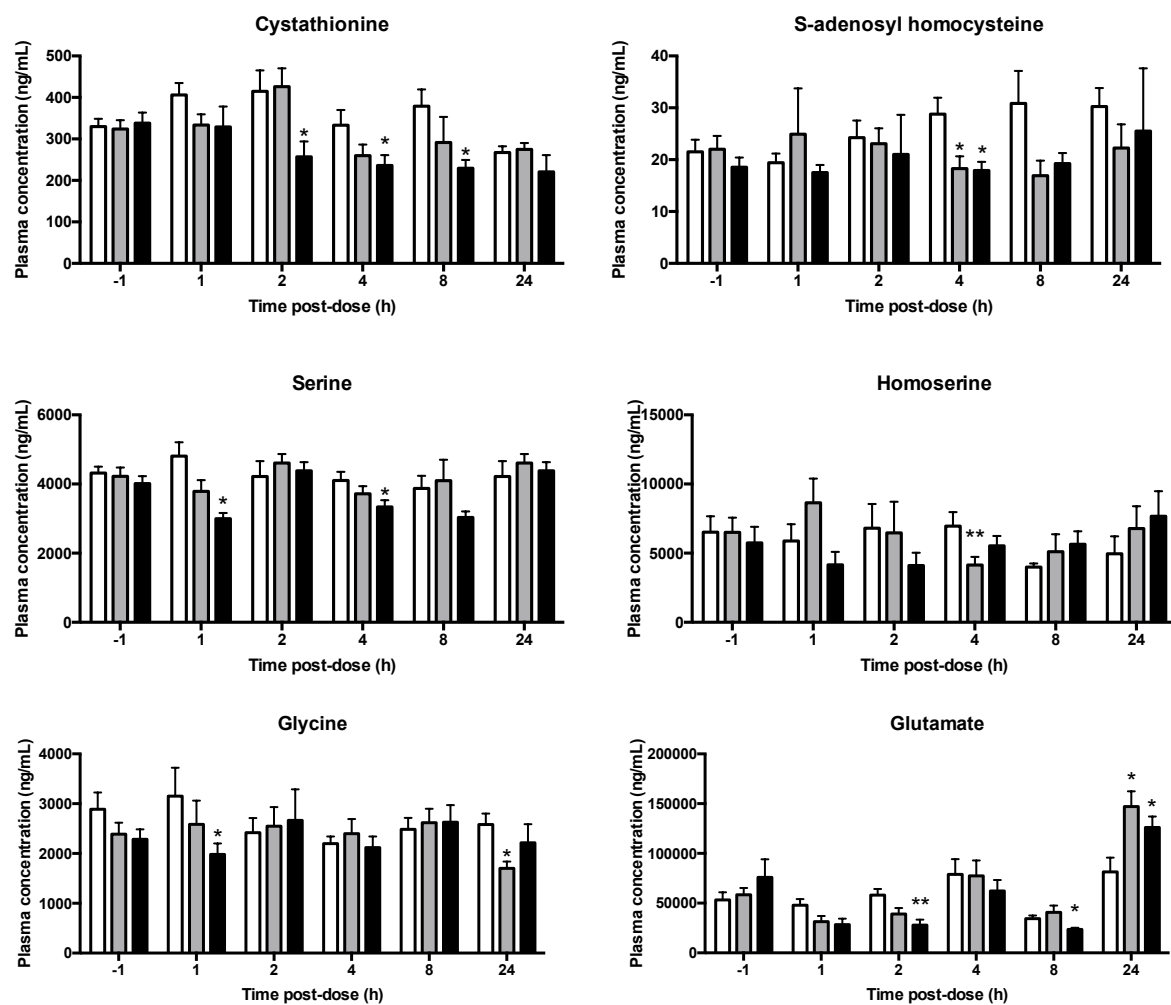


Figure 8.6-2 Selected metabolites from the quantification of 14 metabolites in plasma following APAP dosing, significance Two tailed Mann-Whitney test compared to vehicle treated at each time-point. Dots= individual animals. Bars represent the mean, error bars indicate $\pm$ S.E.M. ($n=12$ at -1 and 4h, remaining time-points $n=6$).

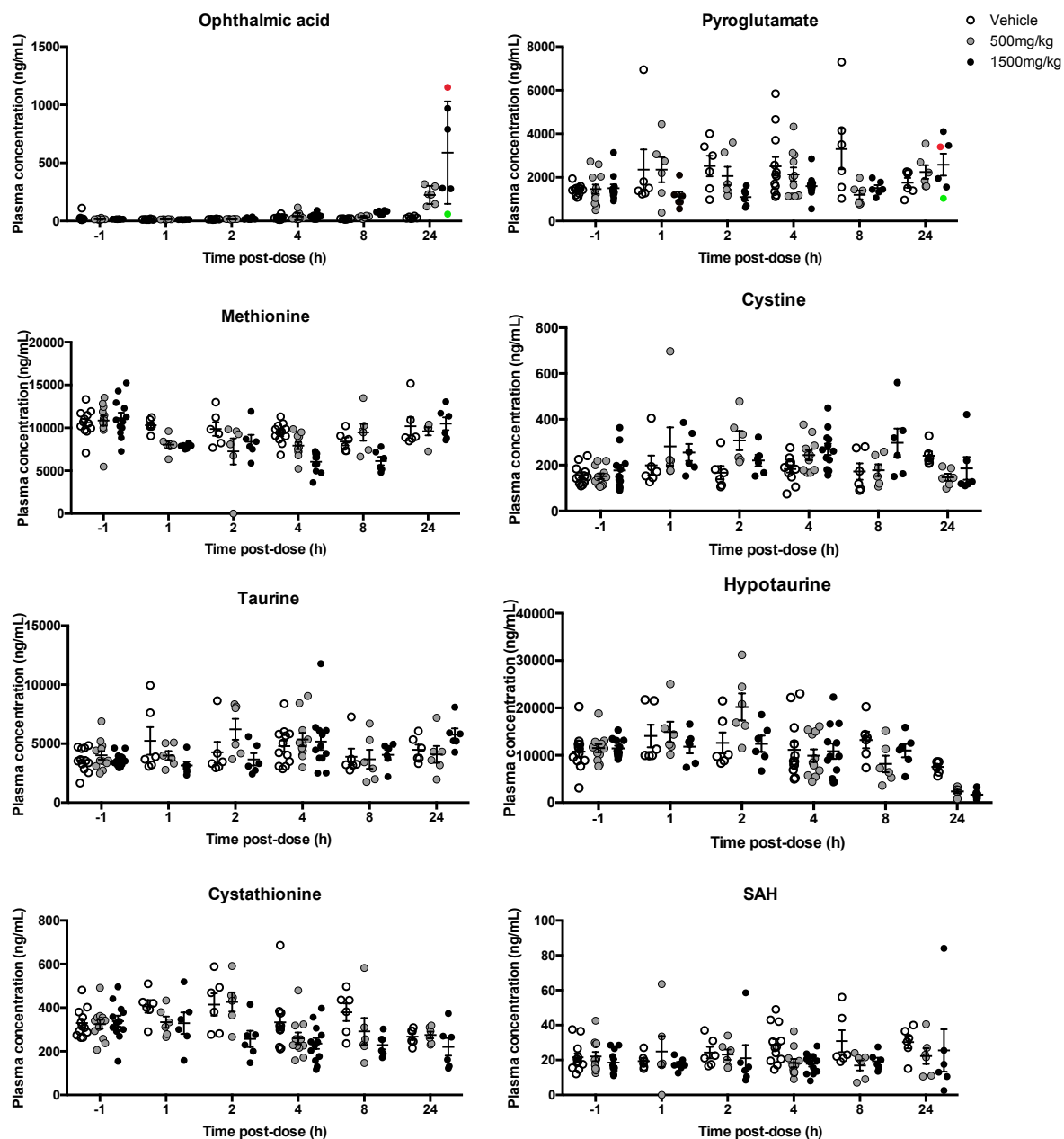


Figure 8.6-3 Part 1 of 2. Selected metabolites from the quantification of 14 metabolites in plasma following APAP dosing, significance Two tailed Mann-Whitney test compared to vehicle treated at each time-point. Dots= individual animals. Lines represent the mean, error bars indicate $\pm$ S.E.M. (n=12 at -1 and 4h, remaining time-points n=6).

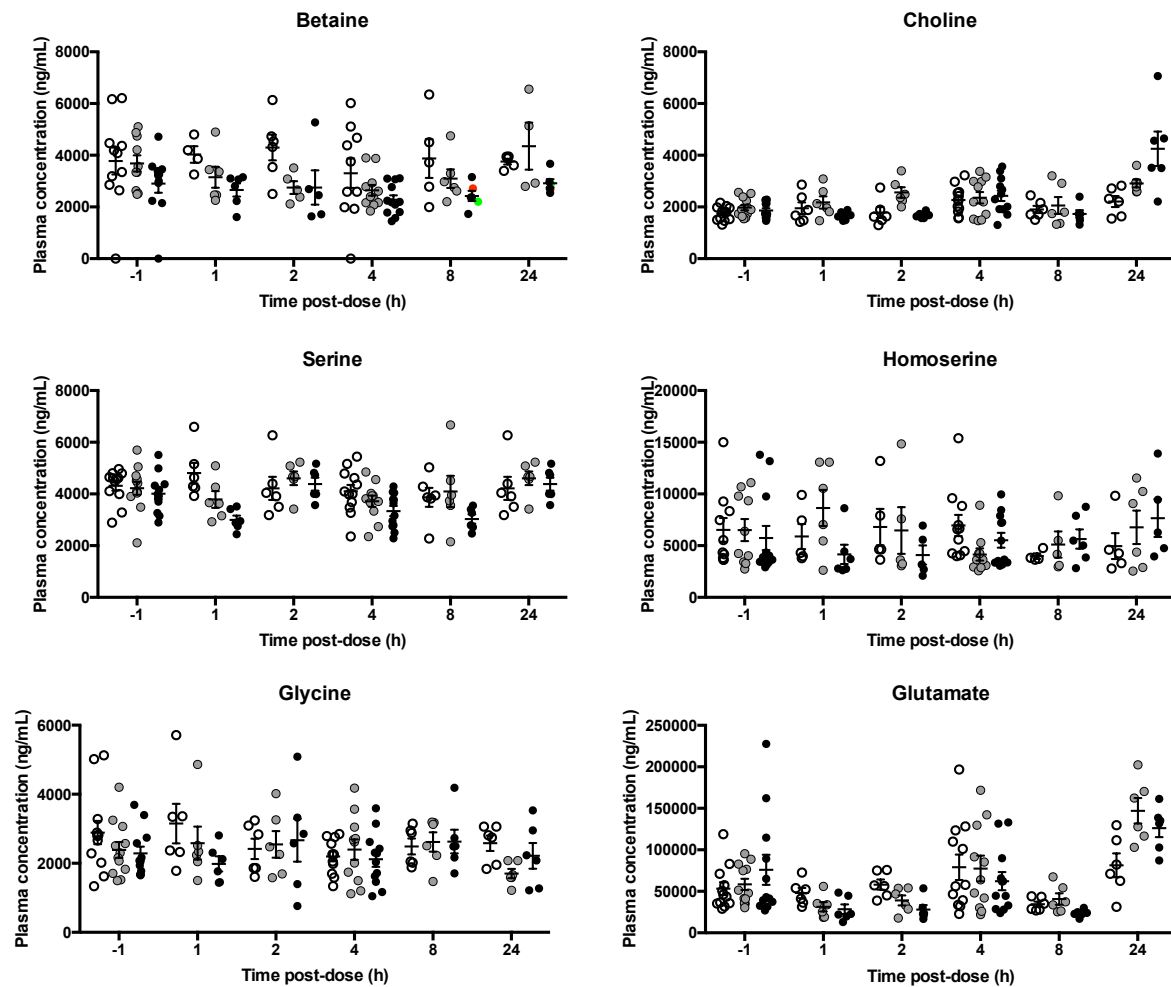


Figure 8.6-4 Part 2 of 2. Selected metabolites from the quantification of 14 metabolites in plasma following APAP dosing, significance Two tailed Mann-Whitney test compared to vehicle treated at each time-point. Dots= individual animals. Lines represent the mean, error bars indicate $\pm$ S.E.M. ($n=12$ at -1 and 4h, remaining time-points $n=6$).

Thank you.

*My patient supervisors,
generous collaborators,
challenging CSM staff, and
motivational CSM students.*

*My faithful family
and loyal friends.*

I would not have come close to finishing without you.



This thesis is dedicated to the eighty-two rats featured in this work.